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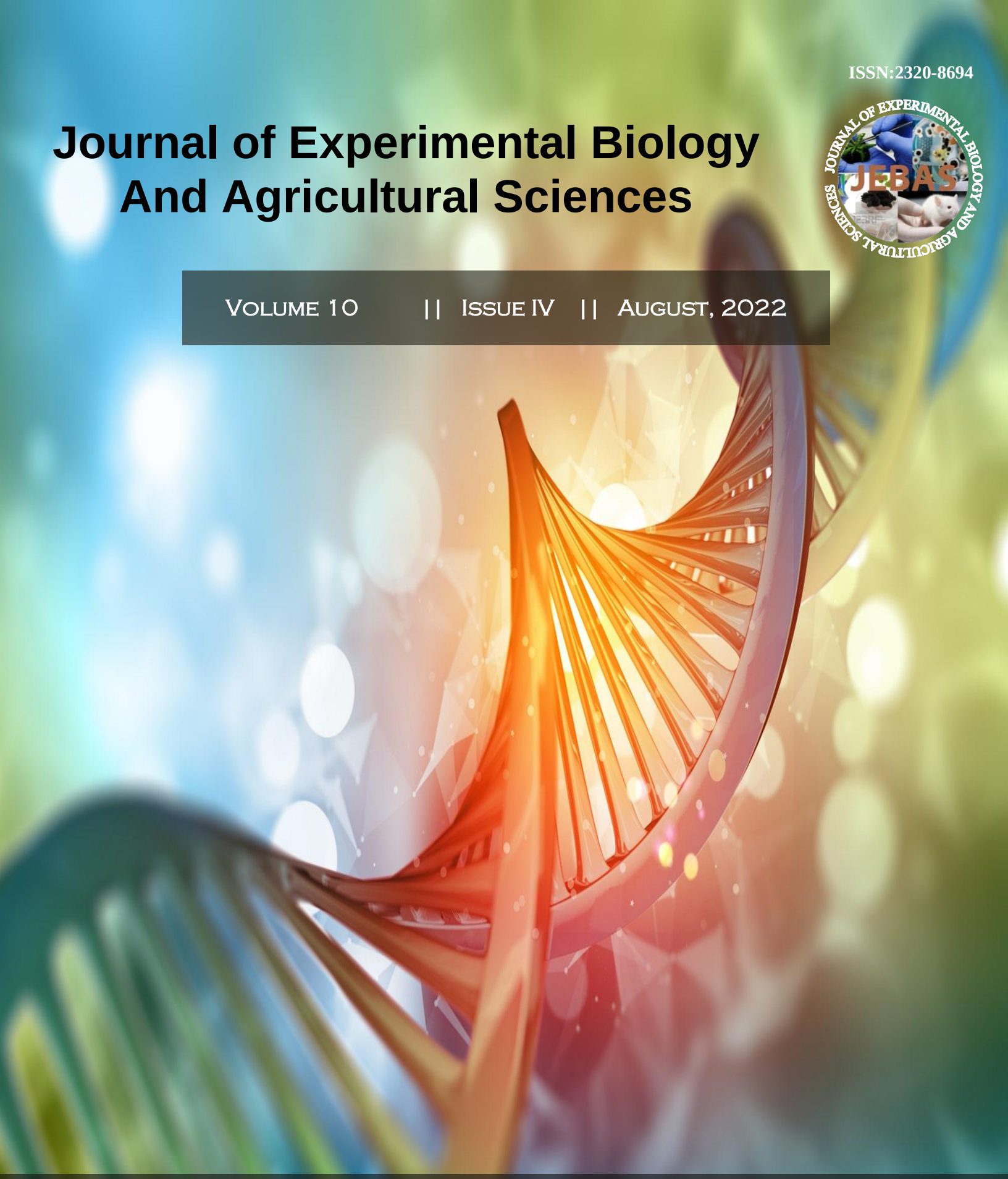

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Monkeypox: An Update on Current Knowledge and Research Advances

Deepak Chandran[1*], Kuldeep Dhama[2*], Muhammad Aslam M K[3], Sandip Chakraborty[4], Ranjan K. Mohapatra[5], Mohd Iqbal Yatoo[6], Md. Aminul Islam[7,8], Mahmoud Alagawany[9], Anil K. Sharma[10], Pran Mohankumar[11], Anupama Das Panalil[12], Diljith Chandran[13]

[1]Department of Veterinary Sciences and Animal Husbandry, Amrita School of Agricultural Sciences, Amrita Vishwa Vidyapeetham University, Coimbatore, Tamil Nadu –642109, India
[2]Division of Pathology, ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh- 243122, India
[3]Base Farm, Kerala Veterinary and Animal Sciences University, Kolahalamedu, Idukki, India-685501
[4]Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, R.K. Nagar, West Tripura, Tripura—799008, India
[5]Department of Chemistry, Government College of Engineering, Keonjhar, Odisha,-758002, India
[6]Division of Veterinary Clinical Complex, Faculty of Veterinary Sciences and Animal Husbandry, Shuhama, Alusteng Srinagar, Sher-E-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Srinagar-190006, Jammu and Kashmir, India
[7]COVID-19 Diagnostic Lab, Department of Microbiology, Noakhali Science and Technology University, Noakhali-3814, Bangladesh
[8]Advanced Molecular Lab, Department of Microbiology, President Abdul Hamid Medical College, Karimganj, Kishoreganj- 834001, Bangladesh
[9]Poultry Department, Faculty of Agriculture, Zagazig University, Zagazig 44511, Egypt
[10]Department of Biotechnology, Maharishi Markandeshwar University (Deemed to be University) Mullana-Ambala-133207, Haryana, India
[11]School of Agriculture and Biosciences, Karunya Institute of Technology and Sciences, Coimbatore, Tamil Nadu- 641114, India
[12]Dental Speciality Centre, Kulappully, Shoranur, Palakkad, Kerala- 679122, India
[13]KVG Dental College & Hospital, Kurunjibhag, Sullia, Karnataka – 574327, India

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ABSTRACT

The resurgence of the disease in humans that is very similar to smallpox called monkeypox (MPX) disease, caused by the monkeypox virus (MPXV), is the dominant topic of discussion in the scientific and popular press around the world right now. This is taking place as the world celebrates the historic accomplishments made in the fight against the Coronavirus Disease (COVID-19) pandemic MPX is currently thought to pose a risk to the general public's health, particularly in areas with high rates of MPXV infection and close human-wild animal contact. Despite the rarity of MPX outbreaks, they are often caused by human-to-human transmission, especially in households and healthcare settings. Recent decades have seen recurrent outbreaks of the MPX after the smallpox disease was declared eliminated and the

* Corresponding author
E-mail: c_deepak@cb.amrita.edu (Deepak Chandran);
kdhama@rediffmail.com (Kuldeep Dhama)

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Vaccination

Treatment

Prevention

Control

consequent cessation of smallpox vaccination programs. MPX has presently spread to several countries throughout the world and posed a global public health emergency, with nearly 45000 confirmed cases in 96 countries and locations, and 12 deaths as of August 24, 2022. Even though this viral illness is thought to be self-limiting, its consequences and feasible pandemic potential seriously jeopardize public health. The main approach to avoiding MPX is to adopt appropriate prevention and control measures, increase awareness of risk factors, and inform the public of the steps they may take to reduce viral exposure. Scientific studies are currently looking at the viability and suitability of the MPX vaccination. This article presents a general introduction to MPXV / MPX along with progress in diagnosis, treatment, vaccination, and prevention and control strategies for tackling this global health emergency.

1 Introduction

Monkeypox (MPX) is a viral zoonosis illness caused by the monkeypox virus (MPXV) that has been linked to detrimental effects on both human and animal health, and currently posed a global public health emergency (Banerjee et al. 2022; Lai et al. 2022; Meo and Jawaid 2022; Mohapatra et al. 2022; Saied et al. 2022a). Its symptoms are similar to those of smallpox, although it is clinically less severe. Even though smallpox was eradicated over 40 years ago and smallpox immunization was discontinued, MPXV has emerged as the most important Orthopoxvirus for public health (Adler et al. 2022; Kozlov 2022). Monkeypox virus (MPXV), a member of the family Poxviridae, is the zoonotic pathogen responsible for MPX. Chordopoxvirinae and Entomopoxvirinae are two subfamilies of the Poxviridae family. The Chordopoxvirinae subfamily is comprised of 18 genera, including those that are known to infect vertebrates (Orthopoxvirus, Capripoxvirus, Parapoxvirus, Avipoxvirus, Suipoxvirus, Cervidpoxvirus, Yatapoxvirus and Leporipoxvirus). The Entomopoxvirinae subfamily consists of four genera that cause disease in arthropods (Gammaentomopoxvirus, Betaentomopoxvirus, Deltaentomopoxvirus, and Alphaentomopoxvirus). There are 10 recognized species of Orthopoxviruses at present, including MPXV and variola (smallpox). However, despite being a DNA virus, MPXV does not replicate outside of the cytoplasm of infected cells (Cheema et al. 2022; Harris 2022).

It was first identified in 1958, after an outbreak of the virus among monkeys in a Danish research facility. A nine-month-old boy in the Democratic Republic of the Congo (DRC) contracted the disease in 1970, despite smallpox having been eradicated there in 1968. This brought attention to the disease for the first time (Mohapatra et al. 2022; WOAH 2022). The genetic lineage of MPXV can be broken down into two subgroups: the West African clade and the Congo Basin clade. Although MPXV is mainly prevalent in the Congo Basin, incidences of MPX in both humans and wildlife have also been documented in other Central and West African countries. However, conducting effective surveillance in endemic areas is difficult due to a lack of epidemiological and ecological research infrastructure. The fatality rate in the West African clade is 3.6 percent, while it is 10.6 percent in the Congo basin clade (Bunge et al. 2022; Kumar et al. 2022; WHO 2022a). Patients with immunodeficiencies may have a higher case fatality rate. The incubation phase normally lasts 6 to 13 days, although in exceptional situations it can last up to 21 days. The most likely source of MPXV is rodents. Close contact with an infected person or animal, or with contaminated objects or surfaces, is regarded to be the major route of viral transmission to humans. Human-to-human transmission may occur via droplets, bodily fluids, or infected surfaces. Importantly, MPX may not always be correctly diagnosed. Concurrent infections with varicella zoster virus (VZV) and MPXV are thought to be very common, although they have only been reported seldom (Kozlov 2022; Okyay et al. 2022; WHO 2022b).

On May 7, 2022, a confirmed case of the West African lineage of MPXV was discovered in the United Kingdom (UK), and subsequently, the MPXV has drawn considerable interest worldwide. United States of America (USA), India, Australia, Canada, Israel, and many European countries, including the UK, Portugal, Sweden, Spain, Italy, France, Germany, Netherlands, and Belgium have all reported cases (CDC 2022a; Sah et al. 2022). Since January 1, 2022, nearly 45000 confirmed MPX cases from 96 countries and locations along with 12 deaths have been reported as of August 24, 2022 (Adalja and Inglesby 2022; Adegboye et al. 2022; CDC, 2022a; Mohapatra et al. 2022). We still do not fully understand the natural history of the virus, its origins, or which animals serve as its reservoir hosts. We will be able to better understand how the MPXV spread from animals to humans by closely monitoring it in regions where it is prevalent. The current review updates knowledge on MPX's early pandemic transmission pathways, pathophysiology, clinical manifestation, therapy, and prevention.

2 Etiology

In the same family as cowpox (CPX), variola (VARV), and vaccinia (VACV) viruses, MPXV is among the Orthopoxviruses. Typically, the structure of these viruses often has a lipoprotein

envelope and are either oval or brick-shaped, measuring 200–400 nm in size when viewed under an electron microscope (Mohapatra et al. 2022). MPXV shares various features with other members of the *Orthopoxvirus* genus, including variable number tandem repeats and putative telomere resolution sequence, with its 6379-bp terminal inverted repetition (Yang et al. 2020). There are two ways in which MPXV might enter the host cell: first, by binding to chondroitin or heparan sulfate on the viral envelope and then dispersing into the plasma membrane; or second, through macropinocytosis, which uses actin to enter the cell. Viral proteins and enzyme factors are released from the virus into the cytoplasm and weaken cell defenses, encouraging early gene transcription and DNA replication and the formation of intermediate transcription factors (Kumar et al. 2022).

The literature suggests that the MPXV clade that originated in the Congo Basin is more lethal than its West African counterpart. This is due to differences in genomic architecture, which result in greater virulence and higher fatality rates in the Congo Basin clade (Kabuga and El Zowalaty 2019). Notable virulence features include an inability to replicate in human cells and a propensity to inhibit inflammatory cytokine production by human cells (including interferon-gamma (IFN-γ) and tissue necrosis factor-alpha (TNF-α)). Another important immune-modulating component that contributes to the higher virulence of this strain comes from a gene in the Congo Basin lineage that inhibits complement enzymes. However, contrary to what was previously believed, studies have shown that neither a decrease in major histocompatibility complex (MHC) expression nor a decrease in cellular transport is connected to MPX virulence (Cheema et al. 2022).

3 Transmission routes and infectivity

MPXV has been proven to be transmitted from animals to people, but how it gets from humans to humans remains a mystery. Rats, squirrels, and dormice, as well as a wide variety of monkey species, are the most common vectors of the virus. It has been shown, however, that the virus can be passed from person to person, both within and outside of Africa. The most common route of MPX transmission from animals to people is believed to be through contact with infected animals, either indirectly or directly (touch, bite, or scratch). Bushmeat consumption and open wounds in the skin, mouth, or throat are probable avenues for the virus to infiltrate and infect the human body. Direct or indirect contact with bodily fluids or lesions, contaminated surfaces, or materials (e.g., clothing or linens) is the most common means of human-to-human transmission (Parker and Buller 2013; Angelo et al. 2019; Bunge et al. 2022). MPXV can also be transmitted from mother to fetus by vertical transmission, resulting in congenital MPX (Fahrni et al. 2022). As reported, the pregnant women who contracted MPX experienced spontaneous early miscarriages (Khalil et al. 2022). Human MPX infections may carry a high risk of spontaneous miscarriage, premature birth, and fetal death (Fahrni et al. 2022). However, limited data is available to support the probability of vertical transmission of MPX in pregnant women. Males who have sex with other males are likewise more prone to get the disease. Even though MPX can be spread by direct physical contact, this does not qualify as evidence that it is sexually transmitted. Close contact with patients over a long period makes hospital employees and their families more susceptible to illness. Nosocomial transmission has been shown to take place, according to the available evidence (Bisanzio and Reithinger 2022; Heskin et al. 2022). Without proof to the contrary, the human-to-human transmission alone cannot sustain MPX infections in the broader human population. The R0 value for MPX is between 1.10 and 2.40 in areas with low levels of exposure to Orthopoxviruses; this value suggests that an outbreak is likely if imported human or animal cases are present (Okyay et al. 2022).

4 Clinical symptoms

The incubation period for MPX is 8 days (average), and the duration of symptoms is 2 to 4 weeks. Headache, back pain, malaise, tiredness, lethargy, and low-grade fever are all symptoms that typically appear during the prodromal phase of a viral infection. The vesiculopustular rash begins on the face and trunk and then moves outward in a circular pattern to the hands and feet 12 to 16 days after exposure. The rash then radiates outward to affect various parts of the body, including the palms and soles. Macular, papular, vesicular, and pustular lesions are the morphologically distinct stages of the rash. The pustules will crust over in a few days, and then they will fall off in a week or two. In contrast to smallpox, MPXV infection is characterized by painful maxillary, cervical, and inguinal lymphadenopathy, when it is found in 84 percent of unvaccinated people and only 54 percent of vaccinated patients (Petersen et al. 2019; Adler et al. 2022). Lymphadenopathy suggests that MPXV may elicit a stronger immune response and be more easily identified than VARV. Patients with immunocompromised conditions, prolonged viral particle exposure, and other sequelae, such as bronchopneumonia, encephalitis, and corneal infection-induced blindness, have worse clinical outcomes. Additionally, scarring, hypo-hyperpigmentation, dehydration (as a result of nausea and vomiting), and septicemia are all possible side effects (Bragazzi et al. 2022).

Unvaccinated people (74 percent) are more likely than vaccinated people (39.5 percent) to suffer from the side effects of monkeypox infection. The routine vaccination against smallpox has been discontinued in the modern world due to the disease's eradication (Hofer 2022). Cross-immunity protects persons who received smallpox vaccinations before the 1970s against the adverse effects of MPXV infection. Additionally, septic shock and necrotizing fasciitis would arise because of highly exaggerated immune

responses. Lifelong consequences are quite unusual in MPX because the sickness is self-limiting (Petersen et al. 2019; Okyay et al. 2022).

5 Animals susceptible to MPXV

As evidence of acute or past infection in many animals continues to mount, it is difficult to pinpoint the MPXV's primary reservoir species. Mice, rats, woodchucks, jerboas, and numerous rat and raccoon species have all tested positive for MPXV infection (WHO 2022a). MPXV was discovered by Kabuga and El Zowalaty (2019) in two different wild animals: a sooty mangabey (*Cercocebus atys*) and a rope squirrel (*Funisciurus anerythrus*). Multiple species of non-human primates (NHPs) are susceptible to infection by MPXV. These include short-tailed opossums (*Monodelphia domestica*), southern opossums (*Didelphia marsupialis*), prairie dogs (*Cynomys laudovicianus*), and African hedgehogs (*Atelerix* spp.). The main primary hosts of the African orthopoxvirus include the sun squirrel (*Helioscuitius* spp), giant pouched rat (*Cricetomys* spp.), the rope squirrel (*Funisciurus* spp.), and the African dormice (*Graphiurus* spp.) (Kumar et al. 2022). Chimpanzees (*Pan troglodytes*), macaques (*Macaca fascicularis*), marmosets (*Callithrix jacchus*), orangutans (*Pongo pygaeus*), and sooty mangabeys (*Cercocebus atys*) are all susceptible to infection after receiving an intravenous injection of MPXV. Pet prairie dogs close to ill exotic animals brought from Ghana, West Africa, caused an outbreak of encephalitis in the USA in 2003. Despite this, MPXV has made its way to the cause of a multi-country outbreak, causing concern. African rats, which are commonly kept as pets, are known to be susceptible to MPXV, raising fears that the virus could be passed to humans (Adler et al. 2022; Bragazzi et al. 2022). Although many mammals are susceptible to MPXV however the actual animal host for human transmission is unclear. But recently, an Italian male dog is reported to be infected first time with MPX from an infected MSM patient which suggests human-to-dog transmission of MPX virus. The MPX-infected people should avoid close contact with their pets, and domestic animals to prevent further spreading of the MPX virus (Seang et al. 2022). Zoonosis and reverse zoonosis of MPXV as observed for severe acute respiratory syndrome coronavirus -2 (SARS-CoV-2) causing the ongoing coronavirus disease 2019 (COVID-19) pandemic demands advanced global surveillance and tracking system for emerging and re-emerging viruses for limiting animal-to-human and human-to-animal transmissions (Dhama et al. 2020; Pramod et al. 2021; Sharun et al. 2021a; Sharun et al. 2021b; Afrooghe et al. 2022; Chakraborty et al. 2022a).

6 Public health threats

MPX, a zoonotic disease with animal spillover events is a moderate risk to human health and is currently a global public health threat creating high alert (Banerjee et al. 2022; Kumbhar and Agarwala 2022; Raheel et al. 2022). Since its discovery more than six decades ago (1958), MPX had received little attention because it was assumed to be a rare and self-limiting disorder, however, the recent re-emergence with posing global health concerns has speeded up the research on this virus (Meo and Jawaid 2022; Mohapatra et al. 2022). MPX is on the rise as a serious zoonotic health threat. Many more people are becoming infected with MPX as a result of increased human-wild animal contact in recent years, and the virus is now recognized as a significant threat to public health. There is a risk of international spread if there is unregulated trading in wildlife or its products (CDC 2022b; Kozlov 2022; Zumla et al. 2022). The onset, timing, and distribution of smallpox are all comparable to those of MPX, however, the scarring, complications, and mortality of MPX are often less severe than those of smallpox. While the smallpox vaccine successfully eradicated the disease around the world around 40 years ago, a startling similarity between smallpox and MPXV has lately emerged. During outbreaks, differentiating MPX from chickenpox, another herpesvirus illness, has been challenging. The potential for zoonotic infection from other Orthopoxviruses is something that must constantly be considered (Harris 2022; WHO 2022c). As reported, people having uncontrolled HIV had worse MPX outcomes. A Nigeria-based study reported that four HIV patients with features of AIDS died due to MPX (Yinka-Ogunleye et al. 2019). Another study of MPX cases with HIV patients suggested significant longer-lasting skin rashes, genital ulcers, and secondary bacterial infection (Ogoina et al. 2020). Keeping in mind, the British HIV Association suggests that HIV patients should be considered at higher risk for MPX (Ortiz-Martınez et al. 2022). Moreover, the coinfection of MPX and syphilis have been reported in HIV patients (Bízova et al. 2022). It is highly recommended to investigate the possibility of a combination of MPX with COVID-19 or other diseases which might be dangerous and may increase the fatalities (Farahat et al. 2022).

7 Diagnosis

An ideal specimen for laboratory testing is a sample of dried and sterile exudate or crust taken from skin lesions and kept at a low temperature (without the use of any viral transport media). The best way to get a viral culture is via an oropharyngeal or nasopharyngeal swab. An intact vesicular lesion, or at least a portion of its roof, is an excellent source of skin biopsies for research. An electron microscope, PCR, culture, and sequencing are all necessary for high-containment laboratories to make a conclusive diagnosis of MPX infection (Petersen et al. 2019; McCarthy 2022). Acute and convalescent samples must be matched within 5 days after the presentation for serologic detection of MPXV-specific immunoglobulin M (IgM) or IgG. Papular lesions may exhibit necrosis of keratinocytes, acanthosis, and basal

vacuolization in addition to a superficial and deep dermal perivascular lymphohistiocytic infiltration. Cell degeneration and signs of vasculitis appear in the vessels (e.g., multinucleated epithelial cells, necrosis of the epidermis). Squash-shaped intracytoplasmic aggregates of 200 to 300 mm in diameter are seen in the cytoplasm (Guarner et al. 2022; Haris 2022).

Real-time PCR and Light Cycler quantitative PCR (targeting the ATI/ A-type inclusion body gene) have substantially improved the detection of MPXV reliably and rapidly. Real-time PCRs, especially TaqMan probe-based assays, have considerably improved our ability to detect a variety of Orthopoxviruses, including MPXV. It is not just that such assays are useful in distinguishing MPXV from another pox (ie., Variola) and herpes viruses (viz., Varicella-zoster) (Maksyutov et al. 2016; Petersen et al. 2019). In addition to PCR, genomic sequencing of viral deoxyribonucleic acid (DNA) will aid in the diagnosis and other elements of the disease. West and Central African clades can be detected using a recombinase polymerase amplification (RPA) assay that targets the MPXV G2R gene specifically. The test has high sensitivity and a considerable detection limit, which is advantageous (16 DNA molecules can be detected per microlitre). When electron imaging reveals stages of virion assembly in the cytoplasm of keratinocytes, immunohistochemistry aids in the detection of viral antigen in afflicted epidermal keratinocytes, follicular and eccrine epithelium, and a few mononuclear cells of the dermis (Bunge et al. 2022; Mauldin et al. 2022).

8 Therapeutics and vaccines

As of yet, the Food and Drug Administration (FDA) has not approved a therapy intended to alleviate the symptoms of MPX. Most cases of MPX infection, fortunately, have a mild and self-limiting course. This results in treatment that is often supportive rather than requiring any sort of specialized care. Some examples of supportive treatment include the use of analgesics to alleviate pain, antipyretics to reduce fever, and antibiotics to treat any secondary bacterial infections that may develop (Adler et al. 2022; WHO 2022b). It is possible, however, that certain patients will require unique treatment. Specialized care may be needed for patients with severe diseases and those with impaired immune systems, pregnant women, and children. Smallpox-era medicines and vaccines have shown promising results against MPXV because of their resemblance to smallpox. Despite a lack of proof, FDA and European Medicines Agency (EMA) have approved the antiviral drug Tecovirimat for the treatment of smallpox in humans. Tecovirimat can be administered intravenously or orally. It is approved for use in the USA for the treatment of MPX by the Centers for Disease Control and Prevention (CDC) (Chakraborty et al. 2022b; Hofer 2022; Rodrguez-Cuadrado et al. 2022). Antiviral medications for cytomegalovirus (CMV) and human smallpox diseases, such as cidofovir and brincidofovir, are also an option. Vaccinia Immune Globulin Intravenous (VIGIV) is an intravenous infusion of a vaccinia-specific immunoglobulin intended to alleviate the symptoms of vaccinia vaccination. As part of an enhanced access protocol, the CDC approves it for the treatment of MPX disease (CDC 2022a; CDC 2022c; Keckler 2022). Of note, considering the prophylactic and therapeutic potential of medicinal herbs, plant metabolites, phytochemicals, immunomodulatory foods, and nutritious dietary elements as well as newer and effective chemical ligands, antiviral medicines, broadly neutralizing antibodies (nAbs), these need to be exploited for managing MPX patients, as found promising for many infectious emerging and/or re-emerging pathogens including deadly viruses affecting humans and animals (Dhama et al. 2018a; Dhama et al. 2018b; Tiwari et al. 2018; Anand et al. 2021; Singh et al. 2021; Calder 2022; Chakraborty et al. 2022b; Saied et al. 2022b).

MPX has surpassed smallpox as the most common human Orthopoxvirus infection since smallpox was declared eradicated in 1980, and as a consequence of the cessation of smallpox vaccination, risk factors got triggered for increasing infection with MPXV, especially among the younger population (less than 40-50 years of age) those who did not receive smallpox vaccination (Simpson et al. 2020; Mohapatra et al. 2022). Presently, humans are protected by a vaccine made from a highly attenuated strain of smallpox for up to six weeks following vaccination. While smallpox and MPX vaccinations are now legally available, they are not yet extensively distributed (Simpson et al. 2020). There are now two MPXV vaccines on the market: ACAM2000® (alive, replication-competent vaccinia virus) and JYNNEOSTM (alive, replication-incompetent vaccinia virus). Due to viral replication that goes uncontrolled with ACAM2000®, some persons experience an extremely painful and uncomfortable cutaneous reaction at the injection site, however, this is not the case with JYNNEOSTM. While ACAM2000® has the potential for accidental and self-inoculation, JYNNEOSTM is safe (Keckler 2022; WHO 2022b). The World Health Organization (WHO) has issued a global alert about the current multi-nation MPX outbreak, urging all countries to consider the situation and convene their national immunization technical advisory groups (NITAGs) to examine the available information and formulate vaccine use recommendations suitable to each country's specific circumstances. Before deciding to undergo smallpox or MPX vaccination, a healthcare professional and the person who is being vaccinated should assess the risks and benefits of each immunization individually. This vaccine should be administered in all nations at risk of MPX, to expedite the development of evidence regarding the vaccine's safety and efficacy (Okyay et al. 2022; WHO 2022c).

9 Prevention strategies

The primary strategy for MPX prevention is to raise knowledge of risk factors and educate the public about the measures they may

take to minimize their exposure to the virus. MPX vaccines are now being tested for their ability to prevent and control the disease. People who may be in danger, like health workers, laboratory personnel, and fast reaction teams, are offered vaccines in several countries (Cohen 2022; Saied et al. 2022a). The ability to quickly identify new cases and contain an outbreak is essential. People who come into close contact with infected MPX victims are the most likely to contract the illness. Those in the medical field, as well as their families, are at greater risk of contracting an illness. Health care workers caring for patients with MPXV infection or specimens from them should follow normal infection control procedures (Bunge et al. 2022). People who have been vaccinated against smallpox should be chosen to care for the patient if at all possible. People and animals suspected of having MPXV infection should only be handled by trained laboratory workers (CDC 2022b; CDC 2022c). A patient specimen must be packaged in conformity with WHO guidelines for the transportation of infectious substances to ensure its safety during shipment (WHO 2022a). Non-endemic countries with no direct travel linkages to an endemic area were found in the year 2022 to have clusters of MPX cases. Further study is under place to identify the source of the infection and limit its spread. When determining the origin of an outbreak like this, it is critical to look at all conceivable channels of transmission (CDC 2022d; Haider et al. 2022). Transmission and spread of human monkeypox virus infection have been earlier linked to travelers during previous outbreaks and travelers' perspectives (Bhattacharya et al. 2022).

The majority of human illnesses have transferred from animals to people through personal contact. It is best to stay away from wild animals, especially if they are sick or dead. This also includes avoiding their flesh, blood, and any other body parts. Meat and other animal products must be thoroughly cooked before being consumed. NHPs and rodents are subject to import restrictions in some countries. There should be an urgent quarantine for any animals that may be infected with MPX in captivity. All animals suspected of having come into contact with an infected animal should be quarantined for 30 days and observed for MPX symptoms (CDC 2022e; Saied et al. 2022a; WHO 2022b).

Conclusion and future prospects

Our understanding of MPX is limited since it is based primarily on sporadic reports of cases or outbreaks and on passive intermittent surveillance. In light of the devastation wrought by the COVID-19 pandemic, we must conduct in-depth research into the public health implications of MPX and its potential for a pandemic. Preparedness for public health issues and priority research requires community-led, locally coordinated, interdisciplinary programs centered on capacity building and education. A higher sense of urgency exists in the need to fortify national healthcare systems and develop international rules, regulations, and response mechanisms. Underreporting of MPX may occur in impoverished countries due to inadequate health care infrastructure and scarce resources. To prevent this disease from becoming a source of dread and stigma, swift political and financial backing is required. Healthcare workers and residents alike are worried about the possibility of the disease spreading from one person to another. To better prepare for and respond to future threats to public health, a deeper knowledge of the mechanisms that shaped MPXV's epidemiology, transmission patterns, clinical presentation, and natural habitat is required. Proactive, continuous, and comprehensive surveillance, rapid risk assessments, response measures, early detection, and contact tracing will be required to successfully control emerging or reemerging viral risks. To keep up with the rapidly changing epidemiology of this reemerging disease, there must be concerted efforts on a global scale to enhance the detection and surveillance of MPX cases.

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References

Adalja, A., & Inglesby, T.A. (2022). Novel international monkeypox outbreak. *Annals of Internal Medicine*. doi: 10.7326/M22-1581.

Adegboye, O.A., Castellanos, M.E., Alele, F.O., Pak, D., Ezechukwu, H.C., Hou, K., & Emeto, T.I. (2022). Travel-related monkeypox outbreaks in the era of COVID-19 pandemic: Are we prepared?. *Viruses*, *14*(6), 1283. doi: 10.3390/v14061283.

Adler, H., Gould, S., Hine, P., Snell, L.B., et al. (2022). Clinical features and management of human monkeypox: a retrospective observational study in the UK. *Lancet Infectious Diseases*, *22*(8), 1153-1162. doi: 10.1016/S1473-3099(22)00228-6.

Afrooghe, A., Rayati Damavandi, A., & Ahmadi, E. (2022). Reverse zoonosis and monkeypox: Time for a more advanced global

surveillance system for emerging pathogens. *New microbes and new infections*, *48*, 101013. https://doi.org/10.1016/j.nmni.2022.101013

Anand, A.V., Balamuralikrishnan, B., Kaviya, M., Bharathi, K., et al. (2021). Medicinal plants, phytochemicals, and herbs to combat viral pathogens including SARS-CoV-2. *Molecules*, *26*(6):1775. doi: 10.3390/molecules26061775.

Angelo, K.M., Petersen, B.W., Hamer, D.H., Schwartz, E., & Brunette, G. (2019). Monkeypox transmission among international travellers-serious monkey business? *Journal of Travel Medicine*, *26*(5), p.taz002. doi: 10.1093/jtm/taz002.

Banerjee, I., Robinson, J., & Sathian, B. (2022). Global re-emergence of human monkeypox: Population on high alert. *Nepal journal of epidemiology*, *12*(2), 1179–1181. https://doi.org/ 10.3126/nje.v12i2.45974

Bhattacharya, M., Dhama, K., & Chakraborty, C. (2022). Recently spreading human monkeypox virus infection and its transmission during COVID-19 pandemic period: A travelers' prospective. *Travel Medicine and Infectious Disease*, *49*, 102398. Advance online publication. https://doi.org/10.1016/ j.tmaid.2022.102398

Bisanzio, D., & Reithinger, R. (2022). Projected burden and duration of the 2022 Monkeypox outbreaks in non-endemic countries. *The Lancet Microbe*, *S2666-5247*(22), 00183-5. doi: 10.1016/S2666-5247(22)00183-5.

Bízova, B., Veselý, D., Trojanek, M., & Rob, F. (2022). Coinfection of syphilis and monkeypox in HIV positive man in Prague, Czech Republic. *Travel Medicine and Infectious Disease*, *49*, 102368. doi: 10.1016/j.tmaid.2022.102368.

Bunge, E.M., Hoet, B., Chen, L., Lienert, F., Weidenthaler, H., Baer, L.R., & Steffen, R. (2022) The changing epidemiology of human monkeypox-A potential threat? A systematic review. *PLoS Neglected Tropical Diseases*, *16*(2), e0010141. doi: 10.1371/journal.pntd.0010141.

Bragazzi, N.L., Kong, J.D., Mahroum, N., Tsigalou, C., Khamisy-Farah, R., Converti, M., & Wu, J. (2022). Epidemiological trends and clinical features of the ongoing monkeypox epidemic: A preliminary pooled data analysis and literature review. *Journal of Medical Virology*, 1-15. doi: 10.1002/jmv.27931.

Calder, P. C. (2022) Foods to deliver immune-supporting nutrients. *Current Opinion in Food Science*. *43*:136-145. doi: 10.1016/j.cofs.2021.12.006.

Dhama, K., Patel, S. K., Sharun, K., Pathak, M., et al. (2020). SARS-CoV-2 jumping the species barrier: Zoonotic lessons from SARS, MERS and recent advances to combat this pandemic virus. *Travel medicine and infectious disease*, *37*, 101830. https://doi.org/10.1016/j.tmaid.2020.101830

CDC. (2022a). 2022 Monkeypox Outbreak Global Map. https://www.cdc.gov/poxvirus/monkeypox/response/2022/world-map.html Accessed on August 24, 2022.

CDC. (2022b). Monkeypox and smallpox vaccine guidance. https://www.cdc.gov/poxvirus/monkeypox/clinicians/smallpox-vaccine.html#:~:text=Receiving%20Vaccine%20After%20Exposure%20to%20Monkeypox%20Virus,-The%20sooner%20an&text=CDC%20recommends%20that%20the%20vaccine,may%20not%20prevent%20the%20disease. Accessed 3rd August 2022.

CDC. (2022c). Monkeypox in multiple countries. https:// wwwnc.cdc.gov/travel/notices/alert/monkeypox. Accessed 4th August 2022.

CDC. (2022d). Laboratory procedures: Routine chemistry, hematology, and urinalysis in hospitals or clinical laboratories. https://www.cdc.gov/poxvirus/monkeypox/lab-personnel/lab-procedures.html#Clinical%20Pathology,%20Molecular%20Testing,%20and%20Analysis. Accessed 4th August 2022.

CDC. (2022e). Preparation and collection of specimens. https:// www.cdc.gov/poxvirus/monkeypox/clinicians/prep-collection-specimens.html#:~:text=Vigorously%20swab%20or%20brush%20lesion,in%20a%20separate%20sterile%20container. Accessed 4th August 2022.

Chakraborty, C., Bhattacharya, M., Nandi, S.S., Mohapatra, R.K., Dhama, K., & Agoramoorthy, G. (2022a). Appearance and re-appearance of zoonotic disease during the pandemic period: long-term monitoring and analysis of zoonosis is crucial to confirm the animal origin of SARS-CoV-2 and monkeypox virus. *Veterinary Quarterly*,*42*(1), 119-124. doi: 10.1080/01652176.2022.2086718.

Chakraborty, S., Chandran, D., Mohapatra, R. K., Alagawany, M., et al. (2022b). Clinical management, antiviral drugs and immunotherapeutics for treating monkeypox. An update on current knowledge and futuristic prospects. *International journal of surgery (London, England)*, 106847. Advance online publication. https://doi.org/10.1016/j.ijsu.2022.106847

Cheema, A.Y., Ogedegbe, O.J., Munir, M., Alugba, G., & Ojo, T.K. (2022). Monkeypox: A review of clinical features, diagnosis, and treatment. *Cureus*, *14*(7), e26756. doi: 10.7759/cureus.26756.

Cohen, J. (2022). Monkeypox outbreak questions intensify as cases soar. *Science*, *376*(6596), 902-903. doi: 10.1126/science.add1583.

Dhama, K., Karthik, K., Khandia, R., Chakraborty, S., et al. (2018a). Advances in designing and developing vaccines, drugs, and therapies to counter Ebola virus. *Frontiers in Immunology*, *9*, 1803. doi: 10.3389/fimmu.2018.01803.

Dhama, K., Karthik, K., Khandia, R., Munjal, A., et al. (2018b). Medicinal and Therapeutic Potential of Herbs and Plant Metabolites / Extracts Countering Viral Pathogens - Current Knowledge and Future Prospects. *Current Drug Metabolism*. *19*(3):236-263. doi: 10.2174/1389200219666180129145252.

Fahrni, M.L., Priyanka, & Choudhary, O.P. (2022). Possibility of vertical transmission of the human monkeypox virus. *International Journal of Surgery*, *105*, 106832. doi: 10.1016/j.ijsu.2022.106832.

Farahat, R.A., Ali, I., AL-Ahdal, T., Benmelouka, A.Y., et al. (2022). Monkeypox and human transmission: Are we on the verge of another pandemic? *Travel Medicine and Infectious Disease*, *49*, 102387. doi: 10.1016/j.tmaid.2022.102387.

Guarner, J., Del, R.C., & Malani, P.N. (2022). Monkeypox in 2022-What clinicians need to know. *The Journal of the American Medical Association*, *328*(2), 1-2. doi: 10.1001/jama.2022.10802.

Haider, N., Guitian, J., Simons, D., Asogun, D., et al. (2022). Increased outbreaks of monkeypox highlight gaps in actual disease burden in Sub-Saharan Africa and in animal reservoirs. *International Journal of Infectious Diseases*, *122*, 107-111. doi: 10.1016/j.ijid.2022.05.058.

Harris, E. (2022). What to know about monkeypox. *The Journal of the American Medical Association*, *327*(23), 2278-2279. doi: 10.1001/jama.2022.9499.

Heskin, J., Belfield, A., Milne, C., Brown, N., et al. (2022). Transmission of monkeypox virus through sexual contact - A novel route of infection. *The Journal of infection*, *85(3)*, 334–363. https://doi.org/10.1016/j.jinf.2022.05.028.

Hofer, U. (2022). Case series of monkeypox infections. *Nature Reviews Microbiology*, *20*, 445. doi: 10.1038/s41579-022-00757-2.

Kabuga, A.I., & El Zowalaty, M.E. (2019). A review of the monkeypox virus and a recent outbreak of skin rash disease in Nigeria. *Journal of Medical Virology*, *91*(4), 533-540. doi: 10.1002/jmv.25348.

Keckler, M.S., Salzer, J.S., Patel, N., Townsend, M.B., et al. (2020). IMVAMUNE® and ACAM2000® Provide different protection against disease when administered postexposure in an intranasal monkeypox challenge Prairie dog model. *Vaccines (Basel)*, *8*(3), 396. doi: 10.3390/vaccines8030396.

Khalil, A., Samara, A., O'Brien, P., Morris, E., Draycott, T., Lees, C., & Ladhani, S. (2022). Monkeypox and pregnancy: what do obstetricians need to know? *Ultrasound in Obstetrics & Gynecology*, *60*, 22-27. doi: 10.1002/uog.24968.

Kozlov, M. (2022). Monkeypox goes global: why scientists are on alert. *Nature***,** *606***,** *15-16*.Doi: 10.1038/d41586-022-01421-8.

Kumar, S., Subramaniam, G., & Karuppanan, K. (2022). Human monkeypox outbreak in 2022. *Journal of Medical Virology*, 1-7. doi: 10.1002/jmv.27894.

Kumbhar, N., & Agarwala, P. (2022). The lurking threat of monkeypox in current times. *Indian Journal of Medical Microbiology*, S0255-0857(22)00139-6, Advance online publication. https://doi.org/10.1016/j.ijmmb.2022.07.016

Lai, C. C., Hsu, C. K., Yen, M. Y., Lee, P. I., Ko, W. C., & Hsueh, P. R. (2022). Monkeypox: An emerging global threat during the COVID-19 pandemic. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi*, S1684-1182(22)00102-5. Advance online publication. https://doi.org/10.1016/j.jmii.2022.07.004

Maksyutov, R.A., Gavrilova, E.V., & Shchelkunov, S.N. (2016). Species-specific differentiation of variola, monkeypox, and varicella-zoster viruses by multiplex real-time PCR assay. *Journal of Virological Methods*, *236*, 215-220. doi: 10.1016/ j.jviromet.2016.07.024.

Mauldin, M.R., McCollum, A.M., Nakazawa, Y.J., Mandra, A., et al. (2022). Exportation of monkeypox virus from the African continent. *The Journal of infectious diseases*, *225*(8), 1367-1376. doi: 10.1093/infdis/jiaa559.

McCarthy M. W. (2022). Recent advances in the diagnosis monkeypox: implications for public health. *Expert Review of Molecular Diagnostics*, 10.1080/14737159.2022.2116979. Advance online publication. https://doi.org/10.1080/14737159.2022.2116979

Meo, S. A., & Jawaid, S. A. (2022). Human Monkeypox: Fifty-Two Years based analysis and Updates. *Pakistan Journal of Medical Sciences*, *38*(6), 1416–1419. https://doi.org/10.12669/pjms.38.6.6775

Mohapatra, R.K., Tuli, H.S., Sarangi, A.K., Chakraborty, S., Chandran, D., Chakraborty, C., & Dhama, K. (2022). Unexpected sudden rise of human monkeypox cases in multiple non-endemic countries amid COVID-19 pandemic and salient counteracting strategies: Another potential global threat?. *International Journal of Surgery*, *103*, 106705. doi: 10.1016/j.ijsu.2022.106705.

Ogoina, D., Iroezindu, M., & James, H.I. (2020).Clinical course and outcome of human Monkeypox in Nigeria. *Clinical Infectious Diseases*, *71*(8), e210–e214. doi: 10.1093/ cid/ciaa143.

Okyay, R., Bayrak, E., Kaya, E., Sahin, A., Kocyigit, B., & Tasdogan, A. (2022). Another epidemic in the shadow of covid 19 pandemic: A review of monkeypox. *Eurasian Journal of Medicine and Oncology*, *6*(2): 95-99. doi: 10.14744/ejmo.2022.2022.

Ortiz-Martınez, Y., Zambrano-Sanchez, G., & Rodrıguez-Morales, A.J. (2022). Monkeypox and HIV/AIDS: When the outbreak faces the epidemic. *International Journal of STD & AIDS*, 9564624221114191. DOI: 10.1177/09564624221114191.

Parker, S., & Buller, R.M. (2013). A review of experimental and natural infections of animals with monkeypox virus between 1958 and 2012. *Future Virology*, *8*, 129-157. doi: 10.2217/fvl.12.130.

Petersen, E., Kantele, A., Koopmans, M., Asogun, D., Yinka-Ogunleye, A., Ihekweazu, C., & Zumla, A. (2019). Human monkeypox: epidemiologic and clinical characteristics, diagnosis, and prevention. *Infectious Disease Clinics*, *33*(4), 1027-1043. doi: 10.1016/j.idc.2019.03.001.

Pramod, R. K., Nair, A. V., Tambare, P. K., Chauhan, K., et al. (2021). Reverse zoonosis of coronavirus disease-19: Present status and the control by one health approach. *Veterinary world*, *14*(10), 2817–2826. https://doi.org/10.14202/vetworld.2021.2817-2826

Raheel, H., Raheel, M., Ali Fahim, M. A., & Naeem, U. (2022). Monkeypox and spillover effects: Stigmas, solutions and strategies. *Annals of Medicine and Surgery (2012)*, *81*, 104346. https://doi.org/10.1016/j.amsu.2022.104346

Rodríguez-Cuadrado, F.J., Pinto-Pulido, E.L., & Fernández-Parrado, M. (2022). Potential treatments for monkeypox. *Actas Dermosifiliograficas*, S0001-7310(22)00601-9. doi: 10.1016/ j.ad.2022.06.013.

Sah, R., Mohanty, A., Siddiq, A., Singh, P., Abdelaal, A., Alshahrani, N. Z., & Dhama, K. (2022). Monkeypox reported in India - South East Asia Region: Health and economic challenges. *The Lancet regional health. Southeast Asia*, *4*, 100063. https://doi.org/10.1016/j.lansea.2022.100063

Saied, A.A., Priyanka, M.A.A., & Choudhary, O.P. (2022a). Monkeypox: An extra burden on global health. *International Journal of Surgery*, *104*, 106745. doi: 10.1016/j.ijsu.2022.106745.

Saied, A. A., Nascimento, M., do Nascimento Rangel, A. H., Skowron, K., et al. (2022b). Transchromosomic bovines-derived broadly neutralizing antibodies as potent biotherapeutics to counter important emerging viral pathogens with a special focus on SARS-CoV-2, MERS-CoV, Ebola, Zika, HIV-1, and influenza A virus. *Journal of Medical Virology*, *94*(10), 4599–4610. https://doi.org/10.1002/jmv.27907

Seang, S., Burrel, S., Todesco, E., Leducq, V., Monsel, G., Le Pluart, D., Cordevant, C., Pourcher, V., & Palich, R. (2022). Evidence of human-to-dog transmission of monkeypox virus, *The Lancet*, S0140-6736(22)01487-8.doi: 10.1016/ S0140-6736(22)01487-8.

Sharun, K., Tiwari, R., Natesan, S., & Dhama, K. (2021a). SARS-CoV-2 infection in farmed minks, associated zoonotic concerns, and importance of the One Health approach during the ongoing COVID-19 pandemic. *The Veterinary Quarterly*, *41*(1), 50–60. https://doi.org/10.1080/01652176.2020.1867776

Sharun, K., Dhama, K., Pawde, A. M., Gortázar, C., et al. (2021b). SARS-CoV-2 in animals: potential for unknown reservoir hosts and public health implications. *The Veterinary Quarterly*, *41*(1), 181–201. https://doi.org/10.1080/01652176.2021.1921311

Simpson, K., Heymann, D., Brown, C.S., Edmunds, W.J., et al. (2020). Human monkeypox - After 40 years, an unintended consequence of smallpox eradication. *Vaccine*, *38*(33), 5077-5081. doi: 10.1016/j.vaccine.2020.04.062.

Singh, S., Kola, P., Kaur, D., Singla, G., et al. (2021). Therapeutic Potential of Nutraceuticals and Dietary Supplements in the Prevention of Viral Diseases: A Review. *Frontiers in nutrition*, *8*, 679312. https://doi.org/10.3389/fnut.2021.679312

Tiwari, R., Latheef, S. K., Ahmed, I., Iqbal, H., et al. (2018). Herbal Immunomodulators - A Remedial Panacea for Designing and Developing Effective Drugs and Medicines: Current Scenario and Future Prospects. *Current drug metabolism*, *19*(3), 264–301. https://doi.org/10.2174/1389200219666180129125436

World Organisation for Animal Health (2022). Monkeypox. https://www.woah.org/en/disease/monkeypox/. Accessed 3rd August 2022.

WHO. (2022a). Monkeypox. Retrieved from https://www.who.int/news-room/fact-sheets/detail/monkeypox#:~:text=Monkeypox%20is%20transmitted%20to%20humans,contaminated%20materials%20such%20as%20bedding on 3rd August 2022.

WHO. (2022b). Multi-country monkeypox outbreak: situation update. Retrieved from https://www.who.int/emergencies/disease-outbreak-news/item/2022-DON393 on 4th August 2022.

WHO. (2022c). Laboratory testing for the monkeypox virus: Interim guidance 23rd May 2022. Retrieved from https://www.who.int/publications/i/item/WHO-MPX-laboratory-2022.1 on 6th August 2022.

Yang, L., Tian, L., Li, L., Liu, Q., Guo, X., Zhou, Y., Pei, R., Chen, X., & Wang, Y. (2022). Efficient assembly of a large fragment of monkeypox virus genome as a qPCR template using dual-selection based transformation-associated recombination. *Virologica Sinica*, *37*(3), 341-347. doi: 10.3201/eid2608.191387.

Yinka-Ogunleye, A., Aruna, O., Dalhat, M., Ogoina, D., et al. (2019). Outbreak of human monkeypox in Nigeria in 2017-18: a clinical and epidemiological report. *The Lancet Infectious Diseases*, *19*(8), 872–879. doi: 10.1016/S1473-3099(19)30294-4.

Zumla, A., Valdoleiros, S.R., Haider, N., Asogun, D., Ntoumi, F., Petersen, E., & Kock, R. (2022). Monkeypox outbreaks outside endemic regions: scientific and social priorities. *Lancet Infectious Diseases*, *22*(7), 929-931. doi: 10.1016/S1473-3099(22)00354-1.

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Marburg Virus Disease – A Mini-Review

Sandip Chakraborty[1*], Deepak Chandran[2], Ranjan K. Mohapatra[3], Mahmoud Alagawany[4], Mohd Iqbal Yatoo[5], Md. Aminul Islam[6,7], Anil K. Sharma[8], Kuldeep Dhama[9*]

[1]Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, R.K. Nagar, West Tripura, Tripura, Pin-799008, India
[2]Department of Veterinary Sciences and Animal Husbandry, Amrita School of Agricultural Sciences, Amrita VishwaVidyapeetham University, Coimbatore – 642109, Tamil Nadu, India
[3]Department of Chemistry, Government College of Engineering, Keonjhar-758002, Odisha, India
[4]Poultry Department, Faculty of Agriculture, Zagazig University, Zagazig 44511, Egypt
[5]Mycoplasma Laboratory, Division of Veterinary Clinical Complex, Faculty of Veterinary Sciences and Animal Husbandry, Sher-E-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Shuhama, Alusteng, Srinagar 190006, Jammu and Kashmir, India
[6]COVID-19 Diagnostic Lab, Department of Microbiology, Noakhali Science and Technology University, Noakhali-3814, Bangladesh
[7]Advanced Molecular Lab, Department of Microbiology, President Abdul Hamid Medical College, Karimganj, Kishoreganj- 834001, Bangladesh
[8]Department of Biotechnology, Maharishi Markandeshwar University (Deemed to be University) Mullana-Ambala-133207, Haryana, India
[9]Division of Pathology, ICAR-Indian Veterinary Research Institute, Bareilly, Izatnagar, Uttar Pradesh- 243122, India

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ABSTRACT

Marburg virus disease (MVD) is a highly fatal disease caused by the Marburg virus (MARV) which belongs to the family Filoviridae. The disease has been recently reported from Ghana, an African country, and nearly 15 outbreaks of MVD have been reported in the past five decades. Various species of bats viz., *Rousettus aegyptiacus*, *Hipposideros caffer*, and certain Chiroptera act as the natural source of infection. Pathophysiology of the disease reveals severe antiviral suppression due to changes in gene expression and interferon-stimulated gene (ISG) production in the hepatic cells. With the progression of the disease, there may be the development of pain in the abdomen, nausea, vomition, pharyngitis, and diarrhea along with the onset of hemorrhagic manifestations which may lead to the death of a patient. The advent of molecular detection techniques and kits viz., reverse transcription polymerase chain reaction (RT-PCR) kit has greatly aided in the diagnosis of MVD. Identification of the virus in the specimen with great accuracy can be done by whole viral genome sequencing. The use of a combination of MR-186-YTE

* Corresponding author

E-mail: chakrabortysandip22@gmail.com (Sandip Chakraborty);
kdhama@rediffmail.com (Kuldeep Dhama)

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(monoclonal antibody) and an antiviral drug named remdesivir in the NHP model is greatly effective for eliminating MARV. The protective effect of a Vesicular stomatitis virus (VSV) (recombinant) - based vaccine expressing the glycoprotein of MARV has been revealed through animal model studies, other vaccines are also being developed. Proper health education, personal hygiene and precautions by health care workers while handling patients, good laboratory facilities and service along with the establishment of enhanced surveillance systems are the need of the hour to tackle this highly fatal disease. This article presents an overview of different aspects and salient features of MARV / MVD, and prevention and control strategies to be adopted.

1 Introduction

Marburg virus (MARV), a member of the family Filoviridae that also contain the Ebola virus, causes Marburg virus disease (MVD) which is zoonotic in nature as well as a very fatal disease with up to 88 percent case fatality rate (Singh et al. 2017; Asad et al. 2020; WHO 2021; Zhao et al. 2022). There are five distinct lineages of MARV as is revealed by phylogenetic analysis of the data regarding genomic sequences. There has been a reclassification of these lineages into two viruses separately viz., MARV and Ravn virus (RAVV). Initially, the disease was recognized in Germany and Serbia after outbreaks occurred simultaneously in the year 1967. In these cases, the outbreak occurred due to the handling of tissues obtained from African green monkeys (*Chlorocebus aethiops*) imported from Uganda by scientists and technicians in the laboratory who were conducting experiments for producing polio vaccines (Ristanović et al. 2020). There are previous reports of an outbreak of MVD from African countries viz., Democratic Republic of Congo, Angola, Kenya, Uganda, South Africa, Guinea, and also from the USA, Netherlands, Yugoslavia, and Russia. The most recent outbreak has been reported from Ghana in the current year 2022 (July month) (Koundouno et al. 2022; Okonji et al. 2022; Sah et al. 2022; WHO 2022; Hussain 2022; Zhao et al. 2022). MARV being highly contagious can spread very rapidly and is responsible for causing high mortality (Abir et al. 2022).

It is to be noted that most of the outbreaks of MVD have occurred in Africa. MDV is seldom treatable in non-human primates (NHPs) and humans. The disease results in hemorrhagic fever and dysfunctions of organs viz., hepatic failure; infection of brain; spleen, and tissues of the renal system along with problems concerning coagulation (Mehedi et al. 2011; van Paassen et al. 2012). There may be a presence of fresh blood in the vomitus and feces along with frequent bleeding through the nostrils, gums, and vagina. There is an increased risk of venepuncture providing fluids or obtaining blood samples from the patients infected as there may be spontaneous bleeding at the venepuncture site. The involvement of the central nervous system results in confusion, irritability, and aggression in the behavior (WHO 2021). Since widespread MARV outbreaks are unusual, clinical studies may not always generate enough data to adequately evaluate the treatment options for MVD. In light of this, a thorough in-depth disease investigations and analysis may facilitate future medical research and help to improve the therapeutic management of MVD. In this mini-review, we reviewed and collated key data on MARV and the disease it causes (MVD), and counteracting management approaches to tackle this highly virulent virus.

2 Etiology

MARV is an enveloped virus belonging to the Filoviridae family and is having a negative sense, non-segmented RNA genome that is single-stranded. The virus is having a single species viz., *Marburg marburgvirus* which comprises two strains i.e. MARV and Ravn virus (RAVV). The strains are divergent from one another by 20 percent at the amino acid level (Towner et al. 2006; Kortepeter et al. 2020). The nucleocapsid core of MARV is composed of viral protein (VP) 30; VP35; nucleoprotein (NP); and the L-polymerase protein. VP40 and VP24 constitute the viral matrix protein. The viral proteins are transcribed from monocistronic RNA (Martin et al. 2016; Gordon et al. 2019). Morphologically, MARV is filamentous. There are certain variants of MARV like Musoke; Angola and Ci67 out of which the variant Angola is the most pathogenic one (Kortepeter et al. 2020).

3 Transmission

MVD is a zoonotic disease, and the major infection source (natural) of MARV is the *Rousettus aegyptiacus* species of fruit bat. Apart from this, *Hipposideros caffer* along with certain Chiroptera can also act as an infection source. The transmission of strains of MARV from bat to bat can occur in various ways. In a recent study, detection of viral shedding in oral and rectal samples along with urine of bats inoculated with MARV has been reported, and interestingly it has been found that there is the presence of MARV in the blood and oral samples of bats in contact. Thus this study proves the horizontal transmission of the virus from bats infected to in-contact bats (Schuh et al. 2017). Intermediate hosts like NHPs and animals killed for obtaining bushmeat may also act as vectors (primary) of transmission of the virus. It is however possible that the infection may probably be transmitted to humans and NHPs from the secretions (viz., saliva) and excretions (feces

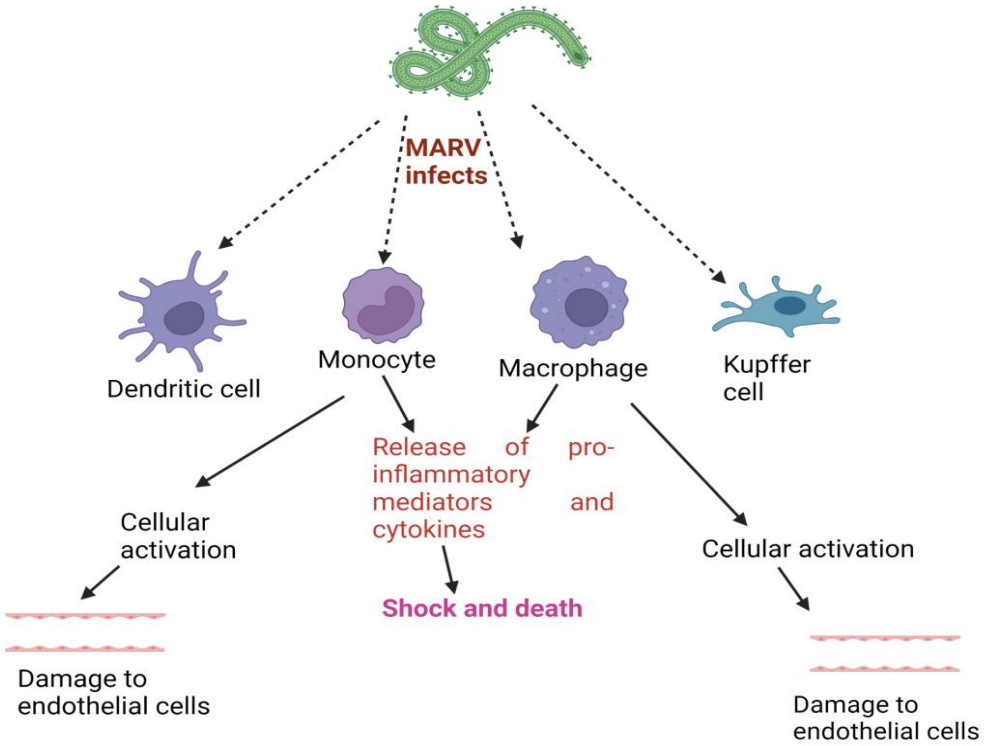

Figure 1 Pathophysiology of Marburg virus (MARV) infection.

and urine) of bats and also from fruits contaminated with MARV (Schuh et al. 2017; Kortepeter et al. 2020; Amman et al. 2021). There may be spread of MARV to humans at the early stages of infection through intermediate animals that are infected. Sexual intercourse may also transmit MVD as already the antigens of MARV have been found in the semen of males infected. Direct contact with blood along with other body fluids viz., urine; feces; tears; breast milk, etc can also facilitate human-to-human transmission. The chances of transmission increase due to the provision of services related to healthcare to the patients infected by MARV, health care workers can be infected, and the handling of corpses of humans inappropriately (Bausch et al. 2003; Kortepeter et al. 2020).

4 Marburg virus disease

The incubation period of the disease varies from 2 days to 3 weeks. Non-specific symptoms like the high rise of temperature; myalgia or arthralgia; malaise and headache appear quickly. There may be an appearance of a maculopapular rash located centrally around the fifth day of MARV infection. As there is a progression of the disease there may be the development of pain in the abdomen; nausea; vomition; pharyngitis and diarrhea. Hemorrhagic manifestations like petechiae and bleeding from mucosa at multiple locations can occur (Miraglia 2019). Severe hemorrhages that prove to be fatal mainly occur in the gastrointestinal tract. It is important to note that during the disease, hemorrhage may not be exhibited in certain patients. Ultimately the patient may die within a few days from the period of onset of clinical signs in fatal cases (Kortepeter et al. 2011; Miraglia 2019). There is a penetration of MARV in the body via broken skin thereby damaging various types of cells and organs. Ultimately there is a development of hemorrhagic fever (Abir et al. 2022). An overview of the pathophysiology of MARV infection is depicted in Figure 1.

5 Diagnosis

MVD should be differentiated from malaria; septicaemia caused by gram-negative organisms; plague; leptospirosis and typhoid (Miraglia 2019). Enzyme-linked immunosorbent assay (ELISA), serum neutralization tests, electron microscopy, virus isolation, and nucleic acid detection methods such as reverse transcriptase polymerase chain reactions (RT-PCR) is used for the diagnosis of MVD (Park et al. 2016; Racsa et al. 2016; Zhao et al. 2022). However, in many areas where MVD outbreaks are most likely to occur, these diagnostic techniques are not readily available (Olejnik et al. 2019; Yu et al., 2022). Biosafety level-4 (BSL-4) facilities are required for the diagnosis and research on MARV. A higher degree of sensitivity, as well as specificity, is obtained by nucleic acid detection methods and with such detection facilities testing can be carried out quickly at the outbreak site (Racsa et al. 2016). The creation of a TaqMan Array card has been done to

screen for outbreaks of acute febrile illness and to use for surveillance purposes. The virus in the specimen can be more accurately identified by sequencing of the full viral genome. Comparison of the full genome of the virus in the specimen with the genome sequences of MARV stored in a database can be done by a microarray assay that uses MARV-specific probes (Miraglia 2019).

6 Treatment and vaccines

No antiviral therapy or vaccine has been approved yet for MVD but supportive care can be given. This includes balancing the levels of fluid and electrolyte; maintaining oxygen level and blood pressure; replacement of blood and clotting factors that are lost due to infection. Attempts have been made to develop efficacious therapies (post-exposure) which include antiviral drugs, antibodies, monoclonal antibodies (mAbs), antiviral small molecules, viral inhibitors-based therapies such as MR-186-YTE (mAb), synthetic anti-VP35 antibody, remdesivir, broad-spectrum nucleoside analogue BCX4430, an inhibitory molecule known as FC-10696a, as well as AVI-7288, host-targeted therapeutics and post-exposure vaccine, recombinant vesicular stomatitis virus vectors (Daddario-DiCaprio et al. 2006; Warren et al. 2014; Cross et al. 2018; Amatya et al., 2019; Cross et al. 2021; Bradfute, 2022; Hickman et al. 2022; Kumari et al. 2022; Zhao et al. 2022). It should be emphasized that if the disease is severe or has progressed to an advanced stage, combined therapy utilizing two direct-acting antiviral (DAA) medications may prove effective, such as remdesivir and a MARV-specific mAb candidate (MR186-YTE) combination therapy was found to more effective (Abir et al. 2022; Hickman et al. 2022; Abir et al. 2022). Besides these, efforts are needed to evaluate the efficacy of herbs, plant metabolites, phytochemicals, immune-boosting nutritional foods, and nutraceuticals as well as novel chemical ligands, antiviral drugs, and broadly neutralizing antibodies, that could be used against MARV / MVD as have been found useful for other important emerging and re-emerging viral pathogens such as SARS-CoV-2, filoviruses including Ebola virus, Zika virus and others (Fu et al. 2016; Dhama et al. 2018a; Dhama et al. 2018b; Tiwari et al. 2018; Zhang et al. 2018; Anand et al. 2021; Calder 2022; Saied et al. 2022).

Investigations have been carried out regarding the potential use of various vaccines against MARV infection. For instance, testing in animal models has revealed the protective effect of a Vesicular stomatitis virus (VSV) (recombinant) based vaccine that expresses the glycoprotein (GP) of MARV (rVSVΔG-MARV-GP) (Marzi et al. 2021). MVA-BN-Filo is another vaccine candidate that contains the antigens of both Ebola and Marburg virus, found to have a potential protective effect against both viruses (Anywaine et al. 2022). Progress has been made in the development of vaccines based on recombinant glycoproteins of filoviruses viz., Ebola virus (EBOV) and Sudan virus (SUDV) along with MARV, for designing monovalent MARV vaccine, monovalent SUDV vaccine, and bivalent formulations. Such a subunit (recombinant) vaccine platform can thereby help in developing multivalent vaccine candidates for protecting against filoviruses while retaining their safety and efficacy (Lehrer et al. 2021). Few other vaccines being tried include multi-epitope vaccine, proteome-based vaccine exploiting computational methods, virus-like particles (VLP), adenoviral vector-based multi-filovirus vaccine, r-protein based filovirus multivalent vaccine, and rVSV-based vesiculovax vector vaccine (rVSV-N4CT1-MARV-GP), and efforts are being made for effective platform designing of preventive MVD vaccines (Reynolds and Marzi 2017; Hasan et al. 2019; Suschak and Schmaljohn 2019; Dulin et al. 2021; Lehrer et al. 2021; Sami et al. 2021; Longini et al. 2022; Sebastian et al. 2020; Soltan et al. 2022; Woolsey et al. 2022; Zhao et al. 2022). Another candidate vaccine (recombinant VSV/ rVSV-based) known as PHV01 has been tested in the guinea pig model for confirming the efficacy (protective effects) against MVD (Zhu et al. 2022).

7 Prevention and control

Effective epidemic control requires many interrelated factors to come together, including case management, strengthening surveillance and tracking, contact tracing, as well as well equipped laboratory facilities, safe and dignified funerals, community mobilization, education, and awareness enhancing of public on the risk factors for catching MARV infection and precautionary measures to be adopted along with adhering to strict standards of hygiene and cleanliness (Aborode et al. 2022; Abir et al. 2022). To avoid MVD it is recommended to avoid contact with NHPs as well as fruit bats, especially in the African setting and outbreaks region. There is a necessity to set up an advanced system of surveillance for interrupting the transmission chain of MARV as a part of a key control strategy by limiting the animal-human interface, following early diagnosis and immediate implementation of mitigation strategies and proactive prevention and control measures. Humans should avoid being too much closeness to fruit bats in mines and caves, and follow personal safety precautions, to lessen the risk of disease transmission. During epidemics, it is crucial that all animal products, including blood and meat, be cooked properly (Baby et al. 2022). Utmost care is required to prevent transmission from human to human by avoiding contact (direct/ close) with people having the symptoms of MVD, especially the contact with body fluid of infected individuals should be avoided. At the hospital, the health care personnel must take care of the ill patients by wearing gloves and personal protective equipment appropriately. After a visit is made to the health care facilities to visit patients, the hands should be washed and sanitized immediately. The use of condoms

is essential for both male and female to reduce any probable chances of possible MARV transmission through sexual contact. The WHO recommends that male survivors of MVD practice safer sex and hygiene for a year or until their semen twice tests negative for the virus (Mehedi et al. 2011; WHO 2021; Wirsiy et al. 2021; WHO 2022). Of note, seeing the rising cases of monkeypox in several countries, now declared a public health emergency of international concern, due attention also needs to be given to MVD which is also presently considered a neglected / rare disease, and high efforts are required to strengthen research, enhance surveillance and develop effective vaccines and therapeutics to counter MVD (Abir et al. 2022; Chakraborty et al. 2022; Sah et al. 2022).

Conclusion & Future perspectives

It is possible to improve the care of patients and reduce death rates, by having a better knowledge of the clinical course as well as the pathology of MVD. Progress has been shown in the field of laboratory diagnosis of the disease and the precision of various diagnostic tests to detect the disease has increased over time. Evaluation of therapeutic compounds as well as vaccines having various mechanisms of action and versatile composition is on the way to reaching any conclusive treatment options and developing vaccines to tackle this deadly virus. Treatment, as well as prevention of MVD, is possible to some extent with some of these compounds and vaccines. However vivid studies are essential to know the exact mechanisms involved in the development of MARV infection (pathogenesis) upon exposure to reservoir animals. Along with this sound knowledge of the mechanism involved in the development of asymptomatic infection is also essential. Trials (clinical) should be carried out in more numbers as far as treatment and vaccination are concerned to earn the approval of the Food and Drug Administration (FDA). High global efforts are required by epidemiologists, diagnosticians, researchers, medicos, veterinarians, health care experts, and health agencies in a coordinated manner so that mankind can get prepared to tackle MVD more efficiently and avoid any probable dangers of global health concerns.

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References

Abir, M. H., Rahman, T., Das, A., Etu, S. N., et al. (2022). Pathogenicity and virulence of Marburg virus. *Virulence*, *13*(1), 609-633. DOI:10.1080/21505594.2022.2054760

Aborode, A. T., Wireko, A. A., Bel-Nono, K. N., Quarshie, L. S., Allison, M., & Bello, M. A. (2022). Marburg virus amidst COVID-19 pandemic in Guinea: Fighting within the looming cases. *The International Journal of Health Planning and Management*, *37*(1), 553-555. DOI: 10.1002/hpm.3332

Amatya, P., Wagner, N., Chen, G., Luthra, P., et al. (2019). Inhibition of Marburg virus RNA synthesis by a synthetic anti-VP35 antibody. *ACS Infectious Diseases*, *5*(8):1385-1396. doi: 10.1021/acsinfecdis.9b00091.

Amman, B. R., Schuh, A. J., Albariño, C. G., & Towner, J. S. (2021). Marburg virus persistence on fruit as a plausible route of bat to primate filovirus transmission. *Viruses*, *13*(12), 2394. DOI: 10.3390/v13122394

Anand, A.V., Balamuralikrishnan, B., Kaviya, M., Bharathi, K., et al. (2021). Medicinal plants, phytochemicals, and herbs to combat viral pathogens including SARS-CoV-2. *Molecules*. *26*(6):1775. doi: 10.3390/molecules26061775.

Anywaine, Z., Barry, H., Anzala, O., Mutua, G., et al. (2022). Safety and immunogenicity of 2-dose heterologous Ad26.ZEBOV, MVA-BN-Filo Ebola vaccination in children and adolescents in Africa: A randomised, placebo-controlled, multicentre Phase II clinical trial. *PLOS Medicine*, *19*(1), e1003865. DOI: 10.1371/journal.pmed.1003865

Asad, A., Aamir, A., Qureshi, N. E., Bhimani, S., et al. (2020). Past and current advances in Marburg virus disease: a review. *Infez Med*, *28*(3):332-345.

Baby, B., Rajalakshmi R., Nair, M. M., & Roshni, P. R. (2022). Sagacious perceptive on Marburg virus foregrounding the recent findings: A critical review. *Infectious Disorders Drug Targets*. DOI: 10.2174/1871526522666220510103618.

Bausch, D. G., Borchert, M., Grein, T., Roth, C., et al. (2003). Risk factors for Marburg hemorrhagic fever, Democratic Republic of the Congo. *Emerging Infectious Diseases*, *9*(12), 1531-1537. DOI: 10.3201/eid0912.030355

Bradfute, S. B. (2022). The discovery and development of novel treatment strategies for filoviruses. *Expert Opinion on Drug Discovery*, *17*(2):139-149. doi: 10.1080/17460441.2022.2013800.

Calder, P. C. (2022). Foods to deliver immune-supporting nutrients. *Current Opinion in Food Science*, *43*, 136-145. doi: 10.1016/j.cofs.2021.12.006.

Chakraborty, S., Chandran, D., Mohapatra, R. K., Alagawany, M., et al. (2022). Clinical management, antiviral drugs and immunotherapeutics for treating monkeypox. An update on current knowledge and futuristic prospects. *International Journal of Surgery*, *106847*. doi: 10.1016/j.ijsu.2022.106847.

Cross, R. W., Bornholdt, Z. A., Prasad, A. N., Borisevich, V., et al. (2021). Combination therapy protects macaques against advanced Marburg virus disease. *Nature Communications*, *12*(1), 1891. DOI: 10.1038/s41467-021-22132-0

Cross, R. W., Mire, C. E., Feldmann, H., & Geisbert, T. W. (2018). Post-exposure treatments for Ebola and Marburg virus infections. *Nature Reviews Drug Discovery*, *17*(6), 413-434. DOI: 10.1038/nrd.2017.251

Daddario-DiCaprio, K. M., Geisbert, T. W., Ströher, U., Geisbert, J. B., et al. (2006). Postexposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment. *The Lancet*, *367*(9520), 1399-1404. DOI: 10.1016/S0140-6736(06)68546-2

Dhama, K., Karthik, K., Khandia, R., Munjal, A., et al. (2018b). Medicinal and Therapeutic Potential of Herbs and Plant Metabolites / Extracts Countering Viral Pathogens - Current Knowledge and Future Prospects. *Current Drug Metabolism*, *19*(3), 236-263. doi: 10.2174/1389200219666180129145252.

Dhama, K., Karthik, K., Khandia, R., Chakraborty, S., et al. (2018a). Advances in designing and developing vaccines, drugs, and therapies to counter *Ebola* virus. *Frontiers in Immunology*, *9*, 1803. doi: 10.3389/fimmu.2018.01803.

Dulin, N., Spanier, A., Merino, K., Hutter, J. N. , Waterman, P. E., Lee, C., & Hamer, M. J. (2021). Systematic review of Marburg virus vaccine nonhuman primate studies and human clinical trials. *Vaccine*, *39*(2), 202-208. doi: 10.1016/j.vaccine.2020.11.042.

Fu, X., Wang, Z., Li, L., Dong, S., et al. (2016). Novel chemical ligands to Ebola virus and Marburg virus nucleoproteins identified by combining affinity mass spectrometry and metabolomics approaches. *Scientific Reports*, *6*, 29680. doi: 10.1038/srep29680.

Gordon, T. B., Hayward, J. A., Marsh, G. A., Baker, M. L., & Tachedjian, G. (2019). Host and viral proteins modulating Ebola and Marburg virus egress. *Viruses*, *11*(1), 25. DOI: 10.3390/v11010025

Hasan, M., Azim, K. F., Begum, A., Khan, N.A., et al. (2019). Vaccinomics strategy for developing a unique multi-epitope monovalent vaccine against Marburg marburgvirus. *Infection, Genetics and Evolution*, *70*, 140-157. doi: 10.1016/j.meegid.2019.03.003.

Hickman, M. R., Saunders, D. L., Bigger, C. A., Kane, C. D., & Iversen, P. L. (2022). The development of broad-spectrum antiviral medical countermeasures to treat viral hemorrhagic fevers caused by natural or weaponized virus infections. *PLoS Neglected Tropical Diseases*, *16*(3), e0010220. DOI: 10.1371/journal.pntd.0010220.

Hussain, Z. (2022). Ghana declares its first outbreak of Marburg virus disease after two deaths. *British Medical Journal* , *378*, o1797. doi: 10.1136/bmj.o1797.

Kortepeter, M. G., Bausch, D. G., & Bray, M. (2011). Basic clinical and laboratory features of filoviral hemorrhagic fever. *The Journal of Infectious Diseases*, *204*(3), S810-S816. DOI: 10.1093/infdis/jir299

Kortepeter, M. G., Dierberg, K., Shenoy, E. S., Cieslak, T. J., & Medical Countermeasures Working Group of the National Ebola Training and Education Center's (NETEC) Special Pathogens Research Network (SPRN). (2020). Marburg virus disease: A summary for clinicians. *International Journal of Infectious Diseases*, *99*, 233-242. DOI: 10.1016/j.ijid.2020.07.042

Koundouno, F.R., Kafetzopoulou, L.E., Faye, M., Renevey, A., et al. (2022). Detection of Marburg virus disease in Guinea. *The New England Journal of Medicine*, *386*(26), 2528-2530. DOI: 10.1056/NEJMc2120183

Kumari, M., & Subbarao, N. (2022). A hybrid resampling algorithms SMOTE and ENN based deep learning models for identification of Marburg virus inhibitors. *Future Medicinal Chemistry*, *14*(10):701-715. doi: 10.4155/fmc-2021-0290.

Lehrer, A. T., Chuang, E., Namekar, M., Williams, C. A., et al. (2021). Recombinant protein filovirus vaccines protect cynomolgus macaques from Ebola, Sudan, and Marburg viruses. *Frontiers in Immunology*, *12*, 703986. DOI: 10.3389/fimmu.2021.703986

Longini, I. M., Yang, Y., Fleming, T.R., Muñoz-Fontela, C., et al. (2022). A platform trial design for preventive vaccines against Marburg virus and other emerging infectious disease threats.

Clinical Trials, *22*, 17407745221110880. doi: 10.1177/17407745221110880.

Martin, B., Hoenen, T., Canard, B., & Decroly, E. (2016). Filovirus proteins for antiviral drug discovery: A structure/function analysis of surface glycoproteins and virus entry. *Antiviral Research*, *135*, 1-14. DOI: 10.1016/j.antiviral.2016.09.001

Marzi, A., Jankeel, A., Menicucci, A. R., Callison, J., et al. (2021). Single dose of a VSV-based vaccine rapidly protects macaques from Marburg virus disease. *Frontiers in Immunology*, *12*, 774026. DOI: 10.3389/fimmu.2021.774026

Mehedi, M., Groseth, A., Feldmann, H., & Ebihara, H. (2011). Clinical aspects of Marburg hemorrhagic fever. *Future Virology*, *6*(9), 1091-1106. DOI: 10.2217/fvl.11.79

Miraglia, C. M. (2019). Marburgviruses: An update. *Laboratory Medicine*, *50*(1), 16-28. DOI: 10.1093/labmed/lmy046

Okonji, O. C., Okonji, E. F., Mohanan, P., Babar, M. S., et al. (2022). Marburg virus disease outbreak amidst COVID-19 in the Republic of Guinea: A point of contention for the fragile health system? (2022). *Clinical Epidemiology and Global Health*, *13*, 100920. DOI: 10.1016/j.cegh.2021.100920

Olejnik, J., Mühlberger, E., & Hume, A. J. (2019). Recent advances in Marburg virus research, *F1000Research*, *8*, 704. DOI: 10.12688/f1000research.17573.1

Park, S. W., Lee, Y. J., Lee, W. J., Jee, Y., & Choi, W. (2016). One-Step Reverse Transcription-Polymerase Chain Reaction for Ebola and Marburg Viruses. *Osong Public Health and Research Perspectives*, *7*(3), 205-209. DOI: 10.1016/j.phrp.2016.04.004

Racsa, L. D., Kraft, C. S., Olinger, G. G., & Hensley, L. E. (2016). Viral Hemorrhagic Fever Diagnostics. *Clinical Infectious Diseases*, *62*(2), 214-219. DOI: 10.1093/cid/civ792

Reynolds, P., & Marzi, A. (2017). Ebola and Marburg virus vaccines. *Virus genes*, *53*(4), 501–515. https://doi.org/10.1007/s11262-017-1455-x

Ristanović, E. S., Kokoškov, N. S., Crozier, I., Kuhn, J. H., & Gligić, A. S. (2020). A forgotten episode of Marburg Virus disease: Belgrade, Yugoslavia, 1967. *Microbiology and Molecular Biology Reviews*, *84*(2), e00095-19. DOI: 10.1128/MMBR.00095-19

Sah, R., Mohanty, A., Reda, A., Siddiq, A., Mohapatra, R. K., & Dhama, K. (2022). Marburg virus re-emerged in 2022: Recently detected in Ghana, another zoonotic pathogen coming up amid rising cases of monkeypox and ongoing COVID-19 pandemic-Global health concerns and counteracting measures. *Veterinary Quarterly*, *22*:1-9. doi: 10.1080/01652176.2022.2116501.

Saied, A. A., Nascimento, M., do Nascimento Rangel, A. H., Skowron, K., et al. (2022). Transchromosomic bovines-derived broadly neutralizing antibodies as potent biotherapeutics to counter important emerging viral pathogens with a special focus on SARS-CoV-2, MERS-CoV, Ebola, Zika, HIV-1, and influenza A virus. *Journal of medical virology*, *94*(10), 4599–4610. https://doi.org/10.1002/jmv.27907

Sami, S. A., Marma, K. K. S., Mahmud, S., Khan, M. A. N., et al. (2021). Designing of a multi-epitope vaccine against the structural proteins of Marburg virus exploiting the immunoinformatics approach. *ACS Omega*, *6*(47), 32043-32071. doi: 10.1021/acsomega.1c04817.

Schuh, A. J., Amman, B. R., Jones, M. E., Sealy, T. K., et al. (2017). Modelling filovirus maintenance in nature by experimental transmission of Marburg virus between Egyptian rousette bats. *Nature Communications*, *8*, 14446. DOI: 10.1038/ncomms14446.

Sebastian, S., Flaxman, A., Cha, K. M., Ulaszewska, M., et al. (2020). A Multi-Filovirus Vaccine Candidate: Co-Expression of Ebola, Sudan, and Marburg Antigens in a Single Vector. *Vaccines* (Basel), *8*(2), 241. doi: 10.3390/vaccines8020241.

Singh, R. K., Dhama, K., Malik, Y.S., Ramakrishnan, M. A., et al. (2017). Ebola virus - epidemiology, diagnosis, and control: threat to humans, lessons learnt, and preparedness plans - an update on its 40 year's journey. *Veterinary Quarterly*, *37*(1), 98-135. doi: 10.1080/01652176.2017.1309474.

Soltan, M. A., Abdulsahib, W. K., Amer, M., Refaat, A. M., et al. (2022). Mining of Marburg virus proteome for designing an epitope-based vaccine. *Frontiers in Immumnology*, *13*, 907481. doi: 10.3389/fimmu.2022.907481.

Suschak, J. J., & Schmaljohn, C. S. (2019). Vaccines against Ebola virus and Marburg virus: recent advances and promising candidates. *Human Vaccines & Immunotherapeutics*, *15*(10), 2359-2377. doi: 10.1080/21645515.2019.1651140.

Tiwari, R., Latheef, S. K., Ahmed, I., Iqbal, H., et al. (2018). Herbal Immunomodulators - A Remedial Panacea for Designing and Developing Effective Drugs and Medicines: Current Scenario and Future Prospects. *Current drug metabolism*, *19*(3), 264–301. https://doi.org/10.2174/1389200219666180129125436

Towner, J. S., Khristova, M. L., Sealy, T. K., Vincent, M. J., et al. (2006). Marburgvirus genomics and association with a large hemorrhagic fever outbreak in Angola. *Journal of Virology*, *80*(13), 6497-6516. DOI: 10.1128/JVI.00069-06

van Paassen, J., Bauer, M. P., Arbous, M. S., Visser, L. G., et al. (2012). Acute liver failure, multiorgan failure, cerebral oedema,

and activation of proangiogenic and antiangiogenic factors in a case of Marburg haemorrhagic fever. *The Lancet Infectious Diseases*, *12*(8), 635-642. DOI: 10.1016/S1473-3099(12)70018-X

Warren, T. K., Wells, J., Panchal, R. G., Stuthman, K. S., et al. (2014). Protection against filovirus diseases by a novel broad-spectrum nucleoside analogue BCX4430. *Nature*, *508*(7496), 402-405. DOI: 10.1038/nature13027

WHO. (2021). Marburg virus disease. Retrieved from https://www.who.int/news-room/fact-sheets/detail/marburg-virus-disease.

WHO. (2022). Marburg virus- Ghana, 22 July, 2022. Retrieved from https://www.who.int/emergencies/disease-outbreak-news/item/2022-DON402.

Wirsiy, F. S., Ako-Arrey, D. E., Nkfusai, C. N., Yeika, E. V., & Bain, L. E. (2021). Marburg virus disease outbreak in Guinea: a SPIN framework of its transmission and control measures for an exemplary response pattern in West Africa. *The Pan African Medical Journal*, *40*, 143. DOI: 10.11604/pamj.2021.40.143.31709

Woolsey, C., Cross, R. W., Agans, K. N., Borisevich, V., et al. (2022). A highly attenuated Vesiculovax vaccine rapidly protects nonhuman primates against lethal Marburg virus challenge. *PLoS Neglected Tropical Diseases*. *16*(5), e0010433. doi: 10.1371/journal.pntd.0010433

Yu, Z., Wu, H., Huang, Q., & Zhong, Z. (2021). Simultaneous detection of Marburg virus and Ebola virus with TaqMan-based multiplex real-time PCR method. *Journal of Clinical and Laboratory Analysis*, *35*(6), e23786. DOI: 10.1002/jcla.23786.

Zhang, X., Liu, Q., Zhang, N., Li, Q. Q., et al. (2018). Discovery and evolution of aloperine derivatives as novel anti-filovirus agents through targeting entry stage. *European Journal of Medicinal Chemistry*, *149*, 45–55. https://doi.org/10.1016/j.ejmech.2018.02.061

Zhao, F., He, Y., & Lu, H. (2022). Marburg virus disease: A deadly rare virus is coming. *Bioscience Trends*, 10.5582/bst.2022.01333. Advance online publication. https://doi.org/10.5582/bst.2022.01333.

Zhu, W., Liu, G., Cao, W., He, S., et al. (2022). A cloned recombinant vesicular stomatitis virus-vectored Marburg Vaccine, PHV01, protects guinea pigs from lethal Marburg virus disease. *Vaccines (Basel)*, *10*(7), 1004. DOI: 10.3390/vaccines10071004

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Improvement of crop and soil management practices through mulching for enhancement of soil fertility and environmental sustainability: A review

Mythili Ravichandran[1], Sumathi C Samiappan[2], Rajesh Pandiyan[3], Rajesh Kannan Velu[4]

[1]Department of Microbiology, Vivekanandha Arts and Science College for Women, Sankagiri, Salem, Tamil Nadu-637303, India.
[2]Srinivasa Ramanujan Centre, SASTRA Deemed University, Kumbakonam, Tamil Nadu, India.
[3]Bharath Institute of Higher Education and Research, Bharath University (Deemed to be University), Selaiyur, Chennai. Tamil Nadu, India.
[4]Rhizosphere Biology Laboratory, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.

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ABSTRACT

The logarithmic phase of the human population creates high food demand near the future throughout the world. On the flip side, improved crop production requires uninterrupted water irrigation. Therefore, sensible agricultural inputs are needed to overcome these concerns. New technology-based innovative agronomic research steps will boost the contemporary agriculture practices in developed and developing countries. Agricultural cropping systems could follow mulching practices as one of the best crop management practices for its water and nutrient management potential. It is primarily to accomplish healthy economic and environmental bonds. By covering the soil's surface with biodegradable resources such as organic and inorganic materials, mulching improves the physicochemical characteristics of the soil. This approach provides a favorable environment for the development of plant growth and fosters the activities of microbial communities. Additionally, it reduces the growth of weeds, manages erosion, gets rid of pesticide residue, and increases soil fertility. Mulching the soil surface has profound benefits in improving the soil moisture levels due to a reduced evaporation rate. This method is a practical agronomic entrance to reduce water scarcity and raise the chance of water conservation, notably in arid and semiarid regions. It can also boost crop security and production to meet the global food requirements. This review significantly focuses on the current influence and advantages of organic mulches for crop establishment in the agriculture sector, which can close the production gap between achievable and actual yield.

* Corresponding author

E-mail: sumathisamiappan@gmail.com (Sumathi C Samiappan)

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1 Introduction

India is one of the agriculture-based countries that is grounded it's economy and environmental effect. It has a rich biome through a dryland farming-based food production system (Schimel 2010; Ghimire et al. 2017). In productive agro-management techniques, water is one of the governing limiting factors among various known factors. Furthermore, it represents a captain's role in active plant growth and productivity (Iqbal et al. 2019; Silva et al. 2019). It is the most desirable candidate to determine the essence of agriculture (Hanjra and Qureshi 2010).

Our current agriculture system has continuously faced limited water resources due to global warming and low irregular rainfall patterns, drastic changes in seasonal and climatic conditions, water limitation, and drought. These are the imperative factors that affect and maintain soil and aid in crop health and development (Kader et al. 2019). To cope with this dilemma, more resourceful and well-organized water usage in agriculture should be the top priority in Indian agronomy particularly, water deficit in drylands (arid and semi-arid regions), seasonally dry tropical forest (SDTF) areas, and rainfed agricultural lands (Barajas-Guzman et al. 2006). To combat this scourge, water conservation needs primary concern to promote crop production with drought management systems and sustain agricultural production (Barron et al. 2003; Zhu et al. 2015). In recent years, the reduced groundwater level from 0.5 m to 1.0 m has brought about truncated rainfall (Ranjan et al. 2017). However, water reaches the deeper soil horizon above 15% under mulched conditions (Zhang et al. 2007). A systematic water conservation practice is necessary to conserve natural resources, food development, food security, and solid farming practices. Recently, modern agricultural practices depend on low-cost inputs. Systemic removal of crop residues reduces the biomass in croplands. Consequently, soil fertility and soil organic carbon (SOC) exhausts. Mulching, mowing, and composting are economically valid soil inputs that could be an alternative to the management of agroecosystems (Uhlirova et al. 2005).

Various mulching innovations are currently being added to agricultural practices to boost crop production. But, it is still in the early stages of development. Mulching breakdowns severely limit the soil ecosystem's long-term vitality, which is hampered by many obstacles. Very little research information is available about the biochemical and molecular mechanisms and interactions of soil microbiota with mulch that need further examination. There exists inadequate exploration of soil physiochemical parameters before and after mulch treatments on the surface. Therefore, more research is required to enhance the sustainability and viability of the agriculture systems through various types of mulching. Continuous and dynamic efforts are required, to accomplish feasible agroelements of future green agriculture sustainability. It will be fascinating to explore attainable potentials for agrotherapeutics (Figure 1).

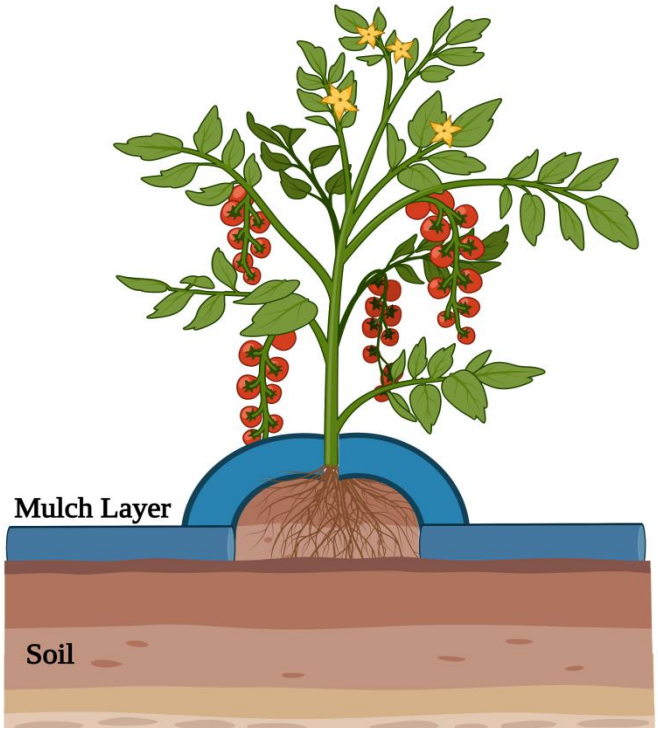

Figure 1 Diagrammatic representation of mulching for improved crop production

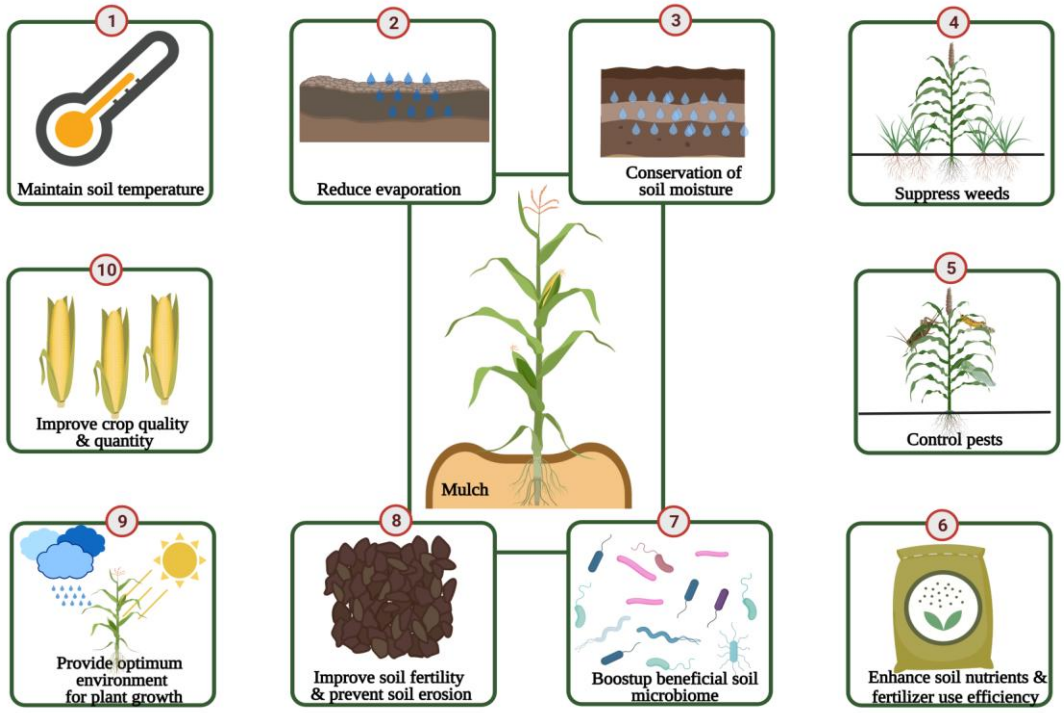

Figure 2 Beneficial spectrum of mulching in agriculture

2 Mulching for Contemporary Agriculture

In modern agriculture practices, mulching is one of the most important economic forces. It is an upward trend for effective water scarcity management through agronomic water conservation measures and enhanced rain-fed crop productivity with more benefits (World Bank 2002; Kader et al. 2019). Mulching refers to covering the soil, which acts as a physical barrier to crop productivity and soil health management. The term mulch means soft to decay. It is an agronomic practice of covering the soil surface with fresh or dry leaves or straw or plastic film, as displayed in figure 3. The positive effects of mulching primarily depend on the plant's response to water. The mulching practice provides essential nutrients, suppresses weed, maintains soil temperature, reduces evaporation, control pest, and enhances soil moisture (Figure 2) to address low crop production due to drought conditions (Patil et al. 2013). It also contends food demand for ever budding populations (Serrano-Ruiz et al. 2021).

3 Mulching and Microbiome

Various mulching practices have a tremendous impact on the soil microbiome like soil bacteria, fungi communities, and the ecosystem (Wu et al. 2022). It also enhances the soil microbial diversity and its functional properties, which interact with the plant rhizosphere region. In addition, it also promotes interrelations of soil microbiome, which improves soil quality (Wang et al. 2020). Its metabolic activity directly influences plant growth. Zhang et al. (2020) followed high throughput 16S rRNA and rDNA gene Illumina sequencing methods in tea plantation soil to assess the variations of the microbiome of plastic ethylene film mulch and peanut null mulch and reported the presence of top four bacterial phyla namely Proteobacteria, Actinobacteria, Acidobacteria, and Chloroflexi and three fungal phyla including Ascomycota, Mortierellomycota, and Basidiomycota. Lee et al. (2021) recently used five various types of mulching, including sawdust, green weed mat, and sawdust on top of the green weed mat. During the mulching process, soil changes the physical and chemical properties that drive microbial communities vary in their structure and metabolic activities. The soil microbial diversity varies according to the types of mulching methods followed. The covering of soil with organic mulches enriched the soil's microbial communities (Zhang et al. 2020).

4 Types of mulching

Various types of mulching followed in agriculture are surface and vertical mulching, mulching with pebble, straw, dust mulching with active organic materials, etc. Based on the utilization of materials, two types of mulches i.e. natural and synthetic are generally found (figure 3). Organic (natural/ biodegradable) mulch consists of organic materials, especially plant residues. The inorganic mulch is made of plastic-based materials (Kader et al. 2017). The agronomic performance profile of mulching is presented in table 1.

Table 1 Agronomic performance profile of mulching

Mulch Type	Beneficial crop	Beneficial Effects	Description	References
Plastic Mulching	*Capsicum frutescens* L., (Tabasco Pepper)	• Enhanced pepper production per unit area • Increased fruit dry mass percentage, water use efficiency, and potassium use efficiency in sandy loam soil	Mulching with drip irrigation	Chaves et al. 2021
Jatropha leaves Mulch	*Triticum* (Wheat (Wadan-17) (rainfed) and (Pirsabaq-2013) (irrigated))	• Preserved soil moisture content • Reduced water stress adverse effects and effectively maintain plant water status.	Mulching alone	Irshad et al. 2021
Transparent and black plastic film mulch	*Zea mays* L. (Spring Maize)	• Improved soil hydrothermal conditions • Enhanced early seedling emergence and silking • Boosted kernel weight and volume • Greater rate of photosynthesis • TM and BM improved grain yields (2019) at 28.1% and 15.1% and 24.6% and 21.1% (2020) respectively.	Mulching alone	Li et al. 2021
Plastic mulch	*Zea mays* L. (Spring Maize)	• Regulated soil hydrothermal traits • Maintained appropriate soil temperature and moisture content to solve the heat stress and water shortage impact	No-tillage with plastic re-mulching	Yin et al. 2021
Biodegradable mulch (BDM) (clear and black)	*Zea mays* L. (Spring Maize)	• Both mulches improved maize yield • Regulated soil temperature and root structure • Maize protein, N, P content, and fat were greater in black BDM.	Ridge-furrow pattern with mulching	Wang et al. 2021
Double mulch with *in-situ* MSM (Maize Stover Mulch) and WHP/RW (White Hoary Pea/ Ragweed)	*Zea mays* L. (Spring Maize)	• Improved soil moisture content and leaf related moisture content	Double mulching alone	Ngangom et al. 2020
Inter-row cornstalk mulch and black ground fabric mulch	*Malus* (Apple)	• Increased bacterial and fungal microbial communities and soil health	Mulching alone	Wang et al. 2020
Organic mulches including rice straw, sorghum straw, sesame straw, and Sudan grass	*Sesame indicum* L. (Sesamum)	• Showed major effect on the conservation of soil moisture content • Improve grain yield	Mulching alone	Teame et al. 2017
Plastic mulch	*Zea mays* L. (Spring Maize)	• Reduced soil evaporation, improved the soil moisture content and its availability • Regulated the soil temperature • Enhanced maize growth and yield	Mulching alone	Zhang et al. 2017
Plastic/ straw mulch	*Triticum* (Wheat) and *Zea mays* L. (Maize)	• Enhanced 60 % of the yield, WUE (yield per unit water) and NUE (yield per Unit N) • Improved yield of wheat at 20% and yield of maize at 60%	Mulching alone	Qin et al. 2015
Plastic and Straw Mulch	*Oryza sativa* (Rice)	• Boosted up the retention potential of the soil moisture • Improved rice water productivity, spikelet fertility, paddy yield, and quality	Mulching alone	Jabran et al. 2014
Cassava starch and poly (butylenes adipate-co-terephthalate)PBAT mulch	*Fragaria* (Strawberry)	Perked up properties of soil, hold soil moisture, magnifies water productivity Improved productivity	Mulching alone	Bilck et al. 2010
Red clover mulch	*Lycopersicon esculentum* (Tomato)	Reduced fruit cracking, improves loner roots	Mulching alone	Bender et al. 2008

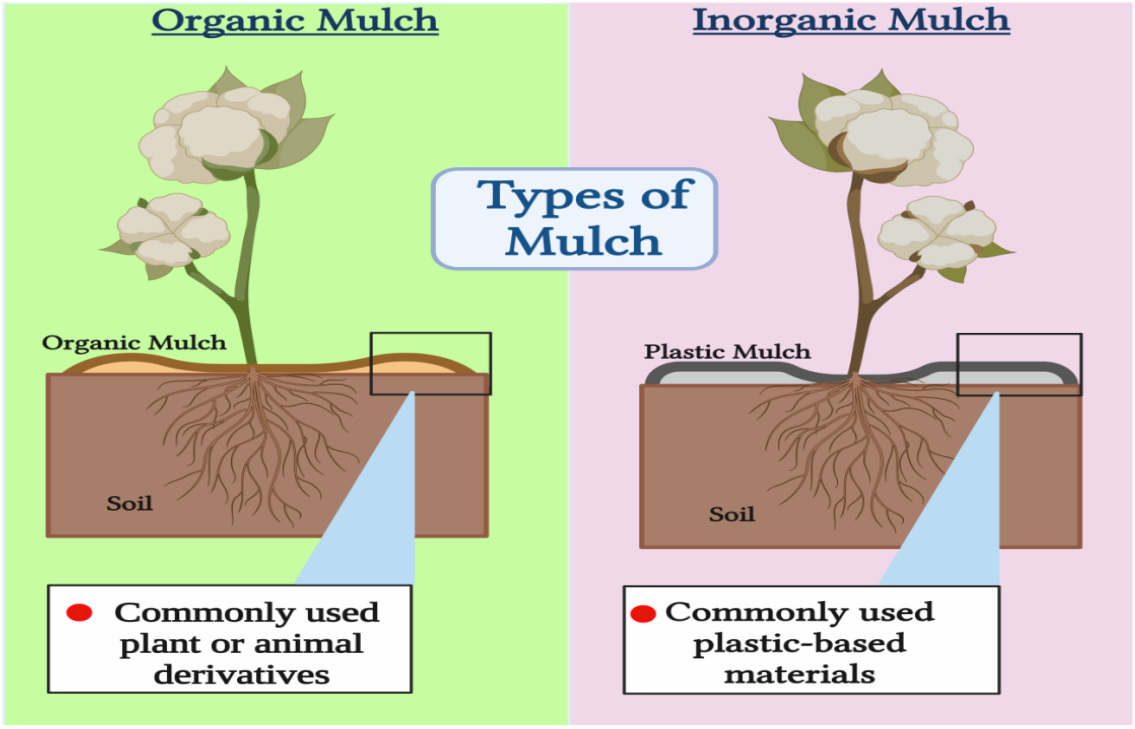

Figure 3 Types of mulch for enhanced crop yield

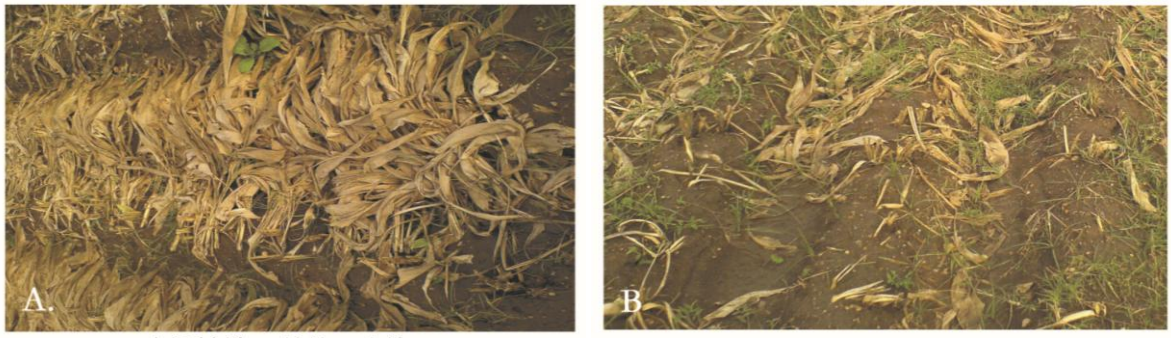

Figure 4 Organic mulched and non-mulched turmeric cultivation plots at Nambiyur, Erode District, Tamil Nadu
(A- Mulched plot and B- non-mulched plot)

4.1 Organic Mulch

Organic mulch is obtainable organically and it undergoes microbial decomposition to release nutrients. For over 60 years, natural materials like leaves, sand, straw, silage, peanut hulls, dung, sawdust, woodchips, and animal manure yielded organic mulches. Sumathi (2010) used turmeric shoots as organic mulch and reported a significant growth in the turmeric (figure 4). Organic mulch could regulate and improve the physical, chemical, and biological properties of the soil (Xu et al. 2022). Mulching with organic materials is found to have many positive effects like soil erosion prevention, increased nutrient cycling, and enhanced biological activities (Yasar and Sahin 2021). It boosts soil health, facilitates minerals, and inhibits weed germination (Zhang et al. 2020). Besides, using organic mulch also reduced the soil temperature to 7°C (Oliveira et al. 2001). In addition, mulching with grass clipping has improved soil nitrogen and expanded the root system. Organic mulching is regarded as a key strategy in managing drought conditions because of the improved water retention capacity of the soil (Chen et al. 2015). The findings of Barajas-Guzma´n et al. (2006) reported that mulching is an effective technique to restore dry soil conditions and help reforestation by supporting plant growth and survival.

Organic mulch increases the sapling survival more than other mulches despite its shelf life of eight months, and it has attracted pests and insects to feed on them to decompose efficiently and needed frequent change. Organic mulch is highly beneficial than

inorganic mulch based on its crop management potential (Ranjan et al. 2017). Mulching showed a positive impact and increased the soil infiltration rate. According to Molla et al. (2022), organic mulching increased the decomposition rate and intense soil nutrient concentration.

Biswas et al. (2015) used paddy straw as an affordable type of organic mulch and straw mulched conditions maintained 55% of soil moisture content. Straw materials mulching significantly improves the soil environment, plant growth, and vegetable crop yield as compared to non-mulched plants. The paddy straw mulch increased potato yield and their starch content. Further, even under reduced irrigation patterns also, paddy straw mulch produced a higher tomato yield. It stands for better water uptake, nutrient utilization, and soil-plant and water relationship. Advantages of using straw mulch are that they are eco-friendly, biodegradable, enhance soil water conservation, retained soil moisture, and increases plant growth development. Further decomposition of straw mulch released organic matter (SOM) into the soil and this type of soil can hold more water, rich in SOM encouraged the binding nature. In addition, the release of nutrients into the soil provided microbial communities with a substrate. Plant growth-promoting chemicals are secreted into the soil by microbial communities that are advantageous to humans to enhance plant growth. In comparison to straw mulch, soil water evaporation was slower under plastic mulch. It is viewed as a flaw in straw mulch (Biswas et al. 2015).

In the top soil layer, rice straw mulching has reduced water use by 30% more than in non-mulching settings (Chaudhary et al. 2004; Teame et al. 2017). None of the mulched crops exhibited signs of water stress, such as leaf rolling and wilting. It showed how mulch promoted nutrient delivery, lessened environmental stress, and boosted phosphorus availability to promote plant growth and development (Sardans and Penuelas 2004).

Use of wheat residue @ 6730kg/ ha increases the average moisture storage capacity up to 1.5m depth as compared to control and increases the 7–13% of root biomass of maize as compared to the non-straw mulched soils. Mulching with maize straw has minimal impact on SOC. At first, straw addition slightly raised the total SOC concentration by 2-3%. Over the years, increased SOC concentration is seen compared to non-straw incorporation (Wang et al. 2016). The grass is also used as a popular mulching material. Newly introduced grasses nourish the soil by supplying nitrogen sources into the soil. Under rainfed conditions, to avoid further root development by grass, dried grass materials are suggested (Patil et al. 2013). According to earlier studies, soil with grass mulch has high soil moisture content up to a depth of 60 cm (Teame et al. 2017) and reduced the loss of soil water due to evaporation by 35-50% (Hatfield et al. 2001). Mulching also helps in suppressing weeds because better rhizo-deposits of the rhizosphere contribute to soil fertility development and moisture conservation (Yin et al. 2013). Mulching increased the yield by producing increased fertilized cob number, seed weight, and more seeds (Niu et al. 2004; Ren et al. 2009). It has reduced irrigation frequency by slowing it down and less water usage (Rui et al. 2020). Mulching with drip irrigation is a good choice for water management practice to improve crop yield (Biswas et al. 2015). Crops use water and nutrients more effectively when the root zone of the soil has high soil moisture (Surya et al. 2000). Thick sheets of newspaper applied as mulch is simple and effective in weed control. Sawdust and bark clippings could be a great source of mulch materials. It is not employed widely because of its poor nutrient and less availability. Additionally, there exist specific restrictions when using organic materials such as mulch. The drawback of organic mulch is making the soil too moist, restricting oxygen entry near the rhizosphere, and encouraging the soil micro-communities to finally damage the plants.

4.1.1 Significant Effects of organic mulch

Soil water evaporation reduces the water use efficiency of crop plants. Agricultural production costs fall, when water use efficiency rises. Organic mulching practice reduces the water limitation by decreasing the soil temperature and soil water evaporation (Chai et al. 2014). Mulching also balances water and energy, buffering action against erosion by water and wind, and recycles soil nutrients to provide food and habitat for soil microflora. Crop residues used for mulching also help to alter microclimate, soil moisture, temperature patterns, etc. It increases soil moisture by modifying the soil water retention capacity. Mulching alters soil organic carbon levels (SOC) because plant-based residues are microbially decomposed (Liu et al. 2015). Indirectly, crop residues alter soil mechanisms based on their microclimate changing pattern, moisture in the soil and soil temperature regimes, water and solute transportation, and soil erosional principle. Mulching with organic materials makes weed control easier and improves soil nutrients. Finally, it promotes the development and production of microorganisms. In contrast, mulching the crop restricts the entry of light or a particular wavelength of light that supports weed growth (Ossomi et al. 2001). As a result of mulching, less soil water evaporated and the soil's moisture was consistently preserved. Retaining soil moisture causes the soil particles to stick together and maintain their close packing. When soil runoff gets reduced, soil erosion is prevented (Haribowo et al. 2021). Due to nutrient cycling, the process of mulching the soil probably improves the soil's nutrient status. The improvement of soil nutrient status promotes agricultural output and soil fertility (Sumathi et al. 2021). Even under rain-fed conditions, mulching boosts the crop yield by 50-60% over non-mulched conditions. Studies conducted by Tiquia et al. (2002)

demonstrated that mulching enhanced the SOM and P content of the soil. Thus, mulching increases soil nutrient content by preventing nutrient loss (Haribowo et al. 2021). Farmers can produce high-quality food in big quantities because of this technology.

Paddy straw as mulch increases the total yield of mulberry leaves more than non-mulched plots. Straw mulching also enhanced the phytochemical components of vegetables like tomatoes, cucumbers, melons, and eggplants. The tomato and okra plants with straw mulch have a higher yield as compared to the control plant. It was established that mulching has a considerable impact on both the quality and quantity of crops. Potato crops get benefited from paddy straw mulching because it raises yield and protects from weeds (Sureshkumar et al. 2021).

4.2 Inorganic mulch

Synthetic mulches are artificial and non-degradable. In the most recent update, mulching using synthetic materials, specifically plastic, has developed into an effective and cutting-edge integrated sophisticated technique in contemporary field agricultural output. It can increase soil moisture, eradicate pests, and treat plant diseases (Zhang et al. 2020).

4.2.1 Plasticulture Technology

Plasticulture is the practice of using plastics in agriculture (plasticization of agriculture). It is generally recommended to reduce water use and increase crop output, particularly in dryland farming (Kasirajan and Ngouajio 2012). In the 1950s and 1960s, plastic films were introduced to agriculture for both research and industrial vegetable production (Hussain and Hamid 2003; Lamont 2004).

Recently, plastic mulches are used worldwide, especially in developed countries, to produce and protect crops against modified weather, insects, weeds, and birds. Every year, the use of approximately 1 million tons of plastic film mulch has increased food productivity worldwide (Yu et al. 2018). In China, it is 40% to conserve water, which increases 53% of crop yield and conserved water by 24-26%. Globally, China is one of the maximum consumers of plastic mulch. It is regarded as the third agricultural revolution in Chinese history (Ingman et al. 2015). It is a cost-effective practice to improve soil temperature, reduce soil evaporation, and employ early harvest (Zhang et al. 2017).

4.2.1.1 Significant Effects of plastic mulch

Plastic mulch directly influences the underground water resources that provide the most favorable environment for plant growth and development. Furthermore, the aids of these mulching materials improved food production with increased quality and quantity (Ramakrishna et al. 2006). Mulch materials act as a soil insulating factor by delivering a buffer system from cold and hot temperatures, which provide decisive activity in creating fine fettle landscapes (Kader et al. 2019). Sizably reduce irrigation demand with conserve water economy. Plastic mulch functions as a barrier to maintain moisture and does not allow any moisture loss from the upper layer of the soil. It condenses on the lower surface of the mulch and returns as droplets were preserved for a long duration and conserve soil moisture at a stable level (Kader et al. 2019). The functioning of water stabilization in the rhizosphere area safeguards roots from excess rainwater impairment. Salinity is considered another serious concern affecting the crops in many countries. High saltwater contents are reduced greatly by water stabilization capacity during the mulching process. Its extended functions include plant uptake, transpiration, percolation into the soil and the crop even losses in water delivery (Ingman et al. 2015). It maintains soil water stress, the major limiting factor in plant growth maintenance and yield. It is one of the effective boosters for maintaining soil temperature and can induce an early crop yield. Besides, it can reduce nitrogen leaching or adds nitrogen to the soil (Frédéric et al. 2009). Soil bulk density and soil compactness was not affected by plastic mulch. The formation of soil aggregates also improved under the influence of soil moisture (Lalitha et al. 2010). The certainty of continuous fertilizer and biocontrol agent's delivery and utilization in the root zone prevent water-soil nutrient-related diseases (Biswas et al. 2022).

The remarkable characteristics of plastic mulch such as reflectivity, transmittance, absorptivity, and solar radiation interaction (Lamont 2005) are the reason for increased temperature in the root zone area of soil. A mechanism that changes the soil's energy balance directly affects the microclimate. However, it is useful for cold climates, which induce and encourage faster germination, plant development, and productivity. Increased soil temperature directly influences nutrient availability, and efficient uptake of nutrients; enhances the activity of beneficial soil microbes. It also induces the soil, root, and air temperature (Tarara 2000; Rangarajan and Ingall 2001; Ruiz et al. 2002). Kwabiah (2004) recorded that under plastic mulching conditions air temperature raised to 11˚C than barren plots. Maintaining soil temperature at the top 20 to 30 cm of the soil profile is another important function of mulching, which promotes root and plant development. Due to changes in surface radiation and minimization in soil water loss, the plastic mulch has an impact on the microclimate inside and around the plant (Tarara 2000). Plastic mulch is crucial for impermeable gaseous flow. It acts as the grander barrier for the fumigants and solarization process along with the pest control mechanism (Chalker-Scoot 2007). Waterer (2010) reported that the characteristic clear mulches increase soil temperature, especially in warm-season vegetable crops grown in temperate climates. Plants are protected by the plastic film from a

variety of environmental elements, including soil, water, wind, and hail damage. It operates for a prolonged period of 2-3 crop seasons (Kader et al. 2019) as it does not undergo decomposition rapidly and frequent replacement becomes unnecessary.

Some of the plastic mulch serves as a reservoir for methyl bromide, a potential fumigant, and ozone-depleting substance (Lalitha et al. 2010). The application of Low water uses efficiencies (WUEs) technology to help some plastic mulches to operate as a barrier and retains methyl bromide. It has led to desired results in productivity and profitability by airproof to prevent moisture and thermal proof to maintain the temperature at night. It provides better coverage than organic mulch as it is lightweight and easy to handle (Haapala et al. 2014). The agrometeorology of plant development and the health of the soil are both maintained by plastic mulch but it has negative effects on pollutants and plant pathogens (Kader et al. 2019).

4.2.1.2 Effect of plastic mulch in the field studies

Much of the existing research spectrum has enormously discussed the positive role of plastic mulch in the research area. Plastic mulch ominously enhanced vegetable production in the last decades worldwide, particularly, in tomato, brinjal, pepper, watermelon, musk melon, cucumber, squash, broccoli, etc. The same profound effects are observed in the crops namely corn, cotton, sugarcane, rice, and watermelon (Figure 5) (Bogiani et al. 2008; Lee et al. 2021). A white revolution was provoked by the rising production of peanuts (*Arachis hypogaea*) in China. The number of pods produced was reduced in black color plastic mulch are higher than the no mulching or straw mulching conditions (Ghosh et al. 2006). Twenty seedlings of three species belonging to Lonchocarpus ericarinalis Micheli (Leguminosae), Caesalpinia enostachys Benth (Leguminosae), and Ipomea wolcottiana Rose (Convolvulaceae) were subjected to experimental testing to assess the effects of plastic mulching under field conditions (Barajas-Guzman et al. 2006). According to their research, plastic mulches outperform other mulches in terms of soil volumetric water content (SVWC) and seedling survival rate. Plastic mulch did not affect the soil's pH, total N, total NH3, or total P organic content. The performance and production of tomatoes and cabbage were improved by the polythene mulch (Branco et al. 2010; Campagnol et al. 2014; Elsayed- Farag et al. 2018).

The Aliyarnagar Research Station reported that the plastic mulching increased the cotton crop productivity due to effective water conservation, and reduced weed growth than control of the cotton plant. The Tamil Nadu Agricultural University (TNAU) also reported that plastic mulching as a water economizing technology increased the height of the plant and the length of the root in red gram and castor crops under semi-arid conditions.

Yaghi et al. (2013) investigated the relationship between maturity time and cucumber (*Cucumus sativus* L.) production using plastic mulch and drip irrigation and enhanced the production rate twofold than the control. Using plastic mulching on vegetable crops such as ladies finger, tomato, and chili for three years produced higher yields, according to research done by the PDC in Coimbatore. Due to the improvement and alteration of soluble solid contents, total phenolics, flavonoids, and anthocyanins, reflection increased the output of Ontario wine grapes, plums, and butter beans. In strawberry and carrot, other color mulches increase the levels of phenolic chemicals, beta carotene, and ascorbic acid (Antonious and Kasperbauer 2002; Coventry et al. 2003; Kasperbauer and Loughrin 2004; Kim et al. 2008). Currently, mulching is a part of integrated agriculture methods that benefit agriculture (Table 2).

Figure 5 Plastic mulched plot for the cultivation of watermelon at Sathyamangalam, Erode District, Tamil Nadu, India

Table 2 Ultimate Mulching for Integrated Agricultural Management

Mulch	Agro-active compound	Crop	Effects	Reference
Paddy straw mulch, maize stubbles, and sawdust mulch	*Rhizobium* Biofertilizer	Pea Plant *(Pisum sativum* L.)	• Mulch with biofertilizer interactions (*Rhizobium* @30g/ kg of seeds with sawdust 210 t/ ha) demonstrated a better response on crop growth and yield	Muhammad et al. 2013
Pine needle mulch and black polythene mulch	NPK and *Azotobacter* fertilizer	Tomato (Naveen 2000, Sun-7711, Solan lalima)	• Studies proved that the three factors such as biofertilizers, variety, and mulch interactions improved fruit yield (1037.33 q/ ha) and quality. This study has employed 75% NPK+ *Azotobacter* (1g/ plant) + PSB (1g/ plant), Sun-7711 and black polythene mulch (V2B2M2).	Singh et al. 2017
Paddy straw and sawdust mulching	*Azotobacter* and PSB biofertilizer	Cauliflower (*Brassica ole*racea var. *Botrytis* L.)	• The combined application of bio-mulching boosts up the growth parameters including plant height, plant spread, and leaf weight. • It also enhanced quality traits such as TSS and ascorbic acid. • It improved curd density, average curd weight, and average curd yield/ ha along with the B: C ratio (3.16: 1).	Singh and Singh 2019
Sun hemp (*Crotalaria juncea* L.) mulch	Organics and *Azotobacter chroococcum* biofertilizers	Mulberry (*Morus Alba* L.	• Combined operations regulated soil moisture and major nutrients, which improved the growth rate, production rate, and quality of mulberry leaves under water stress conditions.	Chakraborty et al. 2016
Black polythene mulch and rice straw mulch	*Azotobacter* and PSB biofertilizer	Garlic (*Allium sativum* L.)	• Mulching along with biofertilizer enhanced the plant height, leaf length, stem diameter, bulb polar diameter and weight, total soluble solids, yield, no. of leaves in the plant, maximum number of cloves per bulb, thickness of bulb neck, length of cloves, pseudo stem and equatorial diameter of the bulb.	Anjali 2021
Silver plastic, black plastic, and wheat straw mulch	Biopesticides includes Chloropyriphos, Cypermethrin, neem oil, and *Trichoderma*	Okra Jassids (*Amrasca biguttula biguttula)*	• It improved fruit yield, pod length and diameter, and plant health.	Dahal et al. 2020
Biodegradable mulch - poly (butylenes adipate-co-terephthalate/ polylactide (PBAT/ PLA)	Herbicide 2-methyl-4-cholorophenoxyacetic acid/poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (MCPA-PHBV)	Fava bean (*Vicia faba*)	• The symbiotic effect of biodegradable mulch with herbicide effectively suppressed the broadleaf weed species and improved crop health and yield.	Khan et al. 2020
Slash mulch	Insecticide	Norway spruce (*Picea abies* L. Karst.)	• The combined impact of insecticide-treated mulch improved crop survival rate. It has improved soil moisture and mineralization. • It enhanced the height, diameter, and volume of the seedlings.	Johansson et al. 2006
Biodegradable mulch - poly (butylenes adipate-co-terephthalate/ polylactide (PBAT/ PLA)	Biodegradable mulch conjugated with herbicide 2-methyl-4-cholorophenoxyacetic acid and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (MCPA) (MCPA-PHBV)	Fava bean (*Vicia faba)*	Biodegradable mulch with agro-active compound (MCPA-PHBV) controlled broadleaf weed species.	Kwiecien et al. 2018
Cover crop mulch	Organic herbicides (capric/caprylic acid, corn gluten meal, and herbicide-free)	Vegetable crops	Reduced weed pressure and need for tillage.	Lewis et al. 2020

5 Factors involved in mulch water-saving system

The effectiveness of mulching for water conservation depends on several parameters, such as the type of material used, the seasons, nutritive value, tendency, and the rate of decomposition. The ability of plastic mulch in water conservation depends heavily on the flexibility and site-specificity of agronomic practices. The reproducibility of the plastic mulch for moisture management, when combined with drip irrigation technology, is based on the crop cultivated, the climate, ecological locations, soil type, installation procedures, holes for transplanting, the color and thickness of the plastic mulch, the cut perforations in the plastic film, the pattern of irrigation, etc. (Yaghi et al. 2013; Ingman et al. 2015). In developed countries, micro-irrigation technology is largely followed for plastic mulching. In farms with sparse rainfall, plastic mulching is used in conjunction with a raised bed system or ridge furrow system to collect rainwater (Gan et al. 2013; Li et al. 2017).

Plastic mulches are clear, thin sheets of plastic film with varying colors and thicknesses, and dimensions. The type of plastics is based on polymers and their intended purpose. Commonly acetate, polyethylene, polymeric substances, low-density polyethylene (LDPE), High-density polyethylene (HDPE), flexible polyvinyl chloride, and ethylene-vinyl acetate have been used (Kader et al. 2019). Generally, PVC is preferred because of its easy process, longwave radiation, more efficient permeability, increased durability and flexibility, and absence of odor and toxicity. Especially black PVC provides better weather and chemical resistance, weed control, and loss for many seasons than opaque white and translucent film. It raised and sustained the soil's temperature throughout the night.

When applied in rows in the field, it seals the upper layer of the soil. The plants are grown by cutting or making holes in the plastic. It is then installed using hand tools or a mechanical process (Ingman et al. 2015). LLDPE is exploited on an economic level (Kasirajan and Ngouajio 2012; Yuan et al. 2022). LLDPE is less than one-third the density of LDPE, flexible, and highly durable. The thinner layer is still very functional and appropriate. LDPE is widely utilized in the USA and has a strong puncture resistance with mechanical stretch applications. HDPE has excellent moisture and vapor barriers (Lamont 2005; Ngouajio et al. 2007). Furthermore, the evaporation rate was influenced by the type of mulch (Chakraborty and Sadhu 1994). Plastic mulch does not change the physical, chemical, and biological properties for short-term applications. However, after the cultivation or harvest, the plastic covers are not completely removed and remain attached to the soil for the long term interfering with ecosystem cycling (Ramos et al. 2015). Soil microbial communities change their structure, biochemical composition, and diversity (Sreejata et al. 2018). Such alterations occur by modification of microclimates primarily.

5.1 Mulch Colour

Mulch color exhibits varied optical and spectral characteristics according to the intensity of light radiation to the soil. However, it directly influences the soil and air temperature, and soil salinity by reducing upward movement and evaporation of water and canopy distribution of the plant. This process used black, silver, white, red, blue, and yellow (Kader et al. 2019). Commonly used plastic film is black due to the greatest absorbing and reradiating warming properties. It does not allow the penetration of sunlight into the soil. Hence, photosynthesis did not take place in the absence of sunlight as it directly controlled and suppressed the weeds. It is better in colder climates. It has better controllability on weed flora. It is widely used in sandy soil, saline water, and weed control in crop plants (Amare and Desta 2021).

Silver mulch acts as an insect repellent when the mulch is used in summer cropped land. Elsayed-Farag et al. (2018) reported that white mulch increased the yield of tomatoes more than black mulch. However, the opaque white film is better in warmer climates, with the golden color it attracts insects through insect controllability. Red mulch-induced aroma compounds and minimizes the early blight on tomatoes (Lamont 1999). The phenolic contents of carrots are induced when yellow and black mulch is used. Yellow and white mulches optimized beta carotene and ascorbic acid contents more than other color mulches. IRT (Infrared Transmitting) mulch is the recent technology used to control the weeds due to warming properties (Lamont 1999).

5.2 Thickness and width of the film

Recent research reported that the thickness of the plastic mulch indirectly affects crop yield. The water vapor flow and thermal conductivity of the heat transfer mechanism depend upon the thickness of the film, commonly used as a thin film. The thickness of the film determines the water vapor flow and thermal conductivity of the heat transfer mechanism and typically a thin film is used. Generally, the recommended thickness of plastic mulch is 15-20µm, which 15µm is an effective one. But the preferred thickness of organic mulch is 4-8cm and under this situation, faster growth and earlier harvesting were achieved as compared to organic mulch. Thick mulches are frequently used in weed control via solarization in orchards and plantations. The width depends on inter-row spacing. Generally, 1-1.5 width is adopted for different conditions. In the rainy seasons, perforations are made to prevent water stagnation in the plants to improve plant growth (Kader et al. 2019).

Conclusion and Future Prospective

This review provides greater clarity about the importance of mulching in the agricultural sector. It is a promising method in contemporary agriculture to improve the soil's physical, chemical,

and biological properties. It is the most economical procedure followed to increase crop production and protection. Both organic and inorganic mulching showed deep physiochemical interactions and significant positive crop responses were reported. When compared with other mulches, plastic mulches are highly effective. The socioeconomic study of plastic mulch demonstrates that it could be implemented for another green revolution. Mulching creates a new rebellion path to develop modern agriculture with plastic culture with colossal future opportunities. Even for the restoration of deforested lands, mulching methods are highly preferred. The mulching process is used for the perfect functioning of the ecosystem, and the soil-water content retention. In a broader sense, our study highlights the necessity of assisting farmers in overcoming the obstacles of integrated weed management to lessen the requirement for soil cultivation and reduce damage to beneficial arthropods. To comprehend the genetic and metabolic connections of the mulched crop, however, and to address the context of climate change and environmental sustainability, more research credentials are required.

The beneficial and controversial information on using plastic mulches is well recorded. The use of plastic mulches is expected to increase at 5.6% per annum by 2030. As part of the benefits of plastic mulching, a detailed study on the persistence and degradation of plastic residues or microplastics by soil microbial communities is ultimately required. This brings an environmental concern. There is no doubt that future agriculture authorities rely on rapidly evolving mulching methods with the progress of ecotoxicological studies. Although the expansion of value-added farming through mulching is desired for the development of an eco-soil regulatory framework. Furtherly, the probe of soil and its floral productivity is also paraphrased for crop productivity. In the future, mulched agriculture will be a dominant platform for the developmental scaffolding of smart agriculture.

Author Contributions

MR and SCS wrote main manuscript text and prepared figures; RP and RKV prepared tables and reviewed the manuscript.

Ethics approval and consent to participate

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Conflict of Interest

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References

Amare, G., & Desta, B. (2021). Coloured plastic mulches: impact on soil properties and crop productivity. Chemical Biological Technologies in Agriculture, 8(4), 1-9. https://doi.org/10.1186/s40538-020-00201-8

Anjali, S. (2021). Impact of organic and inorganic fertilizers on growth, yield and economics of garlic (Allium sativum L.). Annals of Plant and Soil Research, 23, 477-480. 10.47815/apsr.2021.10106.

Antonious, G.F., & Kasperbauer, M.J. (2002). Color of light reflected to leaves modifies nutrient content of carrot roots. Crop Science, 42 (4), 1211–1216. https://doi.org/10.2135/cropsci2002.1211

Barajas Guzman, M. G., Campo, J., & Barradas, V.L. (2006). Soil water, nutrient availability and sapling survival under organic and polyethylene mulch in a seasonally dry tropical forest. Plant and Soil, 287(1), 347-357. https://doi.org/10.1007/s11104-006-9082-7.

Barron, J., Rockström, J., Gichuki, F., & Hatibu, N. (2003). Dry spell analysis and maize yields for two semi-arid locations in east Africa. Agricultural Forests Meteorology, 117(1-2), 23–37. https://doi.org/10.1016/S0168-1923(03)00037-6

Bender, I., Raudseping, M., & Vabrit, S. (2008). Effect of organic mulches on the growth of tomato plants and quality of fruits in organic cultivation. Proceedings of the International symposium on growing media. Acta Horticulturae, 779(1), 341-346. https://doi.org/10.17660/ActaHortic.2008.779.42

Biswas, S.K., Akanda, A.R., & Rahman, M.S. (2015). Hossain Effect of drip irrigation and mulching on yield, water-use efficiency and economics of tomato. Plant Soil Environment, 61(3), 97–102. https://doi.org/ 10.17221/804/2014-PSE.

Biswas, T., Bandyopadhyay, P.K., Nandi, R., Mukherjee, S., et al. (2022). Impact of mulching and nutrients on soil water balance and actual evapotranspiration of irrigated winter cabbage (Brassica oleracea var. capitata L.), Agricultural Water Management, 263, 107456. https://doi.org/10.1016/j.agwat.2022.107456

Bilck, A.P., Grossmann, M.V.E., Yamashita, F. (2010). Biodegradable mulch films for strawberry production. Polymer Testing, 29 (4), 471-476. https://doi.org/10.1016/j.polymertesting.2010.02.007

Bogiani, J.C., Anton, C. S., Seleguini, A. Faria Junior, M.J.A., & Seno, S. (2008). Tip pruning, plant density and plastic mulching in

tomato yield in protected cultivation. Bragantia, 67(1), 145-151. https://doi.org/10.1590/S0006-87052008000100018

Branco R.B.F., Santos L.G.C., Goto, R., Ishimura, I., Schlickmann, S., & Chiarati, S. (2010). Successive organic cultivation of vegetable crops in two irrigation systems and two soil covers. Horticultura Brasileira, 28(1), 75-80. https://doi.org/10.1590/S0102-05362010000100014

Campagnol, R., Abrahao, C., Mello, S.C., Oviedi, V.R.S., & Minami, K. (2014). Impacts of irrigation levels and soil cover on tomato crop. Irrigation, 19(3), 345-357. https://doi.org/10.1590/S0102-05362010000100014

Chai, Q., Gan, Y., Turne, N.C., Zhang, R.Z., Yang, C., Niu, Y., & Siddique, K.H.M. (2014). Water-saving innovations in Chinese agriculture. Advances in Agronomy, 126, 149–201. https://doi.org/10.1016/B978-0-12-800132-5.00002-X

Chakraborty, B., Kundu, M., & Chattopadhyay, R.N. (2016). Organic farming with bio-mulching–a new paradigm for sustainable leaf yield and quality of mulberry (Morus Alba L.) under rainfed lateritic soil condition. International Conference on Inventions & Innovations for Sustainable Agriculture 2016, ICIISA. Agriculture and Agricultural Science Procedia, 11, 31-37. https://doi.org/10.1016/j.aaspro.2016.12.006

Chakraborty, R.C., & Sadhu, M.K. (1994). Effect of mulch type and color on growth and yield of tomato (Lycopersicon esculentum). Indian Journal of Agricultural Science, 64(9), 608–612. http://dx.doi.org/10.46609/IJAER.2020.v06i01.007.

Chalker-Scott, L. (2007). Impact of mulches on landscape plants and the environment- a review. Journal of Environmental Horticulture, 25(4), 239-249. https://doi.org/10.24266/0738-2898-25.4.239

Chaudhry, M.R., Aziz A.M., & Sidh, M. (2004). Mulching Impact on Moisture Conservation ─ Soil Properties and Plant Growth. Pakistan Journal of Water Research, 8(2),1-8.

Chaves, S.W.P., Coelho, R.D., Costa, J.D.O., & Tapparo, S. A. (2021). Vegetative and productive responses of tabasco pepper to fertigation and plastic mulching. Scientia Agricola, 79(5), 10. https://doi.org/10.1590/1678-992x-2021-0084

Chen, Y., Liu, T., Tian, X., Wang, X., Li, M., Wang, S., & Wang, X. (2015). Effects of plastic film combined with straw mulch on grain yield and water use efficiency of winter wheat in Loess Plateau. Field Crops Research, 172, 53-58. https://doi.org/10.1016/j.fcr.2014.11.016

Coventry, J.M., Fisher, K.H., Strommer, J.N., & Reynolds, A.G. (2003). Reflective mulch to enhance berry quality in Ontario wine grapes. VII International Symposium on Grapevine Physiology and Biotechnology. Acta Horticulture, 689, 95-102. http://dx.doi.org/10.17660/ActaHortic.2005.689.7

Dahal, B.R., Rijal, S., Poudel, N., Gautam, B., & Neupane, R. B. (2020). Influence of different bio-pesticides and mulching in management of Okra Jassids Amrasca biguttula biguttula (Hemiptera: Cicadellidae) in Chitwan district of Nepal. Cogent Food and Agriculture, 6(1), 1-9. http://dx.doi.org/10.1080/23311932.2020.1829271

Elsayed-Farag, S., Anciso, J., Marconi, C., Avila, C., Rodriguez, A., Badillo-Vargas, I. E. & Enciso, J. (2018). Appropriate planting dates and plastic mulch for increasing common tomato varieties yield in south texas. Agricultural Research, 13 (26), 1349-1357. http://dx.doi.org/10.5897/AJAR2018.13212

Frédéric, T., Katrine, A.S. & Philippe, S. (2009). Use of Perennial Legumes Living Mulches and Green Manures for the Fertilization of Organic Broccoli, International Journal of Vegetable Science, 15(2), 142-157. https://doi.org/10.1080/19315260802598896

Gan, Y., Siddique, K.H.M., Turner, N.C., Li, X.G., Niu, J.Y., Yang, C., Liu, L., & Chai, Q. (2013). Ridge-Furrow mulching systems – an innovative technique for boosting crop productivity in semiarid rain-fed environments. Advanced Agronomy, 118, 429-476. http://dx.doi.org/10.1016/B978-0-12-405942-9.00007-4

Ghimire, R., Lamichhane, S., Acharya, B.S., Bista, P., & Sainju, U.M. (2017). Tillage, crop residue and nutrient management effects on soil organic carbon in rice-based cropping systems: A review. Journal of Integrated Agriculture, 16(1), 1-15. https://doi.org/10.1016/S2095-3119(16)61337-0.

Ghosh, P.K., Devi, D., Bandyopadhyay, K.K., & Mohanty, M. (2006). Evaluation of straw and polythene mulch for enhancing productivity of irrigated summer groundnut, Field Crops Research, 99(2–3), 76-86. https://doi.org/10.1016/j.fcr.2006.03.004

Haapala, T., Palonen, P., Korpela, A., & Ahokas, J. (2014). Feasibility of paper mulches in crop production: A review. Agriculture and Food Science, 23 (1), 60–79. https://doi.org/10.23986/afsci.8542

Hanjra, M.A., & Qureshi, M.E. (2010). Global water crisis and future food security in an era of climate change. Food Policy, 35 (5), 365–377. https://doi.org/10.1016/j.foodpol.2010.05.006.

Haribowo, R., Asmaranto, R., & Kusuma, L.T.W.N. (2021). Effect of rice straw mulch on surface runoff and soil loss in agricultural land under simulated rainfall. IOP Conference Series: Earth and

Environmental Science, 930(1), 012007. https://doi.org/10.1088/1755-1315/930/1/012007

Hatfield, J. L., Sauer, T. J., & Prueger, J. H. (2001). Managing soils to achieve greater water use efficiency. Agronomy Journal, 93(2), 271–280. https://doi.org/10.2134/agronj2001.932271x

Hussain, I., & Hamid, H. (2003). Plastics in agriculture. In: A.L. Andrady (ed). Plastics and the environment (Pp.185–209). Wiley, Hoboken.

Ingman, M., Santelmann, M.V., & Tilt, B. (2015). Agricultural water conservation in china: plastic mulch and traditional irrigation. Ecosystem Health and Sustainability, 1(4), 1-11. https://doi.org/10.1890/EHS14-0018.1

Iqbal, R., Raza, M.A.S., Saleem, M.F., Khan, I.H., et al. (2019). Physiological and biochemical appraisal for mulching and partial rhizosphere drying of cotton. Journal of Arid Lands, 11 (5), 785-794. https://doi.org/10.1007/s40333-019-0014-9.

Irshad, M., Ullah, F., Fahad, S. Mehmood, S., et al. (2021). Evaluation of Jatropha curcas L. leaves mulching on wheat growth and biochemical attributes under water stress. BMC Plant Biology, 21(1), 303. https://doi.org/10.1186/s12870-021-03097-0

Jabran, K., Ullah, E., Hussain, M., Farooq, M., Zaman, U., Yaseen, M., & Chauhan, B.S. (2014). Mulching improves water productivity, yield and quality of fine rice under water-saving rice production systems. Journal of Agronomy and Crop Science, 201(5), 389-400. http://dx.doi.org/10.1111/jac.12099

Johansson, K., Orlander, G., & Nilsson, U. (2006). Effects of mulching and insecticides on establishment and growth of Norway spruce. Canadian Journal of Forest Research, 36(10), 2377-2385. http://dx.doi.org/10.1139/x06-121

Kader, M.A., Senge, M., Mojid, M.A., & Ito, K. (2017). Recent advances in mulching materials and methods for modifying soil environment. Soil Tillage Research, 168(5), 155-166. http://dx.doi.org/10.1016/j.still.2017.01.001

Kader, M.A., Singha, A., Begum, M.A., Jewel, A., Khan F.H., & Khan, N. (2019). Mulching as water-saving technique in dry land agriculture. Bulletin of the National Research Centre, 43, 147. https://doi.org/10.1186/s42269-019-0186-7.

Kasirajan, S., & Ngouajio, M. (2012). Polyethylene and biodegradeable mulches agricultural applications: A review. Agronomy Sustainable Development, 32(2), 501-529. http://dx.doi.org/10.1007%2Fs13593-011-0068-3

Kasperbauer, M.J., & Loughrin, J.H. (2004). Crop ecology, management and quality: butterbean seed yield, color, and protein content are affected by photomorphogenesis. Crop Science, 44(6), 2123–2126. http://dx.doi.org/10.2135/cropsci2004.2123

Khan, H., Kaur, S., Baldwin, T. C., Radecka, I., et al. (2020). Effective control against broadleaf weed species provided by biodegradable PBAT/PLA mulch film embedded with the Herbicide 2-Methyl-4-Chlorophenoxyacetic Acid (MCPA). ACS Sustainable Chemistry and Engineering, 8(13), 5360-5370. https://doi.org/10.1021/acssuschemeng.0c00991

Kim, E.J., Choi, D.G., & Jin, S.N. (2008). Effect of pre-harvest reflective mulch on growth and fruit of plum (Prunus domestica L.). XXVII International Horticultural Congress—IHC2006: International Symposium on Enhancing Economic and Environmental Sustainability of Fruit Production in a Global Economy. Acta Horticulturae, 772, 323-326. http://dx.doi.org/10.17660/ActaHortic.2008.772.54

Kwabiah, A.B. (2004). Growth and yield of sweet corn (Zea mays L.) cultivars in response to planting date and plastic mulch in a short season environment. Scientia Horiculturae, 102(2), 147–166. http://dx.doi.org/10.1016/j.scienta.2004.01.007

Kwiecien, I., Adamus, G., Jiang, G., Radecka, I., et al. (2018). Biodegradable PBAT/PLA blend with bioactive MCPA-PHBV conjugate suppresses weed growth. Biomacromolecules, 19(2), 511-520. http://dx.doi.org/10.1021/acs.biomac.7b01636

Lalitha, M., Thilagam, V.K., Balakrishnan, N., & Monsour, M. (2010). Effect of plastic mulch on soil properties and crop growth-A Review. Agricultural Reviews, 31 (2), 145-149. https://arccjournals.com/journal/agricultural-reviews/ARCC1349

Lamont, W. (2004). Plastic mulches. In: W. Lamont (ed) Production of vegetables, strawberries, and cut flowers using plasticulture. Natural Resource, Agriculture, and Engineering Service (NRAES-133), Ithaca. Pp. 65. https://hdl.handle.net/1813/69448

Lamont, W.J. (1999). Bulletin on vegetable production using plasticulture. Tapei City, Republic of China on Taiwan: Food and Fertilizer Centre. Pp. 1-9. https://www.fftc.org.tw/htmlarea_file/library/20110808093747/eb476.pdf

Lamont, W.J. (2005). Plastics: modifying the microclimate for the production of vegetable crops. Horticulture Technology, 15(3), 477–481. https://doi.org/10.21273/horttech.15.3.0477

Lee, J.G., Chae, H.G., Cho, S.R., Song, H.J., Kim, P.J., & Jeong, S.T. (2021). Impact of plastic film mulching on global warming in entire chemical and organic cropping systems: life cycle assessment. Journal of Cleaner Production, 308, 12, 7256. https://doi.org/10.1016/j.jclepro.2021.127256

Lewis, D.G., Cutulle, M.A., Schmidt-Jeffris, R.A., & Blubaugh, C.K. (2020). Better together? combining cover crop mulches, organic herbicides, and weed seed biological control in reduced-tillage Systems. Environmental Entomology, 49(4), 1327-1334. http://dx.doi.org/10.1093/ee/nvaa105

Li, C., Wang, Q., Wang, N., Luo, X., Li, Y., Zhang, T., Feng, H., & Dong, Q. (2021). Effects of different plastic film mulching on soil hydrothermal conditions and grain-filling process in an arid irrigation district. The Science of the Total Environment, 795, 148886. https://doi.org/10.1016/j.scitotenv.2021.148886

Li, C., Wang., C, Wen, X., Qin, X., et al. (2017). Ridge-furrow with plastic film mulching practice improves maize productivity and resource use efficiency under the wheat-maize double-cropping system in dry semi-humid areas. Field Crops Research, 203, 201-211. http://dx.doi.org/10.1016/j.fcr.2016.12.029

Liu, L., Hu, C., Yang, P., Ju, Z., Olesen, J.E., & Tang, J. (2015). Effects of experimental warming and nitrogen addition on soil respiration and CH4 fluxes from crop rotations of winter wheat–soybean/fallow. Agricultural Forests Meteorology, 207(1), 38–47. http://dx.doi.org/10.1016/j.agrformet.2015.03.013

Molla, A., Desta, G., Molla, G. A., Desta, G., & Dananto, M. (2022). Soil management and crop practice effect on soil water infiltration and soil water storage in the humid lowlands of Beles Sub-Basin, Ethiopia Getnet Soil Management and Crop Practice Effect on Soil Water Infiltration and Soil Water Storage in the Humid L. Hydrology, 10(1), 1–11. https://doi.org/10.11648/j.hyd.20221001.11

Muhammad, S., Israr, H., Rab, A., Jan, I., Fazal, I.W., Shah, T., & Khan, I. (2013). Influence of organic mulches on growth and yield components of pea's cultivars. Greener Journal of Agricultural Sciences. 3(8), 652-657. http://dx.doi.org/10.15580/GJAS.2013.3.122912351

Ngangom, B., Das A., Lal, R., Idapuganti, R.G., et al. (2020). Double mulching improves soil properties and productivity of maize-based cropping system in eastern Indian Himalayas. International Soil and Water Conservation Research, 8(3), 308-320. http://dx.doi.org/10.1016/j.iswcr.2020.07.001

Ngouajio, M., Goldy, R., Zandstra, B., & Warncke, D. (2007). Plasticulture for Michigan Vegetable Production. Extension Bulletin E-2980 January 2007. Michigan State University, East Lansing, pp 20. https://archive.lib.msu.edu/DMC/extension_publications/e2980/E2980-2007.PDF

Niu, J.Y., Gan, Y.T., & Huang, G.B. (2004). Dynamics of root growth in spring wheat mulched with plastic film. Crop Science, 44(5), 1682–1688. http://dx.doi.org/10.2135/cropsci2004.1682

Oliveira, J.C.M., Timm, L.C., Tominaga, T.T., Cassaro, F.A.M., et al. (2001). Soil temperature in a sugar-cane crop as a function of the management system. Plant and Soil, 230(1), 61–66. http://dx.doi.org/10.1023/A:1004820119399

Ossomi, E. M., Pace, P. F., Rhykerd, R. L., & Rhykerd, C. L. (2001). Effect of mulch on weed infestation, soil temperature, nutrient concentration, and tuber yield in Ipomoea batatus (L.) Lam. In Papua New Guinea. Tropical Agriculture (Trinidad), 78(3), 144–151.

Patil, S., Kelkar-Tushar, S., & Bhalerao, S. (2013). Mulching: A Soil and water conservation practice. Research Journal of Agriculture and Forestry Sciences, 1(3), 26–29.

Qin, W., Hu, C., & Oenema, O. (2015). Soil mulching significantly enhances yields and water and nitrogen use efficiencies of maize and wheat: a meta-analysis. Scientific Reports, 5, 16210. http://dx.doi.org/10.1038/srep16210

Ramakrishna, A., Hoang, M.T., Suhas, P.W., & Tranh, D.L. (2006). Effect on mulch on soil temperature, moisture, weed infestation and yield of groundnut in North Vietnam. Field Crops Research, 95(2-3), 115-125. http://doi.org/ 10.1016/j.fcr.2005.01.030

Ramos, L., Berenstein, G., Hughes, E. A., Zalts, A., & Montserrat, J. M. (2015). Polyethylene film incorporation into the horticultural soil of small periurban production units in Argentina. Science Total Environment, 523, 74–81. http://doi.org/ 10.1016/j. scitotenv.2015.03.142

Rangarajan, A., & Ingall, B., (2001). Mulch color effects radicchio quality and yield. Horticultural Science, 36(7), 1240–1243. https://doi.org/10.21273/HORTSCI.36.7.1240

Ranjan, P., Patle, G. T., Prem, M., & Solanke, K R. (2017). Organic Mulching - A Water saving technique to increase the production of fruits and vegetables. Current Agricultural Research Journal, 5(3), 371- 380. http://dx.doi.org/10.12944/CARJ.5.3.17.

Ren, X., Chen, X., & Jia, Z. (2009). Ridge and furrow method of rainfall concentration for fertilizer use efficiency in farmland under semiarid conditions. Applied Engineering Agriculture, 25 (6), 905–9130.

Rui, L., Qinggui, L., & Lidong, P. (2020). Review of organic mulching effects on soil and water loss. Archives of Agronomy and Soil Science, 67(1), 136-151. https://doi.org/10.1080/03650340.2020.1718111.

Ruiz, J.M., Hernandez, J., Castilla, N., & Luis, R. (2002). Effect of soil temperature on K and Ca concentrations on ATPase and

pyruvate kinase activity in potato roots. Horticultural Science, 37(2), 325–328. https://doi.org/10.21273/HORTSCI.37.2.325

Sardans, J., & Penuelas, J. (2004). Increasing drought decreases phosphorus availability in an evergreen Mediterranean forest. Plant and Soil, 267(1-2), 367–377. https://doi.org/10.1007/s11104-005-0172-8

Schimel, D.S. (2010). Drylands in the earth system. Science, 327, 418–419. https://doi.org/10.1126/science.1184946.

Serrano-Ruiz, H., Martín-Closas, L., & Pelacho, A. (2021). Biodegradable plastic mulches: Impact on the agricultural biotic environment. Science of The Total Environment, 750, 141228 10.1016/j.scitotenv.2020.141228.

Silva, A. C. C. D., Nascimento, J. M. S. D., Diotto, A. V., Lima, L.A., & Oliveira, M.C.D. (2019). Yield in tomato under two water depths and plastic mulching. Revista Brasileira de Ciencias Agrarias, 14 (3), 1-6. https://doi.org/10.14295/CS.v12.3779.

Singh, G., & Singh, S. K. (2019). Effect of bio-fertilizer and mulching on growth, yield and quality of cauliflower (Brassica oleracea var. botrytis L.) in Punjab. Journal of Crop and Weed, 15(1), 182-185. https://www.cabdirect.org/cabdirect/abstract/20183080306

Singh, S.K., Raturi, H.C., & Kumar, R. (2017). Effect of different mulches and biofertilizers on qualitative and quantitative attributes of tomato. Journal of Plant Development Sciences, 9(11), 999-1005. https://www.cabdirect.org/cabdirect/abstract/20183080306

Sreejata, B., Martin-Closas, L., Pelacho, A.M., & DeBruyn, J.M. (2018). Biodegradable Plastic Mulch Films: Impacts on soil microbial communities and ecosystem functions. Frontiers in Microbiology, 9, 819. https://doi.org/10.3389/fmicb.2018.00819

Sumathi, C.S. (2010). Development of sustainable crop improvement strategies through microbial bioinoculants application in turmeric (Curcuma longa L.) plantation. Unpublished Ph.D., thesis submitted to the Bharathidasan University, Trichy, India. Pp: 35

Sumathi, C.S., Mahalakshmi, P., & Rajesh, P. (2021). Impact of mycorrhizal soil fertility proteins and Arbuscular mycorrhizal application to combat drought stress in maize plants. Journal of Plant Biochemistry and Biotechnology, 30(4), 906–917. http://dx.doi.org/10.1007/s13562-021-00745-2

Suresh Kumar, J., Nedunchezhiyan, M., & Sunitha S. (2021). Weed control approaches for tropical tuber crops - A review. International Journal of Vegetable Science, 27(5), 439-455. https://doi.org/10.1080/19315260.2020.1839156

Surya, J.N., Puranik, J.B., Zadode, S.D., & Deshmukh, S.D. (2000), Effect of wheat straw incorporation on yield of green gram and wheat, soil fertility and microbiota. Journal of Maharastra Agricultural University, 25(2), 158-160.

Tarara, J.M. (2000). Microclimate modification with plastic mulch. Horticultural Science, 35(2), 222–228.

Teame, G., Tsegay, A., & Abrha, B. (2017). Effect of organic mulching on soil moisture, yield, and yield contributing components of sesame (Sesamum indicum L.). International Journal of Agronomy, 6, 1-6. http://dx.doi.org/10.1155/2017/4767509

Tiquia, S.M., Wan, J.H.C., & Tam, N.F.Y. (2002) Microbial population dynamics and enzyme activities during composting. Compost Science Utilization, 10(2), 150–161. https://doi.org/10.1080/1065657X.2002.10702075

Uhlirova, E., Simek, M., & Santruckova, H. (2005). Microbial transformation of organic matter in soils of montane grasslands under different management. Applied Soil Ecology, 28 (3), 225-235. https://doi.org/10.1016/j.apsoil.2004.08.002.

Wang Y.P., Xiao G.L., Taotao, F., Lin, W., Neil, C. T., Kadambot H.M. S., & Feng-Min, L. (2016). Multi-site assessment of the effects of plastic-film mulch on the soil organic carbon balance in semiarid areas of China. Agricultural Forest Meteorology, 228–229, 42–51. https://doi.org/10.1016/j.agrformet.2016.06.016

Wang, Y., Liu, L., Luo, Y., Awasthi, M.K., et al. (2020). Mulching practices alter the bacterial-fungal community and network in favor of soil quality in a semiarid orchard system. The Science of the Total Environment, 725, 138527. https://doi.org/10.1016/j.scitotenv.2020.138527

Wang, Z., Li, M., Flury, M., Schaeffer, S.M., Chang, Y., Tao, Z., & Wang, J. (2021). Agronomic performance of polyethylene and biodegradable plastic film mulches in a maize cropping system in a humid continental climate. The Science of the Total Environment, 786, 147460. https://doi.org/10.1016/j.scitotenv.2021.147460

Waterer, D. (2010). Evaluation of biodegradable mulches for production of warm season vegetable crops. Canadian Journal of Plant Science, 90(5), 737–743. http://dx.doi.org/10.4141/CJPS10031

World Bank. (2002). Agenda for water sector strategy for north china (Number 22040- CHA). World Bank/ Rural Development and Natural Resources Unit, Beijing, China.

Wu, C., Yajie, M., Dan, W., Yongpan, S., et al. (2022). Integrated microbiology and metabolomics analysis reveal plastic mulch film residue affects soil microorganisms and their metabolic functions.

Journal of Hazardous Materials, 423, Part B, 127258, https://doi.org/10.1016/j.jhazmat.2021.127258

Xu, D., Ling, J., Qiao, F., Fang, Q., et al. (2022). Organic mulch can suppress litchi downy blight through modification of soil microbial community structure and functional potentials. BMC Microbiology, 22, 155. https://doi.org/10.1186/s12866-022-02492-3

Yaghi, T., Arslan, A., & Naoum, F. (2013). Cucumber (Cucumis sativus, L.) water use efficiency (WUE) under plastic mulch and drip irrigation. Agricultural Water Management, 128, 149-157. http://dx.doi.org/10.1016/j.agwat.2013.06.002

Yaşar, S., & Şahin, H. (2021). The effects of mulching with organic materials on the soil nutrient and carbon transport by runoff under simulated rainfall conditions. Journal of African Earth Sciences, 176, 104152. https://doi.org/10.1016/j.jafrearsci.2021.104152

Yin, H., Xiao, J., Li, Y., Chen, Z., Cheng, X., Zhao, C., & Liu, Q. (2013). Warming effects on root morphological and physiological traits: the potential consequences on soil C dynamics as altered root exudation. Agricultural Forests Meteorology, 180, 287–296. https://doi.org/10.1016/j.agrformet.2013.06.016

Yin, W., Chai, Q., Guo, Y., Fan, H., et al. (2021). No tillage with plastic re-mulching maintains high maize productivity via regulating hydrothermal effects in an arid region. Frontiers in plant science, 12, 649684. http://dx.doi.org/10.3389/fpls.2021.649684

Yu, Y.Y., Turner, N., Gong, Y., Li, F.M., Fang, C., Ge, Li, J., & Ye, J. (2018). Benefits and limitations to straw- and plastic-film mulch on maize yield and water use efficiency: A meta-analysis across hydrothermal gradients. European Journal of Agronomy, 99, 138- http://dx.doi.org/10.1016/j.eja.2018.07.005

Yuan, Y., Zu, M., Zuo, J., Jiajia, Z., Runze, L., & Jun, T. (2022). What will polyethylene film mulching bring to the root-associated microbial community of Paeonia ostii? Applied Microbiology Biotechnology, 106, 4737–4748. https://doi.org/10.1007/s00253-022-11986-z

Zhang, J., Sun, J., Duan, A., Wang, J., Shen, X., & Liu, X. (2007). Effects of different planting patterns on water use and yield performance of winter wheat in the Huang-Huai-Hai plain of China. Agricultural Water Management, 92 (1), 41–47. https://doi.org/10.1016/j.agwat.2007.04.007

Zhang, P., Wei, T., Cai, T., Ali, S., Han, Q., Ren, X., & Jia, Z. (2017). Plastic-film mulching for enhanced water-use efficiency and economic returns from maize fields in semiarid China. Frontiers in Plant Science, 8, 512. https://doi.org/10.3389/fpls.2017.00512

Zhang, S., Wang, Y., Sun, L., Qiu, C., et al. (2020). Organic mulching positively regulates the soil microbial communities and ecosystem functions in tea plantation. BMC Microbiology, 20, 103. https://doi.org/10.1186/s12866-020-01794-8

Zhu, L., Liu, J., Luo, S., Bu, L., Chen, X., & Li, S. (2015). Soil mulching can mitigate soil water deficiency impacts on rainfed maize production in semiarid environments. Journal of Integrative Agriculture, 14 (1), 58–66. https://doi.org/10.1016/S2095-3119(14)60845-5

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Male Fertility Preservation: A boon for young cancer survivors

Vickram A S[1*], Nibedita Dey[1], Kuldeep Dhama[2]

[1]Department of Biotechnology, Saveetha School of Engineering, Saveetha Institute of Medical and Technical Sciences, Chennai, Tamil Nadu, India
[2]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

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ABSTRACT

Diagnosis of any ailment especially cancer is found to be pivotal to evaluating the type of treatment that needs to be administered to man. It aids in subsequent prognosis and timely recovery in patients. When concerned with male cancer survivors, the emphasis on their fertility health is always an issue. As the numbers of survivors are increasing day by day due to the advanced medical and technological approaches, man could look with confidence to a life of ease from cancer. To review and compile all the feasible as well as relevant information about the preservation of male fertility from published resources. Reputed databases were searched for content based on specific keywords like “fertility preservation after cancer treatment", "methods of male gamete preservation", "methods of semen collection for preservation", "fertility preservation", "erectile dysfunction" and "testicular cancer and fertility". The year of publication for articles under study was restricted from 2016-2021 in most of the databases. It was found that oncologists generally recommended preservation of the male fertility before the commencement of the cancer treatment procedures. Preservation of fertility among young men should be considered in all patients before initiating any kind of prognosis related to the disease.

* Corresponding author
E-mail: vickramas.16@gmail.com (Vickram A S)

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1 Introduction

It is very important to maintain the overall reproductive potential of a cancer survivor post-treatment. The ability to sustain the quality of the same also is an essential parameter (Wollersheim et al. 2022). The increase in facilities and technologies available to cure cancer has raised the number of cancer survivors below the age of 65 years (Gordon et al. 2017). As the diagnosis is becoming easier and more effective against cancer, the magnitude of cancer survivors is also increasing (Breidenbach et al. 2022). If put in numbers, around 1-3% are diagnosed with cancer within the age group of 20 years and 9-12% by the age of 45 (Gordon et al. 2017). Statistics have provided evidence that these survivors tend to lead a normal life post-recovery if the treatment or the disease did not affect them in an adverse manner (Miller et al. 2019).

The advancement in therapeutic values has supported medical professionals to heal many cancer patients in the past 20 years (Breidenbach et al. 2022). The treatment generally consists of many harsh measures and approaches with severe side effects, especially on male fertility quality (Arafa and Elbardisi 2020). The process of spermatogenesis can be easily altered as it is sensitive to many potential toxins (Anyanwu and Orisakwe 2020). The techniques involved like radiation therapy, chemotherapy, and other surgical practices impair the normal functionality of ejaculation and could lead to erectile dysfunction (Green et al 2019). Numerous factors affect the quality of male gonads after cancer treatment. Firstly, the effect of the therapy on gametogenesis (Zapata-Restrepo et al. 2019), secondly the quantity and the quality of the sperm pre- and post-treatment, thirdly the medications administered during the prognosis, and fourthly susceptibility of the patient post-treatment and the type of cancer with its correlation with male fertility play an essential role in determining the fate of the reproductive life of a patient after recovery. The overall regime opted by the medical practitioner also influences the sperm count and quality (Byrne et al. 2018). Prediction of the same is also a challenging aspect of the cancer treatment process. Doctors predict a variety of conditions ranging from azoospermia to partial recovery of the patient after cancer therapy. Hence the Ethics Committee of the American Society for Reproductive Medicine has set some norms that need to be followed and intimated well in advance to the male patient before initiating the treatment regime (Chen et al. 2020). The options available to preserve the fertility of the patient should be discussed well in advance to take appropriate measures during the therapy (Lake et al. 2020). The adverse effects should be jotted down clearly and explained well to the recipient (Bhasin et al. 2018). For the 1% of the cases that get detected in young cancer patients hailing in their early reproductive years should consider preservation of their fertility as recommended by clinicians (Munoz et al. 2016). Especially among unmarried men and those who plan on starting a family (Newton et al. 2021). An emotional war is always evident between cancer patients with the anxieties to fight for their survival and later to lead a normal life. This is feasible by cryopreservation, which could protect their fertility status for later years (Kim et al. 2020).

2 Cryopreservation and assisted reproductive technology

The male reproductive potential majorly depends on sperm health and count, so preservation of the same can help in the effective management of male reproductive quality (Colaco and Modi 2018). Cryopreservation is the most sought-after technique in the medical field to preserve sperm. A cancer survivor can opt for the cryopreservation technique before the treatment regime and later utilize assisted reproductive technologies (ART) if needed in the future to lead a normal life (Appiah 2020). Even if the patient has a previous or current medical condition of low sperm motility, abnormality, and other adverse characteristics, still cryopreservation is found to be the best solution to conserve a man's reproductive capability (Hezavehei et al. 2018). Amidst the numerous developments and success scenarios related to ART, only a handful of patients utilize the cryopreserved samples post recovery to attain parenthood (Morgan et al. 2020). The main goal of this freeze preservation was to utilize the samples after treatment to have patients' progeny if demanded in the future. Around 12% of the stored samples available in ART centers are used to have children by post-cancer survivors (Asafu-Adjei and Jenkins 2020). The low utilization of the conserved and preserved samples might be due to the retaining of the reproductive capability of the cancer survivor, stress, lack of interest in parenthood, or the expensive nature of the ART sample retrieval procedures. Even the success rate of the samples stored through ART also plays an important role while preferring to use these cryo sperms for fatherhood (Klipstein et al. 2020). Of the small number of patients who prefer ART post-recovery, around 65% of them do achieve fatherhood (Paoli et al. 2018). Some suggested that the efficiency can go up to 77% in certain cases. Few patients found the whole process for cryopreservation irrelevant and unnecessary as the reutilization percentage was seen to be very low by the cancer survivors before them (Medrano et al. 2018). But, the surety and prediction as to who would retain their fertility and who would restore the same after treatment cannot be easily pointed out by clinicians and doctors. Hence, the most effective and safest way to father a child post-recovery would be cryopreservation.

The mutation load on the semen due to radiation therapy and chemotherapy is a potential hazard faced by many male cancer patients (Nahata et al. 2019). This will in still doubt about genetically defective progeny born to many cancer survivors. Similarly, the same doubtful scenario is seen around samples stored in ART. The genetic normalcy of the progeny and associated

ailments that might occur due to cryopreservation of sperms do haunt the cancer survivors to a large extent (Grin et al. 2021). The process involved with freezing the sample and then thawing it before use could cause unwanted alternations in the genes that lead to progeny with birth and genetic defects (Gianaroli et al. 2019). Boys in their pre-puberty years are not recommended for cryopreservation before their cancer therapies as the active spermatogenesis process wouldn't have reached its maximum potential at this young age (Lei et al. 2020). Testicular tissue could be a better alternative for these patients instead of for living sperms. Later transplantation can be opted to have effective spermatogenesis in these young cancer survivors (Ibtisham et al. 2017). But the experimental complexity of the process is a major drawback of the whole process. The impending urge to have their biological progeny becomes feasible for pre-pubertal cancer survivors after a certain age post-recovery (Lei et al. 2020). Hence, the SickKids fertility preservation program was organized to counsel pre-puberty patients about testicular cryopreservation (Ibtisham et al. 2017). The tram addresses the risks and other effects associated with infertility induced due to radiation and chemotherapy to the family members of the patients. Later motivating them to opt for this cryo method to conserve the fertility state of young boys (Nagirnaja et al. 2018). Testicular tissue [TT] could be collected from the patient before the therapy and a biopsy should be performed for samples from patients with a previous history of cancer onset in them. This uncertainty related to cancer therapy could primarily lead to gonad damage (Jain 2018).

3 Mechanism of DNA damage for testicular cancer patients

Researchers have previously reported that cancer patients have shown more instances of DNA damage when compared to normal patients. But the difference is not found to be too vast among testicular cancer patients and normal infertile men. The conditions of the sperms are found to be quite similar in both scenarios (Cheng et al. 2018; Moody et al. 2019, Panner Selvam et al. 2021). But these reports are limited to the patients who were yet to start therapy and treatment. Hence, suggesting pre-therapy may cause sperm DNA damage. The damage is found to be evident for 2-3 years in the DNA and chromatin among the cancer survivors' post-treatment, especially for testicular cancer. So, the best option is to go for cryobanks before the commencement of the treatment regime. The post-treatment phase has been found to result in damages varying based on the type, dosage of radiation, etc. (Arda et al. 2020). The actual relationship between testicular cancer and the reproductive potential of the patient is still unclear (Arda et al. 2020).

4 Sperm cryopreservation

The safest method to non-invasively collect samples for cryopreservation would be through masturbation or any other technique. These techniques are especially preferred for post-pubertal cancer patients (Machneva et al. 2020). The rate of success was nearly 92% in most of the freshly ejaculated samples for cryopreservation (Vickram et al. 2015a, Parameswari et al. 2017, Pathy et al. 2017, Kumar and Sridharan 2020). The most important requirement would be that the puberty of the patient lies between the age groups of 12 to 15 years and the testis volume around 10 cm^3 or more. For young patients, their last nocturnal seminal emission is very vital (Hoffmann et al. 2021).

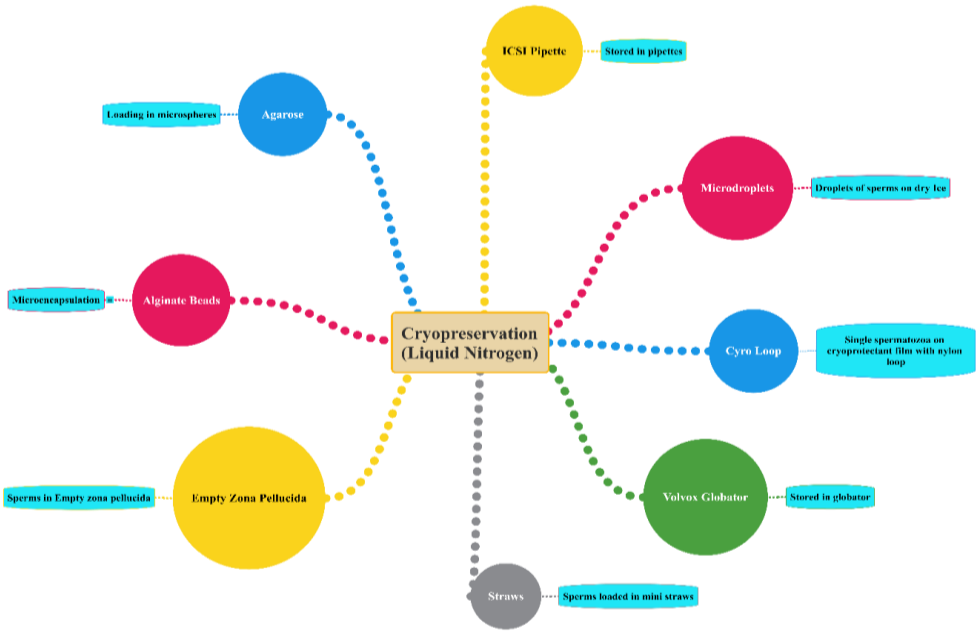

Figure 1 Feasible cryopreservation techniques for human sperm preservation

Cryopreservation of sperms is also possible in azoospermia. This is generally a condition when there is officially nil count of sperms, but pellet assay would show evidence of sperm cells (Pathy et al. 2017). Intracytoplasmic sperm injection can also be opted to transfer a single sperm cell for reproductive purposes (Vickram et al. 2015b). Previously many oncologists weren't suggesting cryopreservation procedures before cancer therapy. If emphasized by the clinicians, around 60% of the patients do agree to deposit their sperm for preservation in banks. Out of these, 70% of them utilize ART to achieve parenthood. This technique is also gaining popularity among patients through mutual contacts and friends (Iannarino and Palmer-Wackerly 2022). Figure 1 gives an outlook on the various feasible techniques for cryopreservation of human sperms.

4.1 Surgery for cryopreservation

The retrieval methods that use surgery as a mode for sperm collection and preservation is mostly considered the last resort (Alouf et al. 2020). This procedure is performed when patients show medical indications of azoospermia, failed ejaculations, and peri-pubertal situations (Talebi et al. 2021). Semen collection is much more effectively done by surgical methods in special cases like non-obstructive and obstructive azoospermia conditions (Hua et al. 2021).

5 Parenthood post testicular cancer therapy

Cancer associated with the testicles is very prevalent and can occur at the peak of the reproductive age man. After successful therapy and treatment of the disease, it is very essential to restore the reproductive status of the individual (Wyns and Kanbar 2021). The ability to father children is an important social and instinctive need of a human male (Ohan et al. 2020). It leads to the overall well-being of the man. The relationship between testicular cancer and its impact on male fertility seems to have a positive correlation that eventually leads to the loss of reproductive traits in men. During the initial stages of diagnosis of testicular cancer, several patients have shown signs of reduced and failed spermatogenesis (Sineath et al. 2021). In these cases, oncologists suggest and prescribe fertility preservation for restoring the existing reproductive status of the cancer patient. In exceptional conditions spermatogenesis has been seen to revive post-therapy. This again purely depends on the amount of radiation being exposed and the extent of therapy based on the severity of cancer (Wyns and Kanbar 2021). If chemotherapy alone is used to cure and treat testicular cancer, 71% of the survivors were able to father children with any ART (Okada and Fujisawa 2019). But the combinational treatment with cisplatin has shown to bring down the conception rate by 54%. This shows that the rate of conception is very much influenced by the protocol utilized for the treatment of testicular cancer. Germ cell testicular cancer is seen on the rise among men in their reproductive years (Goldberg et al. 2019). The efficiency of treating testicular cancer increases to 95% when cisplatin is administered in combination with radiotherapy, but the fate of the sperms becomes a big question mark. Still, it is a known fact that one out of three testicular cancer patients do show defects in their spermatogenesis processes during diagnosis. Hence, it would be wise for oncologists and andrologists to counsel young cancer patients to opt for fertility preservation before undergoing the therapy regime. At least temporarily impaired has been reported due to chemo and radiotherapy on the process of spermatogenesis (McGowan et al. 2017). Dry ejaculation might be very common among post-testicular cancer survivors (Parekh et al. 2020). A longer duration of chemotherapy often leads to this condition in men, which is commonly known as retroperitoneal lymph node disease or failure.

Sympathetic nerves tend to get damaged during the therapy which might lead to hindrance in seminal emission. This phenomenon is also called retrograde ejaculation (Meng et al. 2005). Seminal plasma is very essential for the movement of the sperms in the female reproductive tract. So, ejaculation with seminal plasma will not aid in fertilization (Schoeller et al. 2020). Other possible ways to preserve sperm count and quality naturally in the patient would be limited exposure to the para-aortic area, restricted dosage and duration of radiation therapy for each cycle, dose limitations of chemotherapeutants, etc. (Hamano et al. 2017). The patients feel that attaining parenthood gave a purpose and fulfilment to their life's goals and wishes (Rondhianto et al. 2020). Hence, it is essential to preserve semen before the treatment of testicular cancer (De Roo et al. 2016). It should be a mandatory protocol for patients of their reproductive ages and with no children to perform preservation of their semen and sperm before the commencement of the therapy for cancer (Kyweluk et al. 2018).

6 Neurogenic anejaculation patients

Damage to the spinal cord through injury could be a possible cause of the neurogenic version of anejaculation (Stoffel et al. 2018). These injuries could be related to injuries caused due to accidents or due to an onset of cancer (Brackett et al. 2020). It would create reflex erections usually when oral agents or penile injections which have erectogenic properties are administered (Yafi et al. 2016). A plan for treating testicular cancer would be the categorization of retroperitoneal lymph nodes. This would increase the rate of survival of the patient and then treat infertility (Vaz et al. 2019). The procedure involves the removal of sympathetic chains surgically, that directly leads to seminal emission rectification of normal ejaculation (Butcher and Brannigan 2016). The easiest way among all these techniques would be nerve-sparing protocol. Ejaculation in retroperitoneal lymph node disease is considered to be retrograde ejaculation by many clinicians and researchers (Agha et al. 2017). Failure in the seminal plasma emission is very evident

among the patients while exhibiting dry ejaculation (Andrade-Rocha 2017). If the patient agrees to fertility preservation, then the sperm cells are retrieved through a needle and standard cryopreservation methodologies are followed (Martins et al. 2019).

6.1 Dysfunction in iatrogenic ejaculation

This dysfunction is different from the neurogenic type of failure. It exists due to the intake and administration of a few specific medications like antidepressants and serotonin (Sadowski et al. 2016). These are prescribed for cancer patients who have symptoms of depression, which in turn leads to iatrogenic ejaculation (Shindel et al. 2022). This dysfunction is seen to be irreversible in most cases with a permanent failure in proper ejaculation. Hence, this worsens the situation of cancer survivors as they are unable to initiate a family. Furthermore, drugs administered for genitourinary complications and hypertension also tend to block the sections about prostatic emissions. Therefore, these types are also classified under retrograde ejaculation (Amsterdam et al. 2019).

7 Retrograde ejaculation

At times in some patients, there is a medical condition where the neck of the bladder doesn't close, but other components of the ejaculatory functions are normal (Tanabalan and Ballaro 2019). This is due to the high pressure developed in the bladder. Subsequently leading to adverse anatomic or physiologic abnormality, this is resulting in retrograde ejaculation (Althof and McMahon 2016). The resistance caused due to the high pressure and lack of closure makes the semen flow backward into the bladder (Steuer and Guertin 2019). Their urine exhibits a characteristic cloudy hue due to the presence of semen indicating retrograde ejaculation (Parnham and Serefoglu 2016). Cancer patients might exhibit high pressure and any subsequent surgery-based therapy can affect the closure of the bladder, leading to retrograde ejaculation (Shoshany et al. 2017).

8 Approaches for semen collection for preservation

For an average normal cancer patient, the collection is feasible through masturbation (Kumar et al. 2020). A seven days abstinence is essential before the collection protocol at ART centers (Manna et al 2020). The patient will be provided with a wide container to ejaculate the semen samples after masturbation. Analysis of the sample will be performed to ensure the quality of the sperm. Later cryopreservation techniques will be employed using proper extenders (Skidmore et al. 2018).

9 Penile vibratory simulation

In certain cases of ejaculatory conditions, a penile vibrator will be used to collect semen for fertility preservation (Liu et al 2018). The vibrator will create a stimulus on the tissue when placed on the penis, leading to antegrade ejaculation (Ibrahim et al. 2022). Men with a history of injury or antidepressant medications can opt for this technique for collecting samples for cryopreservation (Chong et al. 2017). The parameters of the vibrator along with continuous adjustment as per the patient's need make this technique very apt for semen collection. 2.7 amplitude at 100 Hz is found to be the optimal parameter to yield maximum semen for collection (Meng et al. 2018). It is advised to keep the vibrator on the frenulum with adequate settings set for ejaculation to occur (Cong et al. 2019). A sterile toxic free wide-mouthed plastic container should be used for collecting the ejaculate. The vibrator should be stopped once ejaculation occurs as continuous usage would lead to dysreflexia (Eldahan and Rabchevsky 2018). Blood pressure should be monitored throughout the process. It generally takes 3 minutes for the entire process to take place. An extra minute is allowed if no ejaculation is seen in the first 3 minutes (Flack and Mellon 2018). Dysreflexia and changes in penile tissues could be one of the main drawbacks that one could face while using this technique. The collection is feasible at the patient's own home if the procedure is clearly explained by the clinicians (Cong et al. 2019). The collected sample will be immediately processed for preservation (Cong et al. 2019).

10 Electroejaculation method - paediatric cancer patients

Patients who failed in collecting semen through vibrators can use rectal probes for the same. These approaches could have improvised results when compared to penile vibrators (Skott et al. 2018). Swagger electro ejaculator is a well-recognized and approved device by the United States Food and drug administration department. Before the procedure nifedipine is given sublingually to avoid any instances of dysreflexia. Around 10mg of the drug is administered at a time (Eldahan and Rabchevsky 2018). The catheter is inserted before the bladder gets stimulated, which aids in emptying the bladder. This is clearing the path for a sperm-friendly environment during this procedure (Furthner et al. 2018). Pre and post-the technique it would be necessary to perform rectoscopy procedure to avoid complications (Barnard et al. 2019). To derive the stimulation a wave pattern seems apt. The rectal probe is inserted into the dorsal lithotomy position to collect anterograde ejaculations effectively (Halpern et al. 2020). A wide-mouthed nontoxic container made of plastic is placed to collect the ejaculate. The container should be proven to be sperm friendly. The initial voltage from r stimulation would be 5V for 5 seconds then discontinued for the 20s. This cycle is repeated until ejaculations occur (Lorenzo et al. 2020). The next cycle commences when the contraction stops. The gradual increase in voltage is done to continue the technique with great efficacy (Anazodo and Ledger 2019). It can go up to 30V if needed. Paediatric cancer patients are administered this technique to

Table 1 Comparison of various cryopreservation techniques used with its pros and cons (Di Santo et al. 2012)

Technique	Mechanism	Merits	Demerits	References
Microdroplets	Droplets of sperms frozen in liquid nitrogen on dry ice	No adhesion to the walls	Cross contamination, difficulty in storage and transportation	Bouamama et al. 2003
Cryoloop	Single spermatozoa on cryoprotectant film with nylon loop frozen in liquid nitrogen	Vitrification, no sample preparation	Cross contamination	Desai et al. 2004; Arraztoa et al. 2022
Straws	Sperms loaded in mini straws	Convenient and simple	Adhesion and loss of sperms	Koscinski et al. 2007; Kaneko et al. 2021
Volvox globator spheres	Stored in globator	Easy thawing	Frequent algae growth	Just et al. 2004
Empty zona pellucida	Sperms in Empty zona pellucida	Less time consuming, easy loading	Cross Contamination	Hassa et al. 2006; Lee et al. 2021
Alginate beads	Microencapsulation	Inert	Less motility	Herrler et al. 2006; AbdelHafez et al. 2009
Agarose microspheres	Loading in microspheres	Non biological carrier	Clinical effect not evaluated	Isaev et al. 2007; Kurihara et al. 2021
Intracytoplasmic Sperm Injection pipette	Stored in pipettes	Simple, convenient	Cross contamination, no long term storage, set up is fragile	Sohn et al. 2003; Baldini et al. 2021

preserve their fertility. Patients with spinal cord injury, spina bifida, multiple sclerosis, situational anejaculation scenarios, retroperitoneal disease, and senile anejac conditions use the electroejaculation technique to collect semen for preservation (Anazodo and Ledger 2019).

11 Collection procedure for idiopathic anejaculation

Infertility due to unknown conditions is referred to as idiopathic infertility. The cause of the existence of the physiological aliment cannot be specifically identified (Perry et al. 2021). 30% of the cases of male infertility fall under this category. Most of the reasons might be due to undiagnosed neurogenic or iatrogenic conditions. But the psychological condition seems to influence this situation the most. Patients with the above-mentioned conditions masturbate normally, but fail to ejaculate during intercourse (Plante et al. 2019). So to preserve semen in these patients' masturbation is the most preferred technique. Cancer patients having similar fertility conditions should opt for masturbation and later cryopreservation. If masturbation doesn't work electroejaculation can be performed (Çayan et al.2017).

12 Methods for preservation

The essential requirement for the preservation of semen is mature spermatozoa. This can be used in *in-vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Sarasa et al. 2020). The most regular method used is masturbation. But in the case of peri-pubertal conditions, immature testicular tissue can be preserved for later. Retrieval of spermatozoa is an issue in these patients (Braye et al. 2019). In recent years, there has been an increase in the preservation of testicular tissue because of continuous counselling and monitoring (Goossens et al. 2020). Hence, 90% of the families of cancer patients agree to preserve mature and immature testicular tissues before starting cancer treatment. After the treatment is completed, a fertility check-up is performed to analyze the status of the patient. If the gonads and the related components and parameters report being normal for conception, then ART is not preferred (Çayan et al. 2017). In case of adverse effects on the male reproductive system are detected, then preserved semen or immature/ mature testicular tissues are utilized. Xenotransplant of the immature tissue to mature spermatozoa is recommended by current clinicians for ART (Zarandi et al. 2018). Problems and hindrances may arise while transferring malignant cells into a cancer survivor. Clinical trials for the xenotransplant of mature spermatozoa from immature testicular tissues are still a long way to go. Still developed countries perform the procedure of preserving immature testicular tissues from cancer patients who are peri-pubertal (Zhang et al. 2009) Table 1 provides a summation of the various cryopreservation techniques used to preserve sperms.

13 Cancer and the sperm characteristics

The correlation between malignancy and baseline sperm characteristics has been discussed well by many researchers previously. The negative effect of cancer on parameters of sperm has been successfully elucidated in various reputed articles (Rives et al. 2017). Most of the patient's sought cryopreservation who had sperm specifications that represented normalcy or intermediate to some extent (Barbaroșie et al. 2021). Table 2 represents the various

Table 2 Comparison of various techniques used before cryopreservation

Age of the patient	Condition	Procedure	Reference
14 years	Hodgkins disease	Transrectal electroejaculation	Skott et al. 2018
peripubertal	Any cancer	masturbation	Tran et al. 2022
	severe necrozoospermia, oligozoospermia, azoospermia	testicular biopsy with testicular sperm extraction	Daudin et al. 2015
Prepubertal or peripubertal with treatment in the process	Azoospermia	spermatogonia stem cells with testicular cell or tissue freezing	Picton et al. 2015
Prepubertal	Tissue banking	vitrification	Poels et al. 2013

techniques that could be opted on a patient before cryopreservation. Patients with testicular cancer exclusively exhibit a reduction in sperm parameters when compared with standard values from the world health organization (Panner et al. 2019). The stage of cancer does not seem to have a proper correlation with semen quality that considers the motility, morphology, and count of the sperm (Ghasemi et al. 2020). The volume of ejaculation was also found to decline in patients suffering from hematopoietic malignancy and testicular cancer. 20% of the cases reported for the previously mentioned conditions had azoospermia irrespective of the presence of obstruction or not (Pelloni et al. 2017). But this group of individuals was not found to be in their reproductive age groups. Chromatin integrity and DNA fragmentation seemed to get worsened in testicular cancer patients (Dave et al. 2021). Aneuploidy was at higher rates in sperms from testicular cancer gonads when compared to healthy control individuals. But the actual mechanism behind cancer and its manipulating effects on sperm characteristics has not been understood well, making the mechanism unclear (Baldi et al. 2021).

14 Nanotechnology and sperm life

The use of nanoparticles to preserve sperms has been of great interest in the past few decades. Through their success in animal models for sperm preservation, utilization of the same is making its way to human clinical trials for sperm conservation. These particles have limited applications for *in vivo* preservations of sperms, but their potential has no bounds when it comes to *in-vitro* based conservation aids. They always act as additional supporting material to the already established system of cryopreservation. The encapsulation of the chemotherapeutic agents within a nanoparticle gives functional features like targeted drug delivery and reduces the potency of the chemo drugs (Ahn et al. 2010, 2013). The lack of free drugs in the plasma decreases the toxicity of these medications on the non-target tissues like sperms and ovaries. The gonads can be well protected while having targeted and specific cancer treatment.

They generally address and rectify the drawbacks found in cryopreservation and thrive to make the whole procedure as efficient as possible. Some nanoparticles are designed to maintain sperm quality for both purification and preservation purposes in cattle. When experimented in boars nano-magnetic iron oxide pods showed results that were compatible and functional with both fresh ejaculates as well as frozen-thawed semen. These particles seemed to increase the rate of conception in cattle too (Huang and Juang 2011; Odhiambo et al. 2014; Ajinkya et al. 2020). High antioxidant features of a few nanoparticles like selenium, zinc, and cerium have been reported to protect semen from reactive oxygen species generated using the cryopreservation procedure (Jahanbin et al. 2015; Falchi et al. 2016; Khalil et al. 2019). But these effects have been seen in animal models to date. Clinical trials on human sperm and nanoparticles still have a long way to travel. Cryopreservation is known to create undesired oxidative stress on the spermatozoa that impair the integrity of spermatozoon membranes. Even mitochondrial and nuclear DNA content gets reduced to a large extent. Hence decreases the aptitude of fertilization in the frozen sperms compared to fresh ejaculates (Falchi et al. 2018a). During the process of freeze preservation, synthetic extenders are used to collect semen samples that provide a nutritional medium and support to the spermatozoa from freeze shock and microbial infestation. If further innovative changes are made to the extenders the negative effects of the cryopreservation procedure can be handled effectively. Nano water as a synthetic extender has promised novel properties of low viscosity, low density, antimicrobial property, high diffusivity, low dielectric constant, and nil thermal coefficients. Thus, claims to be a promising platform to dissolve lipids and a better nutrient and inorganic compound carrier than normal water. Media containing nano water exhibited better fertilization ability in frozen-thawed semen of ram. The ewes impregnated with the same also produced healthy lambs. Nano minerals tend to improve the quality parameters of semen while freezing and thawing. Among animal models' cerium oxide is quite well known for semen storing applications at 4 C for up to four days. This nanoparticle is reported to improve the motility of sperms (ram) after 2 days and maintain the DNA and plasma integrity throughout the 4 days (Falchi et al. 2018b). Selenium nanoparticles also tend to have ROS scavenging activity against sperm cells. 1mg/L of selenium nanoparticles in Holstein bull's frozen sperms manifested improved quality of the same towards fertilization. They reduced apoptosis, damage, and lipid

peroxidation caused due to cryopreservation (Khalil et al. 2019). Similarly, zinc nanoparticles decrease lipid peroxidation and enhance mitochondrial activity. The overall sperm functionality is maintained by optimizing the dosage of the nanoparticle (Afifi 2015). Cryopreservation could also re-localize the phospholipids of the cell membrane of frozen sperms. Stabilizing the membrane using nanoparticles can compensate for the free fatty acids and phospholipids that get altered or removed from the membrane of the spermatozoa during the freezing process (Nadri et al. 2019). Extenders containing nano lecithin (2%) used for diluting goat semen showed traces of improving sperm cryo survival. It lowered the chances of apoptosis and enhanced membrane functionality and motility of the sperm when compared with extenders containing egg yolk (Nadri et al. 2019). The general morphology of the sperm and semen is quite similar in most higher mammals. These particles can also be employed along with cryopreservation on cancer patients to effectively preserve their samples for the future.

15 Futuristic options for fertility revival

Spermatogonial stem cells (SSC) accompanied by autotransplantation can be considered the most effective method to restore fertility in cancer patients. Cryopreserved testicular tissue can be transplanted into the rete testis. SSC can proliferate and colonize by spermatogenesis and provide optimum sperm population to the cancer survivor (Brinster 2007). Xenotransplantation has reported excellent results in *in-vitro* studies (Sadri et al. 2011). A major risk still lurking around this promising technique is the uncertainty of reintroducing the host's cancerous cells again into the recovered patient body. Patients with leukaemia or metastasis of the blood are at high risk of relapse under the given conditions (Sadri et al. 2014). Currently, there are not many references that exclude the possibility of reoccurrence of cancer by autotransplantation, thus re-implantation strategies for real-time clinical application are still under study. SSC cells are maintained in their natural niche and can only be proposed if the presence of tumor cells is excluded (Liu et al. 2020). Xenografts have shown full spermatogenesis in several animal species but never in humans (Goossens et al. 2008). Human testis grafts were found to have better survival and initial differentiation rates in rats when compared to human models (Van Saen et al. 2011). Hence, extensive research is needed to understand the relationship between autotransplantation and the reoccurrence of cancer to put this promising technology into mainstream clinical practice.

Conclusion

Collecting semen samples from cancer patients is a non-invasive and easy procedure that doesn't tend to delay the cancer therapy process in any way. Oncologists recommend and prefer cryopreservation of sperms, especially for post-pubertal patients without much hesitation. The range of azoospermia conditions after chemotherapy or radiation therapy varies from 0 to 65%. Hence it is advisable to preserve the sperms for future progeny and to attain fatherhood. The recent development and improvisation in ART technologies have helped patients to preserve their samples well for childbirth later in life. As the number of cancer survivors is increasing, the desire to attain a normal life makes man hopeful about fatherhood. In our opinion, the preservation of fertility should be made mandatory before starting any cancer-related treatments and therapies.

Conflict of interest

The authors declare no conflicts for this article.

Consent for Publication

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References

AbdelHafez, F., Bedaiwy, M., El-Nashar, S. A., Sabanegh, E., & Desai, N. (2009). Techniques for cryopreservation of individual or small numbers of human spermatozoa: a systematic review. *Human Reproduction Update*, *15*(2), 153-164.

Afifi, M., Almaghrabi, O. A., & Kadasa, N. M. (2015). Ameliorative effect of zinc oxide nanoparticles on antioxidants and sperm characteristics in streptozotocin-induced diabetic rat testes. *BioMed Research International*, *2015*, 153573. doi: 10.1155/2015/153573.

Agha, R. A., Borrelli, M. R., Vella-Baldacchino, M., Thavayogan, R., Orgill, D. P., Pagano, D., & Pidgeon, T. E. (2017). The STROCSS statement: strengthening the reporting of cohort studies in surgery. *International Journal of Surgery*, *46*, 198-202.

Ahn, R. W., Barrett, S. L., Raja, M. R., Jozefik, J. K., et al. (2013). Nano-encapsulation of arsenic trioxide enhances efficacy against murine lymphoma model while minimizing its impact on ovarian reserve in vitro and in vivo. *PloS one*, *8*(3), e58491.

Ahn, R. W., Chen, F., Chen, H., Stern, S. T., et al. (2010). A Novel Nanoparticulate Formulation of Arsenic Trioxide with Enhanced Therapeutic Efficacy in a Murine Model of Breast Cancer Enhanced Antitumor Efficacy of As2O3-Loaded Nanobins. *Clinical cancer research*, *16*(14), 3607-3617

Ajinkya, N., Yu, X., Kaithal, P., Luo, H., Somani, P., & Ramakrishna, S. (2020). Magnetic iron oxide nanoparticle (IONP)

synthesis to applications: present and future. *Materials*, *13*(20), 4644.

Alouf, C. A., Celia, G. F., & Centola, G. (2020). Semen Cryopreservation: A Practical Guide. In G.N. Allahbadia, B. Ata, S.R. Lindheim, B.J. Woodward, B.Bhagavath, (eds) *Textbook of Assisted Reproduction.* Springer, Singapore. https://doi.org/10.1007/978-981-15-2377-9_56

Althof, S. E., & McMahon, C. G. (2016). Contemporary management of disorders of male orgasm and ejaculation. *Urology*, *93*, 9-21.

Amsterdam, S. H., Stanev, T. K., Zhou, Q., Lou, A. J. T., et al. (2019). Electronic coupling in metallophthalocyanine–transition metal dichalcogenide mixed-dimensional heterojunctions. *ACS nano*, *13*(4), 4183-4190.

Anazodo, A., & Ledger, W. (2019). Assessing and Supporting Adolescent Boys Having Fertility Preservation. In T. Woodruff, D. Shah, W. Vitek, (eds) *Textbook of Oncofertility Research and Practice* (pp. 507-512). Springer, Cham.

Andrade-Rocha, F. T. (2017). On the origins of the semen analysis: A close relationship with the history of the reproductive medicine. *Journal of human reproductive sciences*, *10*(4), 242.

Anyanwu, B. O., & Orisakwe, O. E. (2020). Current mechanistic perspectives on male reproductive toxicity induced by heavy metals. *Journal of Environmental Science and Health, Part C*, *38*(3), 204-244.

Appiah, L. C. (2020). Fertility Preservation for Adolescents Receiving Cancer Therapies. *Clinical Obstetrics and Gynecology*, *63*(3), 574-587.

Arafa, M. M., & Elbardisi, H. T. (2020). Fertility Preservation for Boys and Adolescents. In S. Parekattil, S. Esteves, A. Agarwal, (eds) *Male Infertility* (pp. 819-829). Springer, Cham.

Arda, E., Arikan, G., Akdere, H., Akgul, M., & Yuksel, I. (2020). Predictive and prognostic impact of preoperative complete blood count based systemic inflammatory markers in testicular cancer. *International braz j urol*, *46*, 216-223.

Arraztoa, C. C., Miragaya, M. H., Chaves, M. G., Carretero, M. I., et al. (2022). Cryoprotectant-free vitrification of llama spermatozoa: cryoloop vs sphere method, warmed rapidly or ultra-rapidly. *Small Ruminant Research*, *206*, 106576.

Asafu-Adjei, D. A., & Jenkins, L. C. (2020). Ethical Dimensions of Male Infertility. In S. Parekattil, S. Esteves, A. Agarwal, (eds) *Male Infertility* (pp. 839-843). Springer, Cham.

Baldi, E., Muratori, M., Marchiani, S., Tamburrino, L., & Degl'Innocenti, S. (2021). Main Effects of In Vitro Manipulation of Human Spermatozoa. In L. Björndahl, J. Flanagan, R. Holmberg, U. Kvist, (eds) *XIIIth International Symposium on Spermatology* (pp. 263-272). Springer, Cham.

Baldini, D., Ferri, D., Baldini, G. M., Lot, D., Catino, A., Vizziello, D., & Vizziello, G. (2021). Sperm Selection for ICSI: Do We Have a Winner? *Cells*, *10*(12), 3566.

Barbăroșie, C., Agarwal, A., & Henkel, R. (2021). Diagnostic value of advanced semen analysis in evaluation of male infertility. *Andrologia*, *53*(2), e13625.

Barnard, E. P., Dhar, C. P., Rothenberg, S. S., Menke, M. N., Witchel, S. F., Montano, G. T., & Valli-Pulaski, H. (2019). Fertility preservation outcomes in adolescent and young adult feminizing transgender patients. *Pediatrics*, *144*(3), e20183943. doi: 10.1542/peds.2018-3943.

Bhasin, S., Brito, J. P., Cunningham, G. R., Hayes, F. J., et al. (2018). Testosterone therapy in men with hypogonadism: an Endocrine Society clinical practice guideline. *The Journal of Clinical Endocrinology & Metabolism*, *103*(5), 1715-1744.

Bouamama, N., Briot, P., & Testart, J. (2003). Comparison of two methods of cryoconservation of sperm when in very small numbers. *Gynecologie, Obstetrique & Fertilite*, *31*(2), 132-135.

Brackett, N. L., Ibrahim, E., & Lynne, C. M. (2020). Impact of Spinal Cord Injury. In S. Parekattil, S. Esteves, A. Agarwal, (eds) *Male Infertility* (pp. 487-496). Springer, Cham.

Braye, A., Tournaye, H., & Goossens, E. (2019). Setting up a cryopreservation programme for immature testicular tissue: lessons learned after more than 15 years of experience. *Clinical Medicine Insights: Reproductive Health*, *13*, 1179558119886342.

Breidenbach, C., Heidkamp, P., Hiltrop, K., Pfaff, H., Enders, A., Ernstmann, N., & Kowalski, C. (2022). Prevalence and determinants of anxiety and depression in long-term breast cancer survivors. *BMC psychiatry*, *22*(1), 1-10.

Brinster, R.L. (2007) Male germline stem cells: from mice to men. *Science* 316:404–405

Butcher, M. J., & Brannigan, R. E. (2016). Ejaculatory Disorders. In *Contemporary Treatment of Erectile Dysfunction* (pp. 335-359). Humana Press, Cham.

Byrne, J., Grabow, D., Campbell, H., O'Brien, K., Bielack, S., Zehnhoff-Dinnesen, A., & Modan, D. (2018). PanCareLIFE: The scientific basis for a European project to improve long-term care

regarding fertility, ototoxicity and health-related quality of life after cancer occurring among children and adolescents. *European Journal of Cancer*, *103*, 227-237

Çayan, S., Şahin, S., & Akbay, E. (2017). Paternity rates and time to conception in adolescents with varicocele undergoing microsurgical varicocele repair vs observation only: a single institution experience with 408 patients. *The Journal of Urology*, *198*(1), 195-201

Chen, T. W. W., Jan, I. S., Chang, D. Y., Lin, C. H., et al. (2020). Systemic treatment of breast cancer with leptomeningeal metastases using bevacizumab, etoposide and cisplatin (BEEP regimen) significantly improves overall survival. *Journal of neuro-oncology*, *148*(1), 165-172.

Cheng, L., Albers, P., Berney, D. M., Feldman, D. R., Daugaard, G., Gilligan, T., & Looijenga, L. H. (2018). Testicular cancer. *Nature Reviews Disease Primers*, *4*(1), 1-24.

Chong, W., Ibrahim, E., Aballa, T. C., Lynne, C. M., & Brackett, N. L. (2017). Comparison of three methods of penile vibratory stimulation for semen retrieval in men with spinal cord injury. *Spinal cord*, *55*(10), 921-925.

Colaco, S., & Modi, D. (2018). Genetics of the human Y chromosome and its association with male infertility. *Reproductive biology and endocrinology*, *16*(1), 1-24.

Cong, R., Zhang, Q., Wang, Y., Meng, X., Wang, Z., & Song, N. (2019). Two cases of psychogenic anejaculation patients got normal ejaculation ability after penile vibratory stimulation or electroejaculation. *Translational Andrology and Urology*, *8*(6), 758.

Daudin, M., Rives, N., Walschaerts, M., Drouineaud, V., et al. (2015). Sperm cryopreservation in adolescents and young adults with cancer: results of the French national sperm banking network (CECOS). *Fertility and sterility*, *103*(2), 478-486.

Dave, P., Farber, N., & Vij, S. (2021). Conventional semen analysis and advanced sperm function tests in diagnosis and management of varicocele. *Andrologia*, *53*(2), e13629.

De Roo, C., Tilleman, K., T'Sjoen, G., & De Sutter, P. (2016). Fertility options in transgender people. *International Review of Psychiatry*, *28*(1), 112-119.

Desai, N. N., Blackmon, H., & Goldfarb, J. (2004). Single sperm cryopreservation on cryoloops: an alternative to hamster zona for freezing individual spermatozoa. *Reproductive BioMedicine Online*, *9*(1), 47-53.

Di Santo, M., Tarozzi, N., Nadalini, M., & Borini, A. (2012). Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. *Advances in urology*, *2012*, *854837*. doi: 10.1155/2012/854837

Eldahan, K. C., & Rabchevsky, A. G. (2018). Autonomic dysreflexia after spinal cord injury: systemic pathophysiology and methods of management. *Autonomic Neuroscience*, *209*, 59-70.

Falchi, L., Bogliolo, L., Galleri, G., Ariu, F., et al. (2016). Cerium dioxide nanoparticles did not alter the functional and morphologic characteristics of ram sperm during short-term exposure. *Theriogenology*, *85*(7), 1274-1281.

Falchi, L., Galleri, G., Dore, G. M., Zedda, M. T., et al. (2018a). Effect of exposure to CeO2 nanoparticles on ram spermatozoa during storage at 4 C for 96 hours. *Reproductive Biology and Endocrinology*, *16*(1), 1-10.

Falchi, L., Khalil, W. A., Hassan, M., & Marei, W. F. (2018b). Perspectives of nanotechnology in male fertility and sperm function. *International Journal of Veterinary Science and Medicine*, *6*(2), 265-269.

Flack, C. K., & Mellon, M. J. (2018). Current management strategies for autonomic dysreflexia. *Current Bladder Dysfunction Reports*, *13*(4), 224-229.

Furthner, E., Cordonnier, N., Le Dudal, M., Fontbonne, A., & Freiche, V. (2018). Is electroejaculation a safe procedure in cats? An endoscopic and histological prospective blinded study. *Theriogenology*, *119*, 69-75.

Ghasemi, B., Mehrjardi, A. M., Jones, C., & Ghasemi, N. (2020). Semen analysis of subfertility caused by testicular carcinoma. *International Journal of Reproductive BioMedicine*, *18*(7), 539.

Gianaroli, L., Ferraretti, A. P., & Kovačič, B. (2019). Monitoring ART Safety and Biovigilance. In D. M. Kissin, G. D. Adamson, G. Chambers & C. D. Geyter (eds) *Assisted Reproductive Technology Surveillance* (pp. 56 – 68), Cambridge University Press.

Goldberg, H., Klaassen, Z., Chandrasekar, T., Fleshner, N., Hamilton, R. J., & Jewett, M. A. (2019). Germ cell testicular tumors—contemporary diagnosis, staging and management of localized and advanced disease. *Urology*, *125*, 8-19.

Goossens, E., Geens, M., De Block, G., & Tournaye, H. (2008). Spermatogonial survival in long-term human prepubertal xenografts. *Fertility and sterility*, *90*(5), 2019-2022.

Goossens, E., Jahnukainen, K., Mitchell, R. T., Van Pelt, A. M. M., et al. (2020). Fertility preservation in boys: recent

developments and new insights. *Human reproduction open*, *2020*(3), hoaa016. https://doi.org/10.1093/hropen/hoaa016.

Gordon, L. G., Merollini, K., Lowe, A., & Chan, R. J. (2017). A systematic review of financial toxicity among cancer survivors: we can't pay the co-pay. *The Patient-Patient-Centered Outcomes Research*, *10*(3), 295-309.

Green, T. P., Saavedra-Belaunde, J., & Wang, R. (2019). Ejaculatory and orgasmic dysfunction following prostate cancer therapy: Clinical management. *Medical Sciences*, *7*(12), 109.

Grin, L., Girsh, E., & Harlev, A. (2021). Male fertility preservation–Methods, indications and challenges. *Andrologia*, *53*(2), e13635.

Halpern, J. A., Das, A., Faw, C. A., & Brannigan, R. E. (2020). Oncofertility in adult and pediatric populations: options and barriers. *Translational Andrology and Urology*, *9* (Suppl 2), S227.

Hamano, I., Hatakeyama, S., & Ohyama, C. (2017). Fertility preservation of patients with testicular cancer. *Reproductive Medicine and Biology*, *16*(3), 240-251.

Hassa, H., Gurer, F., Yildirim, A., Can, C., Sahinturk, V., & Tekin, B. (2006). A new protection solution for freezing small numbers of sperm inside empty zona pellucida: Osmangazi-Turk solution. *Cell Preservation Technology*, *4*(3), 199-208.

Herrler, A., Eisner, S., Bach, V., Weissenborn, U., & Beier, H. M. (2006). Cryopreservation of spermatozoa in alginic acid capsules. *Fertility and sterility*, *85*(1), 208-213.

Hezavehei, M., Sharafi, M., Kouchesfahani, H. M., Henkel, R., Agarwal, A., Esmaeili, V., & Shahverdi, A. (2018). Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. *Reproductive biomedicine online*, *37*(3), 327-339.

Hoffmann, H. M., Meadows, J. D., Breuer, J. A., Yaw, A. M., et al. (2021). The transcription factors SIX3 and VAX1 are required for suprachiasmatic nucleus circadian output and fertility in female mice. *Journal of Neuroscience Research*, *99*(10), 2625-2645.

Hua, R., Chu, Q. J., Zhou, Y., Zhou, X., Huang, D. X., & Zhu, Y. T. (2021). MicroRNA-449a suppresses mouse spermatogonia proliferation via inhibition of CEP55. *Reproductive Sciences*, *28*(2), 595-602.

Huang, S. H., & Juang, R. S. (2011). Biochemical and biomedical applications of multifunctional magnetic nanoparticles: a review. *Journal of Nanoparticle Research*, *13*(10), 4411-4430.

Iannarino, N. T., & Palmer-Wackerly, A. L. (2022). Fertility preservation decision-making communication between young adult cancer patients and their romantic partners: An application of the DECIDE typology. *Health Communication*, *37*(6), 778-789.

Ibrahim, E., Brackett, N. L., & Lynne, C. M. (2022). Penile Vibratory Stimulation for Semen Retrieval in Men with Spinal Cord Injury: Patient Perspectives. *Research and Reports in Urology*, *14*, 149.

Ibtisham, F., Wu, J., Xiao, M., An, L., Banker, Z., Nawab, A., & Li, G. (2017). Progress and future prospect of in vitro spermatogenesis. *Oncotarget*, *8*(39), 66709.

Isaev, D. A., Zaletov, S. Y., Zaeva, V. V., Zakharova, E. E., Shafei, R. A., & Krivokharchenko, I. S. (2007). Artificial microcontainers for cryopreservation of solitary spermatozoa. *Human Reproduction*, *22* (Suppl 1), i154.

Jahanbin, R., Yazdanshenas, P., Amin Afshar, M., Mohammadi Sangcheshmeh, A., et al. (2015). Effect of zinc nano-complex on bull semen quality after freeze-thawing process. *Animal Production*, *17*(2), 371-380.

Jain, K. K. (2018). A critical overview of targeted therapies for glioblastoma. *Frontiers in oncology*, *8*, 419.

Just, A., Gruber, I., Wöber, M., Lahodny, J., Obruca, A., & Strohmer, H. (2004). Novel method for the cryopreservation of testicular sperm and ejaculated spermatozoa from patients with severe oligospermia: a pilot study. *Fertility and sterility*, *82*(2), 445-447.

Kaneko, R., Kakinuma, T., Sato, S., & Jinno-Oue, A. (2021). Improvement of short straws for sperm cryopreservation: installing an air-permeable filter facilitates handling. *The Journal of reproduction and development*, *67*(3), 235–239. https://doi.org/10.1262/jrd.2021-019

Khalil, W. A., El-Harairy, M. A., Zeidan, A. E., & Hassan, M. A. (2019). Impact of selenium nano-particles in semen extender on bull sperm quality after cryopreservation. *Theriogenology*, *126*, 121-127.

Kim, H., Zhou, E. S., Chevalier, L., Lun, P., Davidson, R. D., Pariseau, E. M., & Long, K. A. (2020). Parental behaviors, emotions at bedtime, and sleep disturbances in children with cancer. *Journal of Pediatric Psychology*, *45*(5), 550-560.

Klipstein, S., Fallat, M. E., Savelli, S., Katz, A. L., Macauley, R. C., Mercurio, M. R., & COMMITTEE ON BIOETHICS. (2020). Fertility preservation for pediatric and adolescent patients with

cancer: medical and ethical considerations. *Pediatrics*, *145(3):*e20193994. doi: 10.1542/peds.2019-3994

Koscinski, I., Wittemer, C., Lefebvre-Khalil, V., Marcelli, F., Defossez, A., & Rigot, J. M. (2007). Optimal management of extreme oligozoospermia by an appropriate cryopreservation programme. *Human Reproduction*, *22*(10), 2679-268

Kumar, A., & Sridharan, T. B. (2020). Oxidative Stress-induced Changes in Fertility Status of Cryopreserved Sperm: A Diagnosis Based on Sperm Chromatin Dispersion Assay. *Cryoletters*, *41*(5), 297-302.

Kumar, A., Vickram, A. S., & Sridharan, T. B. (2020). Oxidation driven surface hydrophobicity in human seminal plasma results in protein structural changes. *Journal of Molecular Liquids*, *316*, 113900.

Kurihara, M., Fukushima, M., Miyata, A., Tanaka, T., Sugimoto, K., & Okada, H. (2021). Comparative study of agarose-gel microcapsules and Cryotop in cryopreservation of extremely small numbers of human spermatozoa. *Systems Biology in Reproductive Medicine*, *67*(3), 244-250.

Kyweluk, M. A., Sajwani, A., & Chen, D. (2018). Freezing for the future: transgender youth respond to medical fertility preservation. *International Journal of Transgenderism*, *19*(4), 401-416.

Lake, P. W., Kasting, M. L., Dean, M., Fuzzell, L., Hudson, J., Carvajal, R., & Vadaparampil, S. T. (2020). Exploring patient and provider perspectives on the intersection between fertility, genetics, and family building. *Supportive Care in Cancer*, *28*(10), 4833-4845.

Lee, H. C., Balough, J. L., Roth, E. W., Vaccari, S., & Duncan, F. E. (2021). A decellularized oocyte-derived scaffold provides a "sperm safe" to preserve mammalian spermatozoa. *Andrology*, *9*(3), 922-932.

Lei, Q., Lai, X., Eliveld, J., de Sousa Lopes, S. M. C., van Pelt, A. M., & Hamer, G. (2020). In vitro meiosis of male germline stem cells. *Stem cell reports*, *15*(5), 1140-1153.

Liu, H. C., Xie, Y., Deng, C. H., & Liu, G. H. (2020). Stem cell-based therapies for fertility preservation in males: current status and future prospects. *World journal of stem cells*, *12*(10), 1097.

Liu, X., Wang, G., Xue, S., Huang, Q., & Yang, S. (2018). High efficient and non-invasive collection of ejaculates from rats using penile vibratory stimulation. *Theriogenology*, *106*, 192-197.

Lorenzo, A. J., Rickard, M., & Santos, J. D. (2020). The role of bladder function in the pathogenesis and treatment of urinary tract infections in toilet-trained children. *Pediatric Nephrology*, *35*(8), 1395-1408.

Machneva, E. B., Panarina, V. Y., Aliev, T. Z., Shevtsov, D. V., et al. (2020). Chronic "graft versus host" disease after allogeneic hematopoietic stem cell transplantation: basic characteristics, pathogenetic mechanisms, treatment strategies and problems of clinical practice. *Russian Journal of Pediatric Hematology and Oncology*, *7*(2), 94-111

Manna, C., Barbagallo, F., Manzo, R., Rahman, A., Francomano, D., & Calogero, A. E. (2020). Sperm parameters before and after swim-up of a second ejaculate after a short period of abstinence. *Journal of Clinical Medicine*, *9*(4), 1029.

Martins, A. D., Agarwal, A., & Henkel, R. (2019). Sperm cryopreservation. In Z. Nagy, A. Varghese, & A. Agarwa, (eds) *In vitro fertilization* (pp. 625-642). Springer, Cham.

McGowan, J. V., Chung, R., Maulik, A., Piotrowska, I., Walker, J. M., & Yellon, D. M. (2017). Anthracycline chemotherapy and cardiotoxicity. *Cardiovascular drugs and therapy*, *31*(1), 63-75.

Medrano, J. V., del Mar Andrés, M., García, S., Herraiz, S., Vilanova-Pérez, T., Goossens, E., & Pellicer, A. (2018). Basic and clinical approaches for fertility preservation and restoration in cancer patients. *Trends in biotechnology*, *36*(2), 199-215.

Meng, M. V., Greene, K. L., & Turek, P. J. (2005). Surgery or assisted reproduction? A decision analysis of treatment costs in male infertility. *The Journal of Urology*, *174*(5), 1926-1931.

Meng, X., Fan, L., Wang, T., Wang, S., Wang, Z., & Liu, J. (2018). Electroejaculation combined with assisted reproductive technology in psychogenic anejaculation patients refractory to penile vibratory stimulation. *Translational Andrology and Urology*, *7*(1), S17.

Miller, K. D., Nogueira, L., Mariotto, A. B., Rowland, J. H., et al. (2019). Cancer treatment and survivorship statistics, 2019. *CA: A Cancer Journal for Clinicians*, *69*(5), 363-385.

Moody, J. A., Ahmed, K., Yap, T., Minhas, S., & Shabbir, M. (2019). Fertility managment in testicular cancer: the need to establish a standardized and evidence-based patient-centric pathway. *BJU international*, *123*(1), 160-172.

Morgan, T. L., Young, B. P., Lipak, K. G., Lehmann, V., Klosky, J., Quinn, G. P., & Nahata, L. (2020). "We can always adopt": perspectives of adolescent and young adult males with cancer and their family on alternatives to biological parenthood. *Journal of Adolescent and Young Adult Oncology*, *9*(5), 572-578.

Muñoz, M., Santaballa, A., Seguí, M. A., Beato, C., de la Cruz, S., Espinosa, J., & Blasco, A. (2016). SEOM Clinical Guideline of fertility preservation and reproduction in cancer patients (2016). *Clinical and Translational Oncology*, *18*(12), 1229-1236.

Nadri, T., Towhidi, A., Zeinoaldini, S., Martínez-Pastor, F., et al. (2019). Lecithin nanoparticles enhance the cryosurvival of caprine sperm. *Theriogenology*, *133*, 38-44

Nagirnaja, L., Aston, K. I., & Conrad, D. F. (2018). Genetic intersection of male infertility and cancer. *Fertility and sterility*, *109*(1), 20-26.

Nahata, L., Morgan, T. L., Ferrante, A. C., Caltabellotta, N. M., Yeager, N. D., Rausch, J. R., & Gerhardt, C. A. (2019). Congruence of reproductive goals and fertility-related attitudes of adolescent and young adult males and their parents after cancer treatment. *Journal of Adolescent and Young Adult Oncology*, *8*(3), 335-341.

Newton, K., Howard, A., Thorne, S., Kelly, M. T., & Goddard, K. (2021). Facing the unknown: uncertain fertility in young adult survivors of childhood cancer. *Journal of cancer survivorship*, *15*(1), 54-65.

Odhiambo, J. F., DeJarnette, J. M., Geary, T. W., Kennedy, C. E., Suarez, S. S., Sutovsky, M., & Sutovsky, P. (2014). Increased conception rates in beef cattle inseminated with nanopurified bull semen. *Biology of reproduction*, *91*(4), 97-1.

Ohan, J. L., Jackson, H. M., Bay, S., Morris, J. N., & Martini, A. (2020). How psychosocial interventions meet the needs of children of parents with cancer: A review and critical evaluation. *European Journal of Cancer Care*, *29*(5), e13237.

Okada, K., & Fujisawa, M. (2019). Recovery of spermatogenesis following cancer treatment with cytotoxic chemotherapy and radiotherapy. *The World Journal of Men's Health*, *37*(2), 166-174.

Panner Selvam, M. K., Ambar, R. F., Agarwal, A., & Henkel, R. (2021). Etiologies of sperm DNA damage and its impact on male infertility. *Andrologia*, 53(1), e13706. https://doi.org/10.1111/and.13706.

Paoli, D., Pallotti, F., Lenzi, A., & Lombardo, F. (2018). Fatherhood and sperm DNA damage in testicular cancer patients. *Frontiers in endocrinology*, *9*, 506.

Parameswari, R., Rao, K. A., Manigandan, P., Vickram, A. S., Archana, K., & Sridharan, T. B. (2017). Tea polyphenol-T. arjuna bark as sperm antioxidant extender in infertile smokers. *Cryoletters*, *38*(2), 95-99.

Parekh, N. V., Lundy, S. D., & Vij, S. C. (2020). Fertility considerations in men with testicular cancer. *Translational andrology and urology*, *9*(Suppl 1), S14.

Parnham, A., & Serefoglu, E. C. (2016). Retrograde ejaculation, painful ejaculation and hematospermia. *Translational andrology and urology*, *5*(4), 592.

Pathy M, R., Vickram, A. S., Sridharan, T. B., Parameswari, R., Archana, K., Nithya, S., & Mishika, A. (2017). Optimization of Human Semen Extender Components for Cryopreservation Using Statistical Tools. *CryoLetters*, *38*(6), 434-444.

Pelloni, M., Coltrinari, G., Paoli, D., Pallotti, F., Lombardo, F., Lenzi, A., & Gandini, L. (2017). Differential expression of miRNAs in the seminal plasma and serum of testicular cancer patients. *Endocrine*, *57*(3), 518-527.

Perry, S. M., Park, T., & Mitchell, M. A. (2021). Sex, drugs and rock iguanas: testicular dynamics and plasma testosterone concentrations could predict optimal semen collection times in Cyclura. *Reproduction, Fertility and Development*, *34*(5), 417-427.

Picton, H. M., Wyns, C., Anderson, R. A., Goossens, E., et al. (2015). A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. *Human reproduction*, *30*(11), 2463-2475.

Plante, M., Gilling, P., Barber, N., Bidair, M., Anderson, P., Sutton, M., & Roehrborn, C. (2019). Symptom relief and anejaculation after aquablation or transurethral resection of the prostate: subgroup analysis from a blinded randomized trial. *BJU International*, *123*(4), 651-660.

Poels, J., Van Langendonckt, A., Many, M. C., Wese, F. X., & Wyns, C. (2013). Vitrification preserves proliferation capacity in human spermatogonia. *Human reproduction*, *28*(3), 578-589.

Rives, N., Walschaerts, M., Setif, V., Hennebicq, S., et al. (2017). Sperm aneuploidy after testicular cancer treatment: data from a prospective multicenter study performed within the French Centre d'Étude et de Conservation des Oeufs et du Sperme network. *Fertility and Sterility*, *107*(3), 580-588.

Rondhianto, R., Nursalam, N., Kusnanto, K., & Melaniani, S. (2020). Development Family Caregiver Empowerment Model (FCEM) To Improve Family Caregiver Capability on Type 2 Diabetes Self-Management. *Systematic Reviews in Pharmacy*, *11*(6):1042-1051

Sadowski, D. J., Butcher, M. J., & Köhler, T. S. (2016). A review of pathophysiology and management options for delayed ejaculation. *Sexual Medicine Reviews*, *4*(2), 167-176..

Sadri-Ardekani, H., Akhondi, M. A., van der Veen, F., Repping, S., & van Pelt, A. M. (2011). In vitro propagation of human prepubertal spermatogonial stem cells. *Jama*, *305*(23), 2416-2418.

Sadri-Ardekani, H., Homburg, C. H., van Capel, T. M., van den Berg, H., et al. (2014). Eliminating acute lymphoblastic leukemia cells from human testicular cell cultures: a pilot study. *Fertility and sterility*, *101*(4), 1072-1078

Sarasa, J., Enciso, M., García, L., Leza, A., Steger, K., & Aizpurua, J. (2020). Comparison of ART outcomes in men with altered mRNA protamine 1/protamine 2 ratio undergoing intracytoplasmic sperm injection with ejaculated and testicular spermatozoa. *Asian Journal of Andrology*, *22*(6), 623.

Schoeller, S. F., Holt, W. V., & Keaveny, E. E. (2020). Collective dynamics of sperm cells. *Philosophical Transactions of the Royal Society B*, *375*(1807), 20190384.

Shindel, A. W., Althof, S. E., Carrier, S., Chou, R., et al. (2022). Disorders of ejaculation: an AUA/SMSNA guideline. *The Journal of Urology*, *207*(3), 504-512

Shoshany, O., Abhyankar, N., Elyaguov, J., & Niederberger, C. (2017). Efficacy of treatment with pseudoephedrine in men with retrograde ejaculation. *Andrology*, *5*(4), 744-748.

Sineath, R. C., Blasdel, G., & Dy, G. W. (2021). Addressing Urologic Health Disparities in Sexual and Gender Minority Communities Through Patient-Centered Outcomes Research. *Urology*, *166*, 66-75.

Skidmore, J. A., Malo, C. M., Crichton, E. G., Morrell, J. M., & Pukazhenthi, B. S. (2018). An update on semen collection, preservation and artificial insemination in the dromedary camel (Camelus dromedarius). *Animal Reproduction Science*, *194*, 11-18.

Skott, M., Schrøder, H., Hindkjaer, J., & Kirkeby, H. J. (2018). Sperm preservation by electroejaculation before anticancer therapy. *Scandinavian Journal of Urology*, *52*(5-6), 461-463.

Sohn, J. O., Jun, S. H., Park, L. S., Kim, E. K., Chung, T. G., & Lee, D. R. (2003). Comparison of recovery and viability of sperm in ICSI pipette after ultra rapid freezing or slow freezing. *Fertility and Sterility*, *80*, 128.

Steuer, I., & Guertin, P. A. (2019). Central pattern generators in the brainstem and spinal cord: an overview of basic principles, similarities and differences. *Reviews in the Neurosciences*, *30*(2), 107-164.

Stoffel, J. T., Van der Aa, F., Wittmann, D., Yande, S., & Elliott, S. (2018). Fertility and sexuality in the spinal cord injury patient. *World Journal of Urology*, *36*(10), 1577-1585.

Talebi, E., Kazemi, L., Rowghani Haghighi Fard, E., Ghazanfarpoor, R., & Rahimi, E. (2021). Evaluation of Sperm Parameters, Reproductive Hormones, Histological Criteria, and Testicular Spermatogenesis Using Turnip Leaf (*Brassica rapa*) Hydroalcoholic Extract in Male Rats: An Experimental Study. *Journal of Medicinal plants and By-product*, *11* (1), 103-109.

Tanabalan, C., & Ballaro, A. (2019). The physiology and pharmacology of the lower urinary tract. *Surgery (Oxford)*, *37*(7), 365-371.

Tran, K. T., Valli-Pulaski, H., Colvin, A., & Orwig, K. E. (2022). Male Fertility Preservation and Restoration Strategies for Patients Undergoing Gonadotoxic Therapies. *Biology of Reproduction*.

Van Saen, D., Goossens, E., Bourgain, C., Ferster, A., & Tournaye, H. (2011). Meiotic activity in orthotopic xenografts derived from human postpubertal testicular tissue. *Human reproduction*, *26*(2), 282-293.

Vaz, R. M., Bordenali, G., & Bibancos, M. (2019). Testicular cancer—surgical treatment. *Frontiers in Endocrinology*, *10*, 308.

Vickram, A. S., Rao, K. A., Archana, K., Jayaraman, G., Kumar S, V., & Sridharan, T. B. (2015a). Effects of various semen extenders on semen parameters for the purpose of human male fertility preservation. *Cryoletters*, *36*(3), 182-186.

Vickram, A. S., Rao, K., Pathy, R. M., Thomas, C., Parameswari, R., & Sridharan, T. B. (2015b). Effect of Semen Extender on Protein Concentration in Each Fraction of Cryopreserved Human Semen. *CryoLetters*, *36*(6), 405-412.

Wollersheim, B. M., van Asselt, K. M., Pos, F. J., Akdemir, E., Crouse, S., van der Poel, H. G., & Boekhout, A. H. (2022). Specialist versus Primary Care Prostate Cancer Follow-Up: A Process Evaluation of a Randomized Controlled Trial. *Cancers*, *14*(13), 3166.

Wyns, C., & Kanbar, M. (2021). Reply: Fertility restoration in azoospermic cancer survivors from testicular VSELs that survive oncotherapy upon transplanting MSCs. *Human Reproduction Update*, *27*(3), 621-622.

Yafi, F. A., Jenkins, L., Albersen, M., Corona, G., Isidori, A. M., Goldfarb, S., & Hellstrom, W. J. (2016). Erectile dysfunction. *Nature reviews Disease primers*, *2*(1), 1-20.

Zapata-Restrepo, L. M., Hauton, C., Williams, I. D., Jensen, A. C., & Hudson, M. D. (2019). Effects of the interaction between temperature and steroid hormones on gametogenesis and sex ratio in the European flat oyster (Ostrea edulis). *Comparative*

Biochemistry and Physiology Part A: Molecular & Integrative Physiology, *236*, 110523.

Zarandi, N. P., Galdon, G., Kogan, S., Atala, A., & Sadri-Ardekani, H. (2018). Cryostorage of immature and mature human testis tissue to preserve spermatogonial stem cells (SSCs): a systematic review of current experiences toward clinical applications. *Stem cells and cloning: advances and applications*, *11*, 23.

Zhang, D., Jiang, W., Liu, M., Sui, X., et al. (2009). Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. *Cell research*, *19*(4), 429-438.

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Application of Fungi as Meat Alternatives in Industry: Mini Review

Wong Kok Kee[1], Ong Ghim Hock[1*], Sabrina Ling Shuet Yee[1], Loh Khye Er[2]

[1]Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN, Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia.
[2]Department of Bioscience, Faculty of Applied Sciences, Tunku Abdul Rahman University College, Jalan Genting Kelang, 53300 Setapak, Kuala Lumpur.

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ABSTRACT

Human consumption has outpaced meat production and manufacturing due to the rising human population and limited land for livestock agriculture. Meat consumption can have negative effects on human health, but meat production can negatively affect the environment by causing global warming and water pollution. Hence, this study produces the idea of using fungus as an alternative to replacing meat. Fungus is an ideal choice as a meat replacement because it has high nutritional content and a fast growth rate. The main objective of this review was to assess the nutritional potential of nine fungal species namely *Fusarium venenatum, Neurospora intermedia, Tuber sp., Xerocomus badius, Ganoderma lucidum, Pleurotuseryngii, Agaricus bisporus, Pleurotus sajor-caju* and *Lentinula edodes* and to determine which species is the best candidate for meat replacement. The nutritional values, toxicity, and growth rate of each fungus were assessed. Comparative data analysis suggests that *F. venenatum, N. intermedia, P. eryngii, A. bisporus, P. sajor-caju,* and *L. edodes* are found suitable for producing fungi-based meat.

* Corresponding author

E-mail: ghimhock.ong@newinti.edu.my (Ong Ghim Hock)

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1 Introduction

Worldwide meat consumption was reported to be 360 million tonnes in 2018 alone (Curtain and Grafenauer 2019) and is steadily increasing annually. The primary reason for increasing meat demand is humans' preference for the superior protein source found in meat. The projected rising human population is believed to outpace the rising food resource (Ritson 2020). This is because livestock agriculture required huge land for the production of animal feed and raising of animals but the land available for livestock agriculture is limited (Nadathur et al. 2017). It was also pointed out by various researchers that the consumption of meat might have some negative impact on humans' health and the environment (González et al. 2020). Although laboratory cultured meat has been reported as an environmentally friendly meat alternative, but its safety for human consumption remains a public concern (Roy et al. 2021). Hence, an alternative solution is needed to overcome the shortcomings.

The fungus can be an excellent meat alternative (Hashempour-Baltork et al. 2020), because of its similarity to animal meat in terms of nutritional value, texture, and taste (Ismail et al. 2020; Michel et al. 2021). Fungus is also high in protein content (Finnigan et al. 2019) and possesses high growth and reproduction rate which shortened the period of conventional meat production (Battilani et al. 2008). The fungus also produces mycoprotein that can be processed into meat alternatives (Hüttner et al. 2020; Souza Filho et al. 2018). Currently, two manufacturing methods i.e. aerial mycelium and treating fungus at high temperature are available for fungi-based meat production (Wiebe 2004; Souza Filho et al. 2019). Although both methods contributed to successful mass production, the quality of the fungi meat varies, and it depends on the type of fungi species used for the meat production. Therefore, this review aims to assess the fungal species that have the potential for alternative meat based on the nutritional values, toxicity safety, and growth rate.

2 Criteria of Potential Fungi as Meat Alternatives

Fungi with high nutritional and similar protein content (%) to animal meat are the best potential species used as meat alternatives. However, species containing mycotoxins will not be safe for human consumption because these may cause disease; therefore, these are not a good candidate. Fungi with a high growth rate are also favored because they can be easily upscale by industry to meet the market demands. Nine fungal species namely *Fusarium venenatum, Neurospora intermedia, Tuber sp.* (edible truffles), *Xerocomus badius, Ganoderma lucidum, Pleurotus eryngii, Agaricus bisporus, Pleurotus sajor-caju* and *Lentinula edodes* were filtered from the available literature. These fungi were selected to be analyzed in this review because they are commonly found in the market, and demonstrate a higher level of acceptance amongst consumers.

2.1 Nutritional Value Evaluation

2.1.1 Protein Content

The protein content is one of the most important criteria when evaluating the nutritional value of potential fungus as meat alternatives. This is because the minimum protein dietary intake for humans is 1.0g per 1kg of body weight per day (Wu 2016). Animal meat has an average of 22% protein value (Ahmad et al. 2018). Data presented in table 1 revealed that among the tested nine species, *F. venenatum* has the highest amount of protein (65% to 76%), followed by. *N. intermedia* (56%), *P. sajor-caju* (29.3 %), and *A. bisporus* (27.1%), all surpassing the protein value found in meat (Kalač 2016). Further, *L. edodes* has a protein content of 20%, which is similar to the average protein content in meat while protein content for *P. eryngii*(16.39%), *X. badius* (1.62%), *Tuber sp* (0.72%), and *G. lucidum* (7% to 8%) was reported lower than the average protein value of meat. Overall, consuming *F. venenatum, N. intermedia, P. sajor-caju, A. bisporus,* and *L. edodes* can provide higher protein value to replace the meat diet. Although *P. eryngii* has slightly lower protein content than animal meat, but a higher intake of *P. eryngii* can easily resolve this minor issue. *G. lucidum, X. badius,* and *Tuber sp.* have too low protein value, relatively compared to meat, and so it is not a choice as a meat alternative.

2.1.2 Fat Content

The dietary reference intake for fats in adults is 25 - 30% of total daily energy intake (Ministry of Health Malaysia 2017). Data given in table 1 suggests that the *Tuber sp* has the highest fat content (4.4%) followed by *A. bisporus* (4.3%), *P. sajor-caju* (0.91%), *L. edodes* (0.8%), *X. badius* (0.71%), *G. lucidum* (3% - 5%), *N. intermedia* (3.5%), *F. venenatum* (2.0% - 3.5%) and *P. eryngii* (0.16%). Both *N. intermedia* (Gmoser et al. 2020) and *A. bisporus* (Kalač 2016) contained α-linolenic acid up to 77.7% in their fats, which can help to reduce cardiovascular diseases (Feeney et al. 2014). Overall, all the selected fungal species have relatively lower fats content compared to meat. The fat content of meat varies according to the type and parts of the animal, for example, pork consists of 31.7% fats (Ahmad et al. 2018).

Based on the protein and fat nutritional value, it can be concluded that *F. venenatum, N. intermedia, P. eryngii, A. bisporus, P. sajor-caju,* and *L. edodes* have the greatest potential to replace meat protein while *Tuber sp., X. badius* and *G. lucidum* have least non-potential of meat alternatives.

Table 1 Potential fungi species and the nutrient content

Species	Ingredients	Amount	References
Fusarium venenatum	Protein	65% - 76%	Reihani and Khosravi-Darani 2018; Wiebe 2002
	Fats	2.0% - 3.5%	Hoseyni and Khosravi-Darani 2010; Rodger 2001
Neurospora intermedia	Protein	56%	Nair et al. 2016
	Fats	3.5%	Karimi et al. 2019
Tuber sp.	Protein	0.72%	Saritha et al. 2016
	Fats	4.4%	
Xerocomus badius	Protein	1.62 ± 0.18%	Jaworska et al. 2015
	Fats	0.71 ± 0.06%	
Ganoderma lucidum	Protein	7% - 8%	Wang et al. 2017
	Fats	3% - 5%	
Pleurotus eryngii	Protein	16.39%	Nie et al. 2019
	Fats	0.16 ± 0.03%	Reis et al. 2012
Agaricus bisporus	Protein	27.1%	Kalač 2016
	Fats	4.3%	
Pleurotus sajor-caju	Protein	29.3%	Gogavekar et al. 2014
	Fats	0.91%	
Lentinula edodes	Protein	20%	Rahman and Choudhury 2012
	Fats	0.8 ± 0.01%	Cohen et al. 2014; Kalač 2016

2.2 Evaluation of Toxicity Safety

Toxicity is a crucial criterion that needs to be assessed while evaluating the possibility of fungus as a meat substitute. Results presented in table 2 suggested the presence of mycotoxin in some fungal species that can harm the human being when consumed.

F. venenatum was approved for sales by the Ministry of Agriculture, Fisheries, and Food in the United Kingdom for human consumption after 12 years of intensive testing (Wiebe 2002). The FDA has granted GRAS (Generally Recognized as Safe) status to a Quorn product produced from *F. venenatum* (FDA 2020). However, allergic reactions like pruritus, breathing difficulties, nausea, and diarrhoea due to allergen in the mycoprotein of *F. venenatum* were reported by Quorn's consumers (Jacobson and DePorter 2018). However, it was argued that based on the systematic evidence the prevalence of allergic responses towards mycoprotein remains extremely low and uncommon. Thus, *F. venenatum* can be accepted as safe for human consumption (Finnigan et al. 2019).

N. intermedia was also classified as GRAS with no detectable mycotoxins (Gmoser et al. 2018). This species is widely used in the production of antibiotics, enzymes, animal foods, and pharmaceutical products (Gmoser et al. 2020). So far, no study has implicated on the disease caused by *Neurospora* species in humans, animals, or plants (Perkins and Davis 2000).

Further, *X. badius* is an edible wild mushroom without mycotoxins (Proskura et al. 2017). Although no cases of allergic reaction were recorded after consuming *X. badius*, this specie can bioconcentrate heavy metals such as mercury from polluted soil (Falandysz et al. 2012). To avoid having heavy metals inside the fungus, it is imperative to choose an unpolluted environment to grow the mushroom.

P. eryngii is also commonly consumed by humans without any reported case of allergies or disease to human beings (Hu et al. 2019). Daily consumption of *P. eryngii* is recommended because it has anti-inflammatory properties that can help prevent acute lung injury caused by a bacterial infection (Kawai et al. 2014). Furthermore, consuming *P. eryngii* was reported to lower blood cholesterol in humans (Alam et al. 2011).

Although *A. bisporus* is an edible mushroom (Usman et al. 2021) but it contains mycotoxin agaritine which is a potential carcinogen (Mohamed 2012). Ingestion of *A. bisporus* can cause anaphylaxis in patients with allergic reactions (Cunha et al. 2020; Gabriel et al.

Table 2 Mycotoxin and the reported effect on human in fungal species

Species	Mycotoxin content	Effect/Response	References
Fusarium venenatum	Absent	Allergic reactions	FDA 2020; Jacobson and DePorter 2018; Wiebe 2002).
Neurospora intermedia	Absent	-	Gmoser et al. 2018
Tuber sp.	Absent	Addiction	Pacioni et al. 2015; Yan et al. 2017
Xerocomus badius	Absent	Bioconcentration potential in fruiting body	Falandysz et al. 2012; Proskura et al. 2017
Ganoderma lucidum	Absent	Adverse effects (insomnia, digestive upsets, skin rashes and hepatotoxicity)	Boa 2004; Jin et al. 2012; Wachtel-Galor et al. 2011
Pleurotus eryngii	Absent	-	Hu et al. 2019
Agaricus bisporus	Present (agaritine)	Stomach bloating, anaphylaxis	Blumfield et al. 2020; Cunha et al. 2020; Gabriel et al. 2015; Usman et al. 2021
Pleurotus sajor-caju	Absent	-	Gogavekar et al. 2014; Mazidi et al. 2020
Lentinula edodes	Absent	Allergic reactions, shiitake dermatitis	Goikoetxea et al. 2009; Grotto et al. 2016; Mendonça et al. 2015

2015). However, the concentration of agaritine in *A. bisporus* is naturally low and the cooking method of *A. bisporus* such as boiling and frying greatly reduces the agaritine content (Mohamed 2012). Consumption of *A. bisporus* did not impair cognitive function in a human, but minor side effect such as stomach bloating has been documented (Blumfield et al. 2020) and attributed to allergy reaction (Cunha et al. 2020). Long-term consumption of *A. bisporus* was able to prevent the development and progression of type 2 diabetes (Calvo et al. 2016).

P. sajor-caju is a popular culinary and medicinal mushroom (Mazidi et al. 2020). Since it does not contain mycotoxin, no cytotoxic effect on cells has been observed and is safe to be consumed by humans (Elhusseiny et al. 2021). Although traces of heavy metals like nickel, lead and chromium was occasionally reported in *P. sajor-caju* (Gogavekar et al. 2014), but the concentrations were within the FAO/WHO safety guideline 2001 (Gogavekar et al. 2014).

L. edodes is one of the most consumed mushrooms worldwide (Grotto et al. 2016; Mao et al. 2021). There were cases of reported allergic reactions, including itching toxicoderma, asthma, rhinitis, and hypersensitivity pneumonitis (Goikoetxea et al. 2009). Raw or undercooked *L. edodes* can cause shiitake dermatitis if consumed (Mendonça et al. 2015).

Tuber sp. or truffles are a non-toxic mushroom delicacy (Yan et al. 2017). However, the species *T. melanosporum* naturally consists of 7.0 ± 5.8 pmol anandamide per 1mg protein (Pacioni *et al*. 2015). The anandamide is similar to tetrahydrocannabinol which is a marijuana's active compound that can cause addiction (Pacioni et al. 2015).

In the end, *G. lucidum* is widely used in various medicinal applications (Boa 2004; Wachtel-Galor et al. 2011). However, there are studies showing patients treated with *G. lucidum* experience adverse effects such as insomnia, digestive upsets, skin rashes, and hepatotoxicity (Jin et al. 2012). Long-term intake of *G. lucidum* was also reported to cause liver failure in humans.

In summary, *F. venenatum, N. intermedia, X. badius, P. eryngii, P. sajor-caju* and *L. edodes* are the least toxic and safest, so they are suitable as meat alternatives since they do not contain mycotoxins, and ingestion of them have no severe side effects. *A. bisporus* contains low concentration mycotoxin but is safe for consumption with the proper cooking method (heat) to reduce the toxin. Although *Tuber sp.* does not contain mycotoxin but it contains anandamide that can cause addiction making the fungus unsuitable to be consumed as a meat alternative. *G. lucidum* also does not produce mycotoxin, but long-term consumption causes liver failure in humans, and thus cannot serve as a meat alternative. However, the conclusion in this review has to be taken with caution, as data on the pattern of consumption, individual body weight, and eating habits might influence the toxicity outcome. Thus, the safety of fungi species discussed earlier can only estimate the real situation for the vast majority of consumers, but not for every individual consumer.

2.3 Evaluation of Growth Potential

Meat producing animals like chickens, pigs, lambs, and cattle needs 48 days, 118 days, 140 days, and 400 days respectively to achieve the standard weight to be slaughtered and marketed (Penn State Extension 2020). In comparison to these, the growth rate of fungus is fast and these can be easily produced on artificial media or other organic compounds. Further, the growth of the fungus is crucial if it is to be used as a meat alternative because it will have a significant impact on cost. Fungus with a slow growth rate will need more nutrients to accelerate the growth, resulting in a higher production cost.

Table 3 Potential fungi species and the growth rate with optimal growth parameters

Species	Growth rate	Optimal condition		References
		Temperature	Substrate	
Fusarium venenatum	0.19g / hours	25°C	Modified Vogel's medium	Farnworth et al. 2003
Neurospora intermedia	7.632mm /day	30°C	PDA agar	Osadolor et al. 2017
Tuber sp.	0.1-0.35mg /90 days	23°C	MMN agar	Nakano et al. 2020
Xerocomus badius	14.3mm / day	25 °C	PDA agar	Liu et al. 2019
Ganoderma lucidum	14.8 ± 0.4 days for 86.1 ± 2.6g /kg	25°C	poplar sawdusts	Atila 2020
Pleurotus eryngii	3.35mm / day	25°C	PDA agar	Uysal andSoylu 2016
Agaricus bisporus	6.17mm / day	25°C	WHS2 medium	Rashid et al. 2018
Pleurotus sajor-caju	7.11mm / day	25°C	PSA medium	Go et al. 1984
Lentinula edodes	7.5mm / day	25°C	PDA agar	Ohga 1990

As indicated in table 3, most of the selected fungi species can grow relatively faster than the chicken (48 days), and in this manner, these fungi grow at the fastest rate among all the poultry animals. The only exception is the *Tuber sp.* (edible truffles), which took 90 to 120 days (Nakano et al. 2020). All the growths were recorded with growing temperature in the optimal range of 23°C to 30°C on potato dextrose agar (PDA).

F. venenatum shows the highest growth rate since the growth rate was calculated per hour. In terms of the day, *X. badius* has the highest growth rate (14.3mm /day), followed by *N. intermedia* (7.63mm /day), *L. edodes* (7.5mm /day), *P. sajor-caju* (7.11mm /day), *A. bisporus* (6.17mm /day) and lastly *P. eryngii* (3.35mm /day). *G. lucidum* takes about 14 days to produce a yield of 86.1 ± 2.6g /kg fungus. The slowest growth is for *Tuber sp* with only 0.1-0.35mg /90 days.

In summary, *F. venenatum*, *X. badius*, *N. intermedia*, *L. edodes*, *P. sajor-caju*, *A. bisporus*, *P. eryngii*, and *G. lucidum* have relatively high growth rates and require less than a month to produce the mycelium. The fungi can produce 300 kg of mycelium in hours, whereas pig, which weighs around 130 kg, takes about 4 months to mature (Wiebe 2002). However, in this review, one of the main limitations while assessing the growth parameters is that the units used to calculate the fungus growth rate are not fixed, making it difficult to compare growth rates between different fungal species.

2.4 Complications of using Fungi as Meat Alternatives

Some difficulties might be encountered when assessing which fungi species is the best to replace meat protein. There are too many fungal species with different growth characteristics or nutritional parameters under a single genus. Under a single genus, some species remain unidentified and assumed to share similar characterises as the other species within genus. This assumption will lead to safety issues since some of these unidentified fungi might produce harmful mycotoxins. Toxicity studies are complex since researchers might only be able to reveal that a particular fungus is toxic only after long-term exposure to humans. Therefore, the toxic fungus might be mistaken as safe to be developed as meat alternatives. Although this review narrows down some of the fungi identify, more information on toxicity is needed to better determine which fungi species have the best potential to be developed as meat alternatives to replace animal meat.

Conclusion

In this review, nine fungi species were examined in terms of nutritional values, toxicity, and growth rate to determine which fungus can be used as meat alternatives. This review concludes that *F. venenatum*, *N. intermedia*, *P. eryngii*, *A. bisporus*, *P. sajor-caju*, and *L. edodes* have the best potential fungi as meat alternatives due to their high nutritional content, high growth rate, and non-toxic. *Tuber sp.*, *G. lucidum* and *X. badius* are not suitable due to relatively low protein content, slower growth rate, and higher toxicity risk.

Although this review allows the identification of fungi as meat alternatives, more empirical studies on the selected species need to be conducted and refine these findings. The primary concern of this review is to access the nutritional value, toxicity, and growth rate of the selected fungi. However, the flavor and texture of fungus are equally important, since differences in taste and texture between alternative meat made from fungus and animal meat may cause consumers to lose faith in purchasing fungi-based meat.

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Conflict of Interest Statement

There are no conflicts of interest.

References

Ahmad, R. S., Imran, A., & Hussain, M. B. (2018). Nutritional composition of meat. In M. S. Arshad (Ed) *Meat science and nutrition*, Intechopen, DOI: 10.5772/intechopen.77045.

Alam, N., Yoon, K. N., Lee, J. S., Cho, H. J., et al. (2011). Dietary effect of *Pleurotus eryngii* on biochemical function and histology in hypercholesterolemic rats. *Saudi Journal of Biological Sciences*, *18*(4), 403–409.https://doi.org/10.1016/j.sjbs.2011.07.001

Atila, F. (2020). Comparative study on the mycelial growth and yield of *Ganoderma lucidum* (Curt.: Fr.) Karst. on different lignocellulosic wastes. *Acta Ecologica Sinica*, *40*(2), 153-157.

Battilani, P., Barbano, C., & Logrieco, A. (2008). Risk assessment and safety evaluation of mycotoxins in fruits. In R. Barkai-Golan, & N. Paster (Eds.) *Mycotoxins in fruits and vegetables* (pp. 1-26). Academic Press.

Blumfield, M., Abbott, K., Duve, E., Cassettari, T., et al. (2020). Examining the health effects and bioactive components in *Agaricus bisporus* mushrooms: A scoping review. *The Journal of Nutritional Biochemistry*, *84*, 108453.

Boa, E. R. (2004). Wild edible fungi: a global overview of their use and importance to people. Non-wood forest products, 17. Rome: FAO.

Calvo, M. S., Mehrotra, A., Beelman, R. B., Nadkarni, G., et al. (2016). A Retrospective Study in Adults with Metabolic Syndrome: Diabetic Risk Factor Response to Daily Consumption of *Agaricus bisporus* (White Button Mushrooms). *Plant Foods for Human Nutrition*, 71(3), 245–251.

Cohen, N., Cohen, J., Asatiani, M. D., Varshney, V. K., et al. (2014). Chemical composition and nutritional and medicinal value of fruit bodies and submerged cultured mycelia of culinary-medicinal higher Basidiomycetes mushrooms. *International journal of medicinal mushrooms*, *16*(3), 273–291.

Cunha, I. M., Marques, M. L., Abreu, C., Bartolomé, B., et al. (2020). Anaphylaxis to Agaricus bisporus ingestion. *Einstein (São Paulo)*, *18*, 1-4.

Curtain, F., & Grafenauer, S. (2019). Plant-based meat substitutes in the flexitarian age: An audit of products on supermarket shelves. *Nutrients*, *11*(11), 2603.

Elhusseiny, S. M., El-Mahdy, T. S., Awad, M. F., Elleboudy, N. S., et al. (2021). Antiviral, Cytotoxic, and Antioxidant Activities of Three Edible Agaricomycetes Mushrooms: *Pleurotus columbinus*, *Pleurotus sajor-caju*, and *Agaricus bisporus*. *Journal of Fungi*, *7*(8), 645.

Falandysz, J., Kojta, A., Jarzyńska, G., Drewnowska, M., et al. (2012). Mercury in bay bolete (*Xerocomus badius*): bioconcentration by fungus and assessment of element intake by humans eating fruiting bodies. *Food Additives & Contaminants: Part A*, *29*(6), 951-961.

Farnworth, N. E., Robson, G. D., Trinci, A. P., & Wiebe, M. G. (2003). Trypsin-like protease (TLP) production in *Fusarium oxysporum* and *Fusarium venenatum* and use of the TLP promoter for recombinant protein (glucoamylase) production. *Enzyme and Microbial Technology*, *33*(1), 85–91.

Feeney, M. J., Dwyer, J., Hasler-Lewis, C. M., et al. (2014). Mushrooms and health summit proceedings. *The Journal of Nutrition*, *144*(7), 1128S-1136S.

Finnigan, T. J., Wall, B. T., Wilde, P. J., Stephens, F. B., et al. (2019). Mycoprotein: the future of nutritious nonmeat protein, a symposium review. *Current developments in nutrition*, *3*(6), nzz021.

Food and Drug Administration. (2020). Generally recognized as safe (GRAS) notice for mycoprotein as a food ingredient. U.S. Food and Drug Administration. Retrieved from https://www.fda.gov/media/145554/download

Gabriel, M. F., González-Delgado, P., Postigo, I., Fernández, J., et al. (2015). From respiratory sensitization to food allergy: Anaphylactic reaction after ingestion of mushrooms (*Agaricus bisporus*). *Medical mycology case reports*, *8*, 14-16.

Gmoser, R., Ferreira, J. A., Lundin, M., Taherzadeh, M. J., et al. (2018). Pigment production by the edible filamentous fungus *Neurospora intermedia*. *Fermentation*, *4*(1), 11.

Gmoser, R., Fristedt, R., Larsson, K., Undeland, I., et al. (2020). From stale bread and brewers spent grain to a new food source using edible filamentous fungi. *Bioengineered*, *11*(1), 582-598.

Go, S. J., Byun, M. O., You, C. H., & Park, Y. H. (1984). Selection of *Pleurotus sajor-caju* as suitable species for cultivation under summer climatic conditions in Korea. *The Korean Journal of Mycology*, *12*(2), 53-58.

Gogavekar, S. S., Rokade, S. A., Ranveer, R. C., Ghosh, J. S., et al. (2014). Important nutritional constituents, flavour components, antioxidant and antibacterial properties of *Pleurotus sajor-caju*. *Journal of food science and technology*, *51*(8), 1483-1491.

Goikoetxea, M. J., Fernández-Benítez, M., & Sanz, M. L. (2009). Food allergy to Shiitake (Lentinus edodes) manifested as oesophageal symptoms in a patient with probable eosinophilic oesophagitis. *Allergologia et immunopathologia*, 333-334.

González, N., Marquès, M., Nadal, M., & Domingo, J. L. (2020). Meat consumption: Which are the current global risks? A review of recent (2010–2020) evidences. *Food Research International*, *137*, 109-341.

Grotto, D., Bueno, D. C. R., de Almeida Ramos, G. K., da Costa, S. R., et al. (2016). Assessment of the safety of the shiitake culinary-medicinal mushroom, *Lentinus edodes* (agaricomycetes), in rats: Biochemical, hematological, and antioxidative parameters. *International journal of medicinal mushrooms*, *18*(10), 861–870.

Hashempour-Baltork, F., Hosseini, S. M., Assarehzadegan, M. A., Khosravi-Darani, K., et al. (2020). Safety assays and nutritional values of mycoprotein produced by *Fusarium venenatum* IR372C from date waste as substrate. *Journal of the Science of Food and Agriculture*, *100*(12), 4433-4441.

Hoseyni, S. M., & Khosravi-Darani, M. M. K. (2010). Production and rheological evaluation of mycoprotein produced from *Fusarium venenatum* ATCC 20334 by surface culture method. *Seed*, *72*, 48.

Hu, Q., Yuan, B., Wu, X., Du, H., et al. (2019). Dietary intake of *Pleurotus eryngii* ameliorated dextran-sodium-sulfate-induced colitis in mice. *Molecular nutrition & food research*, *63*(17), 1801265.

Hüttner, S., Johansson, A., Gonçalves Teixeira, P., Achterberg, P., et al. (2020). Recent advances in the intellectual property landscape of filamentous fungi. *Fungal Biology and Biotechnology*, *7*(1), 1-17.

Ismail, I., Hwang, Y. H., & Joo, S. T. (2020). Meat analog as future food: A review. *Journal of animal science and technology*, *62*(2), 111-120.

Jacobson, M. F., & DePorter, J. (2018). Self-reported adverse reactions associated with mycoprotein (Quorn-brand) containing foods. *Annals of Allergy, Asthma & Immunology*, *120*(6), 626–630.

Jaworska, G., Pogoń, K., Skrzypczak, A., & Bernaś, E. (2015). Composition and antioxidant properties of wild mushrooms *Boletus edulis* and *Xerocomus badius* prepared for consumption. *Journal of food science and technology*, *52*(12), 7944-7953.

Jin, X., Beguerie, J. R., Sze, D. M. Y., & Chan, G. C. (2012). *Ganoderma lucidum* (Reishi mushroom) for cancer treatment. *Cochrane Database of Systematic Reviews*,*6*, 1-33.

Kalač, P. (2016). *Edible mushrooms: chemical composition and nutritional value*. Academic Press.

Karimi, S., Mahboobi Soofiani, N., Lundh, T., Mahboubi, A., et al. (2019). Evaluation of filamentous fungal biomass cultivated on vinasse as an alternative nutrient source of fish feed: protein, lipid, and mineral composition. *Fermentation*, *5*(4), 99.

Kawai, J., Andoh, T., Ouchi, K., & Inatomi, S. (2014). *Pleurotus eryngii* Ameliorates Lipopolysaccharide-Induced Lung Inflammation in Mice. *Evidence-Based Complementary and Alternative Medicine*, eCAM, 2014, 532389. https://doi.org/10.1155/2014/532389.

Liu, B., Liu, X., Liu, F., Ma, H., et al. (2019). Growth improvement of Lolium multiflorum Lam. induced by seed inoculation with fungus suspension of *Xerocomus badius* and *Serendipita indica*. *AMB Express*, *9*(1), 1-11.

Mao, L., van Arkel, J., Hendriks, W. H., Cone, J. W., et al. (2021). Assessing the nutritional quality of fungal treated wheat straw: Compounds formed after treatment with *Ceriporiopsis subvermispora* and *Lentinula edodes*. *Animal Feed Science and Technology*, *276*, 114924.

Mazidi, M. N. I. B. H., Ibrahim, R., & Yaacob, N. D. (2020). The Growth Morphology and Yield of Grey Oyster Mushrooms (*Pleurotus sajor-caju*) Subjected to Different Durations of Acoustic Sound Treatment. *IOP Conference Series: Materials Science and Engineering*, *767*(1), 012013

Mendonça, C. N. D., Silva, P. M. C., Avelleira, J. C. R., Nishimori, F. S., et al. (2015). Shiitake dermatitis. *Anais Brasileiros de Dermatologia*, *90*, 276-278.

Michel, F., Hartmann, C., & Siegrist, M. (2021). Consumers' associations, perceptions and acceptance of meat and plant-based meat alternatives. *Food Quality and Preference*, *87*, 104063.

Ministry of Health Malaysia. (2017). Recommended nutrient intakes for Malaysia. nutrition.moh. Retrieved from https://nutrition.moh.gov.my/wp-content/uploads/2017/05/FA-Buku-RNI.pdf

Mohamed, E. M. (2012). Chemical profile, agaritine and selenium content of *Agaricus bisporus. Brazilian Archives of Biology and Technology*, *55*(6), 911–920.

Nadathur, S. R., Wanasundara, J. P. D., & Scanlin, L. (2017). Proteins in the diet: Challenges in feeding the global population. In S. R. Nadathur, J.P.D. Wanasundara, & L. Scanlin (Eds.) *Sustainable protein sources* (pp. 1-19), Academic Press.

Nair, R. B., Lennartsson, P. R., & Taherzadeh, M. J. (2016). Mycelial pellet formation by edible ascomycete filamentous fungi, *Neurospora intermedia*. *AMB Express*, *6*(1), 1-10.

Nakano, S., Kinoshita, A., Obase, K., Nakamura, N., et al. (2020). Influence of pH on in vitro mycelial growth in three Japanese truffle species: *Tuber japonicum*, *T. himalayense*, and *T. longispinosum*. *Mycoscience*, *61*(2), 58–61.

Nie, Y., Zhang, P., Deng, C., Xu, L., et al. (2019). Effects of *Pleurotus eryngii* (mushroom) powder and soluble polysaccharide addition on the rheological and microstructural properties of dough. *Food Science & Nutrition*, *7*(6), 2113-2122.

Ohga, S. (1990). Growth Rate of Mycelium of Shiitake, *Lentinus edodes*, in Relation to Water Potential of Medium. *Journal of the Faculty of Agriculture, Kyushu University*, *34*(4), 413–420.

Osadolor, O. A., Nair, R. B., Lennartsson, P. R., & Taherzadeh, M. J. (2017). Empirical and experimental determination of the kinetics of pellet growth in filamentous fungi: a case study using *Neurospora intermedia*. *Biochemical engineering journal*, *124*, 115-121.

Pacioni, G., Rapino, C., Zarivi, O., Falconi, A., et al. (2015). Truffles contain endocannabinoid metabolic enzymes and anandamide. *Phytochemistry*, *110*, 104-110.

Penn State Extension (2020, May 28). Adjusting and Monitoring Meat Animal Growth Rate. Retrieved from https://extension.psu.edu/adjusting-and-monitoring-meat-animal-growth-rate

Perkins, D. D., & Davis, R. H. (2000). Evidence for Safety of *Neurospora* Species for Academic and Commercial Uses. *Applied and Environmental Microbiology*, *66*(12), 5107–5109.

Proskura, N., Podlasińska, J., & Skopicz-Radkiewicz, L. (2017). Chemical composition and bioaccumulation ability of *Boletus badius* (Fr.) Fr. collected in western Poland. *Chemosphere*, *168*, 106-111.

Rahman, T., & Choudhury, M. B. K. (2012). Shiitake mushroom: a tool of medicine. *Bangladesh Journal of Medical Biochemistry*, *5*(1), 24-32.

Rashid, H. M., Abed, I. A., & Owaid, M. N. (2018). Mycelia growth performance of *Agaricus bisporus* in culture media of composts supplemented with Sesbania sesban straw and phosphate rock. *Current Research in Environmental & Applied Mycology*, *8*(3), 323–330.

Reihani, S. F. S., & Khosravi-Darani, K. (2018). Mycoprotein production from date waste using *Fusarium venenatum* in a submerged culture. *Applied Food Biotechnology*, *5*(4), 243-352.

Reis, F. S., Barros, L., Martins, A., & Ferreira, I. C. (2012). Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: an inter-species comparative study. *Food and Chemical Toxicology*, *50*(2), 191-197.

Ritson, C. (2020). Population growth and global food supplies. In M. Rutland, A. Turner, (eds) *Food Education and Food Technology in School Curricula* (pp. 261-271). Springer, Cham.

Rodger, G. (2001). Mycoprotein—a meat alternative new to the US Production and properties of mycoprotein as a meat alternative. *Food Technology*, *55*(7), 36-41.

Roy, B., Hagappa, A., Ramalingam, Y. D., & Mahalingam, N. (2021). A review on lab-grown meat: Advantages and disadvantages. *Quest International Journal of Medical and Health Sciences*, *4*(1), 19-24.

Saritha, K., Prakash, B., Khilare, V., Khedkar, G., et al. (2016). Mushrooms and Truffles: Role in the Diet. *Encyclopedia of Food and Health*, 1–8.

Souza Filho, P. F., Andersson, D., Ferreira, J. A., & Taherzadeh, M. J. (2019). Mycoprotein: environmental impact and health aspects. *World Journal of Microbiology and Biotechnology*, *35*(10), 1-8.

Souza Filho, P. F., Nair, R. B., Andersson, D., Lennartsson, P. R., et al. (2018). Vegan-mycoprotein concentrate from pea-processing industry byproduct using edible filamentous fungi. *Fungal biology and biotechnology*, *5*(1), 1-10.

Usman, M., Murtaza, G., & Ditta, A. (2021). Nutritional, medicinal, and cosmetic value of bioactive compounds in button mushroom (*Agaricus bisporus*): a review. *Applied Sciences*, *11*(13), 5943.

Uysal, E., & Soylu, M. K. (2016). Pleurotus eryngii Türünün Farklı İzolatlarına Ait Mantarların Bazı Mineral Besin İçeriklerinin Belirlenmesi. *Turkish Journal of Agriculture-Food Science and Technology*, *4*(3), 139-143.

Wachtel-Galor, S., Yuen, J., Buswell, J. A., & Benzie, I. F. (2011). *Ganoderma lucidum* (Lingzhi or Reishi). *Herbal Medicine: Biomolecular and Clinical Aspects. 2nd edition*, CRC Press.

Wang, J., Cao, B., Zhao, H., & Feng, J. (2017). Emerging roles of *Ganoderma lucidum* in anti-aging. *Aging and disease*, *8*(6), 691.

Wiebe, M. (2002). Myco-protein from *Fusarium venenatum*: a well-established product for human consumption. *Applied microbiology and biotechnology*, *58*(4), 421-427.

Wiebe, M. G. (2004). QuornTM Myco-protein-Overview of a successful fungal product. *Mycologist*, *18*(1), 17-20.

Wu, G. (2016). Dietary protein intake and human health. *Food & Function*, *7*(3), 1251–1265.

Yan, X., Wang, Y., Sang, X., & Fan, L. (2017). Nutritional value, chemical composition and antioxidant activity of three Tuber species from China. *AMB Express*, *7*(1), 1-8.

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Potential COVID -19 Therapeutics in Clinical Trials – A Brief Review

Dinesh Kumar Lakshmi Narayanan[1], Sinouvassane Djearamane[2*], Vinodhkumar Ramalingam[3*], Saminathan Kayarohanam[1], Sivabalan Rajagopal[1], SankaraKumaran Pandian[4], Ashok Kumar Janakiraman[5], Pradeep Balakrishnan[6]

[1]Faculty of Bioeconomics and Health sciences, Geomatika University College, Kuala Lumpur, Malaysia
[2]Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Kampar, Malaysia
[3]Faculty of Health and Life Sciences, INTI International University, Nilai, Malaysia
[4]School of Physiotherapy, Faculty of Allied Health Professions, AIMST University, Bedong, Malaysia
[5]Faculty of Pharmaceutical Sciences, UCSI University, Kuala Lumpur, Malaysia
[6]School of Health Sciences, KPJ University, Nilai, Malaysia

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ABSTRACT

The severe acute respiratory syndrome coronavirus 2 (SARS – CoV2), the causative viral pathogen of the COVID-19 pandemic belongs to the family of Coronaviruses which are positive single stranded RNA viruses. The scientific fraternity has developed and developing various types of vaccines for prevention against COVID-19, such as inactivated virus vaccines, mRNA vaccines, replicating vector protein subunit vaccines, etc., Out of which ten vaccines namely Novovax, Covovax (protein subunit vaccines), Pfizer BNT16b2, Moderna mRNA 1273 (mRNA vaccines), Johnson & Johnson Ad26, Cov2.S, Astrazeneca AZD1222, Covishield (non-replicating viral vector vaccines), Covaxin, Sinopharm BBIBP-CorV, CoronoVac (inactivated vaccines) have been approved for clinical use by WHO. There is an urgent need for SARS-CoV2 specific therapeutics for the treatment of COVID-19 as there is the emergence of various variants such as Alpha, Beta, Gamma, Delta, Omicron, etc. The emergence of variants that possesses immune evading property and spike protein mutation have increased infectivity and more pathogenicity which impelled the need to develop various therapeutics for the treatment of COVID-19.

* Corresponding author

E-mail: vinodh.ramalingam@newinti.edu.my (Vinodhkumar Ramalingam);
sinouvassane@utar.edu.my (Sinouvassane Djearamane)

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This review compiles the information about potential antiviral candidates in preclinical trials intended for the treatment of COVID-19. The clinical development of such antivirals will be very crucial for the treatment of COVID-19 and also to curb the spread as the present scenario depends on the development of effective prophylactic vaccines.

1 Introduction

The outbreak of COVID-19 was caused by the severe acute respiratory syndrome coronavirus 2 (SARS – CoV2), which was initially referred to as novel coronavirus 2 or nCoV2 and was first reported in Wuhan, China in December 2019 (Narayanan et al. 2021). Soon the virus was spread worldwide after which the World Health Organisation (WHO) declared it a pandemic in March 2020 (Jebril 2020). Globally, as of 4 July 2022, the total numbers of confirmed cases were 546,357,444 including 6,336,415 deaths as per World Health Organisation (World Health Organization 2022a). Thanks to the continuing research about the coronaviruses and collaboration among worldwide scientists for very quick sequencing of the SARS-CoV2 genome. This whole genome sequencing was carried out by various organizations worldwide such as NCBI, GISAID, and Gen Bank and the collaboration of these organizations was very useful to know the details about the pathogen, which enabled us to devise protective measures and also research and develop therapeutics to curb the infection (NCBI Resources 2022). This review provides an insight about the current status, preclinical research, and clinical trial progress of the potential candidates for the treatment of COVID-19.

2 Entry & Multiplication of SARS–CoV2 into the Host Cells

Coronaviruses reported in 1966 for the first time by Tyrell and Bynoe are positive single stranded RNA viruses that are known to infect animals and humans (Velavan and Meyer 2020). These viruses belong to the order of *Nidovirales*, suborder *Coronavirineae*, and to the family *Coronaviridae*. The family *Coronaviridae* is further subdivided into *Orthocoronavirinae* subfamily. There are four genera amongst this *Orthocoronavirinae* subfamily viz., alpha coronavirus, beta coronavirus, gamma coronavirus and delta coronavirus. Among these genera, alpha and beta coronavirus are known to infect humans and animals. The pathogenic viruses which are known to infect humans and animals including the severe acute respiratory syndrome coronavirus 2 (SARS – CoV2) and also a causative viral pathogen of the current pandemic belong to the genus beta coronavirus. Severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle East respiratory syndrome (MERS- CoV) are the pathogens from this genus that caused an outbreak in the past. SARS – CoV2 possesses a genome 80% identical to SARS-CoV and approximately 50% identical to MERS- CoV. These pathogenic human coronaviruses (HCoVs) are known to infect the respiratory tract which may develop into life-threatening respiratory tract infections (V'kovski et al. 2021; Narayanan et al. 2022).

The genome length of SARS –CoV2 is found to be approximately in the range of 30Kb with twelve open reading frames (ORFs) encoding for the non-structural proteins, structural proteins, and accessory proteins (Chan et al. 2020). The ORF 1a and ORF 1b replicase genes encode for the polyproteins which are cleaved into sixteen non-structural proteins (nsp1-nsp16). Remaining ORFs encode for the structural proteins namely Spike protein (S), an Envelope protein (E), Membrane protein (M), and Nucleocapsid (N) as well as the accessory proteins (Rahimi et al. 2021). The pathology of COVID-19 initiates with the entry of SARS–CoV2 in the respiratory tract through human angiotensin-converting enzyme 2 (hACE2) which is the primary receptor. This metallocarboxyl peptidase enzyme cleaves the peptides of the renin-angiotensin system which is found in the lungs, especially in type 2 alveolar cells, kidneys, and gastrointestinal system (Batlle et al. 2020). The hACE2 is the receptor site for Spike protein (S) of the SARS-CoV2 pathogen. Both the Spike protein (S) and the hACE2 are heavily glycosylated and possess O-linked glycans (Yang et al. 2020). Spike protein comprises two functional units namely S1 and S2 which are responsible for binding and fusion respectively. The receptor binding domain (RBD) of the spike protein binds with the host hACE2 cell after which the spike protein furin site is cleaved by transmembrane serine protease 2 (TMPRSS2) and the cell surface protein is expressed in the endothelial cells of the respiratory tract. The S1 site is responsible for stabilizing the membrane-anchored S2 subunit which contains the fusion machinery for the fusion of the viral membranes with the host cell (Walls et al. 2020).

Once the virus entered into the host cell, the genome of SARS-CoV2 initiates the viral RNA synthesis by utilizing the host cell machinery. Open reading frame 1a synthesizes the polyproteins pp1 a/ b which is cleaved into non-structural proteins that happens in the host cell cytosol. As mentioned earlier these non-structural proteins are involved in the translation machinery as the replicase and in various processes (Rohaim et al. 2021). The replicases generate the subgenomic mRNAs encode for the four structural proteins viz., spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins as well as the accessory proteins which take part in the assembly and transport of infective viral particles (Sicari et al. 2020; Kumar et al. 2021). The translated viral proteins are translocated in the endoplasmic reticulum of the host cell which is facilitated by the Golgi intermediate compartment

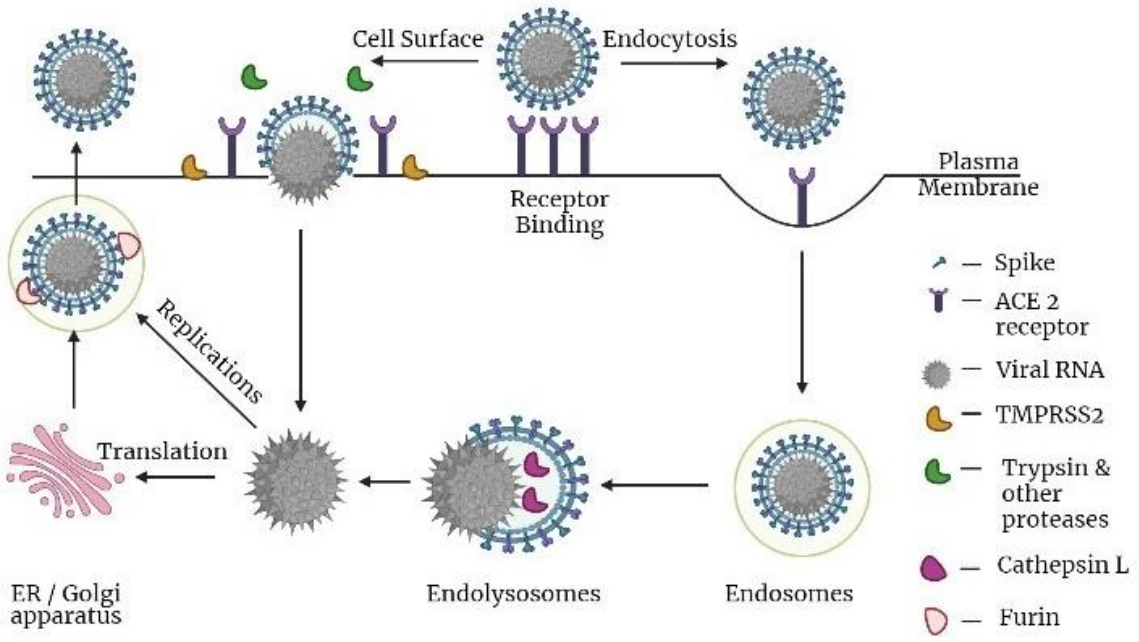

Figure 1 Entry of SARS-CoV-2 into the host cell (ACE2, angiotensin converting enzyme 2, TMPRSS2 – Trans Membrane Protein Serine Protease 2, ER, Endoplasmic reticulum, SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2)

(ERGIC) of the SARS-CoV2 for glycosylation, folding, and assembling of virus budding. The fully assembled virion is then released by exocytosis (Kadam et al. 2021). Figure 1 depicts the entry of the virus into the host cells.

3 Current Status of COVID- 19 Therapeutics

For the prevention of COVID – 19, according to WHO there are 167 vaccines in clinical development which includes the approved vaccines in clinical use, and 198 vaccines in pre-clinical development (World Health Organization 2022b). Apart from the preventive measures and vaccination, current COVID - 19 treatments involve the treatment with FDA-approved antiviral Remdesivir (intravenous) which was approved in October 2020 (Drożdżal et al. 2020). Apart from this antiviral drug, FDA has also approved monoclonal antibodies such as bamlanivimab, casirivimab, and imdevimab which have to be administered together (U.S. Food and Drug Administration 2021; FDA 2022). Other treatment options include IV steroids, anti-clotting medications as well as interleukins as per the WHO (Tim Jewell 2021). According to the latest guidelines of the CDC, monoclonal antibodies bamlanivimab with etesevimab, casirivimab, and orimdevimab can be used for the treatment of COVID-19 (National Institutes of Health 2021). The treatment options presently involve antivirals and immune modulators for aiding the immune system. There has been continuous research for potential antivirals and monoclonal antibodies for the treatment options some of which are discussed in the next section of this review.

3.1 Molnupiravir (MK – 4482) (EIDD-2801)

Developed by Merck and Ridgeback Molnupiravir/ MK – 4482 /EIDD-2801 is an investigational, ribonucleoside analog that inhibits the replication of SARS-CoV-2 and is found to be effective against various mutant strains. The molecule Molnupiravir was researched and innovated by Drug Innovations at Emory (DRIVE), LLC, and is currently developed by Merck and Ridgeback. The oral Molnupiravir is investigated for post-exposure prophylaxis and in Phase 2/ Phase 3 trials and it has shown effective in preclinical trials. The phase 3 trials for Molnupiravir is currently undergoing in Argentina, Brazil, Canada, Chile, Colombia, Egypt, France, Germany, Guatemala, Israel, Italy, Japan, Mexico, Philippines, Poland, Russia, South Africa, Spain, Sweden, Taiwan, Ukraine, the United Kingdom and the United States (Julia Robinson 2021; Merck 2021; U.S. National Library of Medicine 2022).

3.2 AT-527

AT- 527 was researched and developed by Atea Pharmaceuticals and is an orally available double prodrug of guanosine nucleotide analog, which inhibits viral replication by interfering with viral RNA polymerase. AT – 527 is a free base of AT-511 that was proven to inhibit the viral RNA-dependent RNA polymerase (RdRp) selectively in Hepatitis C virus *in-vitro* as well as *in-vivo*. When it was investigated for potent antiviral activity against several human coronaviruses, including SARS-CoV-2, it was

found to be effective in *in-vitro* studies. The active triphosphate metabolite of AT-527 is proven to be in substantial amounts in primary human cells of the respiratory tract and may be an effective treatment option (ATEA Pharmaceuticals 2021; Good et al. 2021; U.S. National Library of Medicine 2021a).

3.3 Ritonavir/PF-07321332

Ritonavir/PF-07321332 is an investigational oral SARS-CoV-2-3CL protease inhibitor that blocks the viral protease needed for viral assembly and budding thereby arresting the viral replication in the host cell, is researched and developed by Pfizer. After an encouraging Pre-clinical trial, the Ritonavir/PF-07321332 started its Phase 1 trial in March 2021 in which it was found that it's safe and well tolerated. Currently, Ritonavir/PF-07321332 is undergoing Phase 2/3 trial EPIC-PEP (Evaluation of Protease Inhibition for COVID-19 in Post-Exposure Prophylaxis) trial after the July 2021 EPIC-HR (Evaluation of Protease Inhibition for COVID-19 in High-Risk Patients) Phase 2/3 trial (Pfizer 2021; U.S. National Library of Medicine 2021b)

3.4 Immunomodulators

Immunomodulators are substances either of biological or synthetic origin for modulating the immune system. Immunomodulators can be used to stimulate (immune stimulators), suppress (immune suppressive), or modulate (biological response modifiers such as GM-CSF) the innate and adaptive immune systems (Catanzaro et al. 2018). Immunomodulators are the potential treatment option for COVID – 19 as they can be used to stimulate an effective B and T cell-based immunity (Zhou and Ye 2021). In addition to the promising antivirals in clinical trials, there are also several immune modulators in trials, namely; AZD7442, Tocilizumab, Sarilumab, Regdanvimab, Canakinumab, Anakinra, Baricitinib, Ruxolitinib, Tofacitinib, Acalabrutinib, Imatinib, Brensocatib, Ravulizumab, Namilumab, Infliximab, Adalimumab, Otilimab, Bamlanivimab, Etesevimab, Sotrovimab, Leronlimab, Risankizumab, Lenzilumab, and IMU-838 (Timothy et al. 2020; Vincent et al. 2021).

Conclusion and future prospects

The scientific community has been on a quest for treatment options since the outbreak of the COVID-19 pandemic in which they have succeeded with the development of vaccines for prevention as well as reducing hospitalizations and mortality. The emergence of SARS-CoV2 variants does pose a greater challenge as the available vaccines even though will still prevent serious illness but may be less effective against the emerging variants as well as there may be more transmission which may lead to more serious implications worldwide. To nullify the present threat situation of variants and to curb the spread of the pandemic, we are in the need of more therapeutic options, especially for post covid-19 treatment. The potential specific antivirals against COVID-19 will not only help the scientific fraternity to treat the patients effectively but also pave the way to curb the pandemic with the available vaccines.

References

ATEA Pharmaceuticals. (2021). Seeking to combat COVID-19 with an oral RNA viral polymerase inhibitor Retrieved from https://ateapharma.com/at-527/

Batlle, D., Wysocki, J., & Satchell, K. (2020). Soluble angiotensin-converting enzyme 2: a potential approach for coronavirus infection therapy? *Clinical science*, *134*(5), 543-545

Catanzaro, M., Corsini, E., Rosini, M., Racchi, M., & Lanni, C. (2018). Immunomodulators Inspired by Nature: A Review on Curcumin and Echinacea. *Molecules*, *23*(11), 2778

Chan, J. F. W., Kok, K. H., Zhu, Z., et al. (2020). Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. *Emerging microbes & infections*, *9*(1), 221-236

Drożdżal, S., Rosik, J., Lechowicz, K., Machaj, F., et al. (2020). FDA approved drugs with pharmacotherapeutic potential for SARS-CoV-2 (COVID-19) therapy. *Drug Resistance Update*s, *53*, 100719

FDA (2022) Coronavirus (COVID-19) Update: FDA Limits Use of Certain Monoclonal Antibodies to Treat COVID-19 Due to the Omicron Variant Retrieved from https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-limits-use-certain-monoclonal-antibodies-treat-covid-19-due-omicron

Good, S. S., Westover, J., Jung, K. H., Zhou, X. J., et al. (2021). AT-527, a double prodrug of a guanosine nucleotide analog, is a potent inhibitor of SARS-CoV-2 in vitro and a promising oral antiviral for treatment of COVID-19. *Antimicrobial Agents and Chemotherapy*, *65*(4), e02479-20

Jebril, N. (2020). World Health Organization declared a pandemic public health menace: a systematic review of the coronavirus disease 2019 “COVID-19”. Available at SSRN 3566298

Julia Robinson (2021) Everything you need to know about the COVID-19 therapy trials Retrieved https://pharmaceutical-journal.com/article/feature/everything-you-need-to-know-about-the-covid-19-therapy-trials

Kadam, S. B., Sukhramani, G. S., Bishnoi, P., Pable, A. A., & Barvkar, V. T. (2021). SARS-CoV-2, the pandemic coronavirus: Molecular and structural insights. *Journal of basic Microbiology*, *61*(3), 180-202

Kumar, S., Singh, B., Kumari, P., Kumar, P. V., et al. (2021). Identification of multipotent drugs for COVID-19 therapeutics with the evaluation of their SARS-CoV2 inhibitory activity. *Computational and Structural Biotechnology Journal*, *19*, 1998-2017

Merck (2021) Merck and Ridgeback's Investigational Oral Antiviral Molnupiravir Retrieved from https://www.merck.com/news/merck-and-ridgebacks-investigational-oral-antiviral-molnupiravir-reduced-the-risk-of-hospitalization-or-death-by-approximately-50-percent-compared-to-placebo-for-patients-with-mild-or-moderat/

Narayanan, D.L., Djearamane, S., Fuloria, S., Kayarohanam, S., et al. (2022). A Review on DNA Vaccines in Pre-Clinical Trials Against SARS-CoV-2. *Journal of Experimental Biology and Agricultural Sciences*, *10*(3), 487 –493

Narayanan, D.L., Kayarohanam, S., Fuloria, S., Fuloria, N., et al. (2021). Covid-19 vaccine candidates under clinical evaluation-a review. *International Journal of Pharmaceutical Research*, *13*(1), 4588-4598

National Center for Biotechnology Information. (2022). Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome Retrieved from https://www.ncbi.nlm.nih.gov/nuccore/1798174254. Accessed on 13 January 2021

National Institutes of Health. (2021). The COVID-19 Treatment Guidelines Panel's Interim Statement on Patient Prioritization for Outpatient Anti-SARS-CoV-2 Therapies or Preventive Strategies Retrieved from https://www.covid19treatmentguidelines.nih.gov/therapies/updated-statement-on-the-prioritization-of-anti-sars-cov-2-mabs/

Pfizer. (2021). Pfizer Starts Global Phase 2/3 EPIC-PEP Study of Novel COVID-19 Oral Antiviral Candidate for Post-Exposure Prophylaxis in Adults Retrieved from https://www.pfizer.com/news/press-release/press-release-detail/pfizer-starts-global-phase-23-epic-pep-study-novel-covid-19. Accessed on 1 January 2022

Rahimi, A., Mirzazadeh, A. & Tavakolpour, S. (2021). Genetics and genomics of SARS-CoV-2: A review of the literature with the special focus on genetic diversity and SARS-CoV-2 genome detection. *Genomics*, *113*, 1221-1232

Rohaim, M. A., El Naggar, R. F., Clayton, E., & Munir, M. (2021). Structural and functional insights into non-structural proteins of coronaviruses. *Microbial pathogenesis*, *150*, 104641

Sicari, D., Chatziioannou, A., Koutsandreas, T., Sitia, R., et al. (2020). Role of the early secretory pathway in SARS-CoV-2 infection. *Journal of Cell Biology*, *219*(9), e202006005

Tim Jewell. (2021). Everything You Should Know About the 2019 Coronavirus and COVID-19. Retrieved from https://www.healthline.com/health/coronavirus-covid-19#treatment

Timothy, A. C., Snow, M.S., & Nishkantha, A. (2020). Immunomodulators in COVID-19: Two Sides to Every Coin. *American Journal of Respiratory and Critical Care Medicine*, *202*, 10

U.S. Food and Drug Administration. (2021). Coronavirus (COVID-19) Update: FDA Authorizes Monoclonal Antibodies for Treatment of COVID-19 Retrieved fromhttps://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-monoclonal-antibodies-treatment-covid-19-0

U.S. National Library of Medicine. (2021a). Study of PF-07321332 in Healthy Participants retrieved from https://clinicaltrials.gov/ct2/show/NCT04756531?term=PF-07321332&draw=2&rank=10

U.S. National Library of Medicine. (2021b). Study to Evaluate the Effects of RO7496998 (AT-527) in Non-Hospitalized Adult and Adolescent Participants with Mild or Moderate COVID-19 Retrieved from https://clinicaltrials.gov/ct2/show/NCT04889040

U.S. National Library of Medicine. (2022). The Safety of Molnupiravir (EIDD-2801) and Its Effect on Viral Shedding of SARS-CoV-2 Retrieved from https://clinicaltrials.gov/ct2/show/NCT04405739

V'kovski, P., Kratzel, A., Steiner, S., Stalder, H., & Thiel, V. (2021). Coronavirus biology and replication: implications for SARS-CoV-2. *Nature Reviews Microbiology*, *19*(3), 155-170

Velavan, T. P., & Meyer, C. G. (2020). The COVID-19 epidemic. *Tropical medicine & international health*, *25*(3), 278

Vincent, F., Bruno, C, & Alain, T. (2021). Combining Antivirals and Immunomodulators to Fight COVID-19. *Trends Immunology*, *42*(1): 31–44

Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., et al. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell*, *181*(2), 281-292

World Health Organisation. (2022a). Covid-19 Dashboard Retrieved from https://covid19.who.int/

World Health Organisation. (2022b). COVID-19 vaccine tracker and landscape Retrieved https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines

Yang, Q., Hughes, T. A., Kelkar, A., Yu, X., et al. (2020). Inhibition of SARS-CoV-2 viral entry upon blocking N-and O-glycan elaboration. *Elife*, *9*, e61552

Zhou, X., & Ye, Q. (2021). Cellular immune response to COVID-19 and potential immune modulators. *Frontiers in Immunology*, *12*, 646333

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Microbial biodegradation of nitrophenols and their derivatives: A Review

Sk Aftabul Alam, Pradipta Saha*

Department of Microbiology, The University of Burdwan, Golapbag, Burdwan-713104, WB, India.

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KEYWORDS

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Recalcitrant

Xenobiotic

ABSTRACT

Today, nitrophenols (NPs) represent chemicals highly in demand not only due to their function in synthetic chemistry but also due to their huge applications in several industries. Such diverse requirements and applications has resulted in a widespread abundance of these chemicals. Improper application and waste disposal practice results in the continuous discharge of these compounds into the environment and causes pollution threat to soil, groundwater, river water, etc. These xenobiotic chemicals are hazardous, toxic, carcinogenic, and mutagenic which results in serious health problems. The Nitro group present in the phenol makes them recalcitrant which causes the persistence of these chemicals in the environment. Although several chemicals, electrochemical, physical, and physicochemical methods have been proposed, bioremediation approaches mainly involving bacteria are considered best. To date, very few successful attempts (related to microbe-assisted bioremediation) have been carried out with environmental habitats for the removal of NPs (both *in-situ* and *ex-situ* attempts). So, as far as the effectiveness of the bioremediation process for NP decontamination is concerned, we are far away. More explorative studies using efficient aerobic-anaerobic NP degrading bacterial consortium (or combination of microbes- plant systems) and advanced techniques including omics approaches and nanotechnologies may help towards developing better practicable bioremediation approaches, in the future. This review article focuses on the list of nitrophenol degrading microorganisms, biodegradation pathways of NPs, bioremediation by immobilized cell technique, and the advantages and disadvantages of bioremediation. This article will increase our knowledge of the biodegradation of NPs.

* Corresponding author

E-mail: psaha@microbio.buruniv.ac.in (Pradipta Saha)

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1 Introduction

NPs are aromatic compounds that contain the nitro group attached to phenol rings. These are used as raw materials in industries for the manufacturing of pesticides, fungicides, paints, explosives, drugs, dyes, and cosmetics (Ju and Parales 2010; Xu et al. 2021). Many of these are considered pollutants of significant importance due to their toxicity to the living world, high mobility, and long persistence in most natural ecosystems (Kulkarni and Chaudhari 2007). The release of these organic compounds from industries, agriculture uses, domestic activities, and medical applications not only pose threat to living systems and the environment (Spain 1995) but are also responsible for contamination of almost all ecosystems on earth (representing soil, air, and water) (Kulkarni and Chaudhari 2007).

Degradation of toxic compounds by microbes has been established as one of the best possible approaches toward decontamination of xenobiotic, hazardous, toxic compounds (Chen et al. 2012). A survey of the literature revealed that reports from the bacterial domain outnumber other representative groups. The reason might be due to their higher abundance, better adaptability due to their long evolutionary ancestry, rapid growth rate, small generation time, ability to utilize diverse compounds (as electron donors and acceptors), and availability of more versatile metabolic resources to utilize newly added xenobiotic compounds (Schleifer 2004). This review focuses on the current understanding of the biodegradation of various nitrophenols over the last two decades. Comprehensive research has been done on the isolation of nitrophenol degrading bacteria in the last two to three decades. But reports documenting catabolic utilization of NPs are very rare. Microorganisms-mediated biodegradation of nitrophenols are summarized in Table 1.

Table 1 List of microorganisms involved in biodegrading of nitrophenols

Name of microorganism	Type of NPs & (conc. of degradation)	Nature of metabolism	Isolation site	Reference
		Bacteria		
Achromobacter xylosoxidans Ns strain	PNP, 1.8mM	Catabolic	Mai Po Nature Reserve in Hong Kong, China	Wan et al. 2007
Alcaligenes eutrophus JMP 134; *Alcaligenes eutrophus* JMP 222	2,6-dinitrophenol; 0.05mM	cometabolic	NR	Ecker et al. 1992
Alcaligenes sp. strain NyZ215	2NP	cometabolic	Activated sludge, China.	Xiao et al. 2007
Arthrobacter aurescens TW17 *Nocardia* sp. Strain TW2	PNP, 0.1mM	cometabolic	The soil of Chico, California	Hanne et al. 1993
Arthrobacter protophormae RKJ100	PNP and 4-Nitrocatechol (NC); 0.3-0.5mM	Catabolic (C, N source)	Agricultural soil of Chandigarh, India	Chauhan et al. 2000
Arthrobacter protophormiae RKJ100	PNP; 0.5mM	cometabolic	An agricultural field containing pesticide	Labana et al. 2005
Arthrobacter sp. HY2	PNP, 100mg/L	cometabolic	The soil of pesticide factory, Anyang, Henan Province, China	Qiu et al. 2009
Arthrobacter sp. JS443	PNP; 100mg/Liter	cometabolic	Soil of Florida	Jain et al. 1994
Arthrobacter sp. SPG	PNP, 0.3mM	Catabolic (C, N source)	Pesticide contaminated site, Hyderabad, India;	Arora 2012b
Arthrobacter sp. Y1	PNP, 100mg/liter	cometabolic	Activated sludge, China	Li et al. 2008
Arthrobacter sp. CN2	PNP, 0.05mM	Catabolic (C source)	Activated sludge, China;	Wang et al. 2016
Bacillus sp. *Pseudomonas* sp.	PNP	Catabolic (C source)	Parathion amended alluvial soil of Orissa, India	Siddaramappa et al. 1973
Bacillus sphaericus JS905	PNP; 0.15mM	cometabolic	Agricultural soil of Chandigarh, India	Kadiyala and Spain 1998
Bacillus subtilis RKJ700	4-Chloro-2-nitrophenol; 1.5mM	cometabolic	The soil of Pesticide contaminated site at Bathinda, Panjab, India	Arora 2012b
Brevibacterium linens	PNP; 100mg/L	cometabolic	Garden soil of Imphal, India	Ningthoujam 2005
Citriococcus nitrophenolicus sp. PNP1	PNP, 0.7mM	Catabolic (C, N source)	The wastewater treatment plant, pesticide factory at cheminova A/S, Denmark	Nielsen et al. 2011

Name of microorganism	Type of NPs & (conc. of degradation)	Nature of metabolism	Isolation site	Reference
Flavobacterium sp.	PNP	cometabolic	Paddy water of Philippines;	Sethunathan and Yoshida 1973
Janthinobacterium sp.	2,4-DNP	Catabolic	Forest soil and Freshwater stream respectively	Hess et al. 1990
Mixed culture: *Enterbacter cloacae, Alcaligens* sp. TK2	4-Chloro-2-nitrophenol	cometabolic	Sludge of sewage plant, Munster	Beunink and Rehm 1990
Moraxella sp.	PNP, 150mg/L	Catabolic (C source)	Activated sludge of Florida	Spain and Gibson 1991
Nocardioides simplex FJ2-1A	2,4,6-trinitrophenol; 2,4,6-trinitrotoluene (0.35mM); 2,4-DNP; (0.35mM)	cometabolic	Picric acid waste water	Ebert et al. 2001
Nocardioides sp. NSP41	PNP	cometabolic	Industrial wastewater	Cho et al. 2000
Nocardioides sp. Strain CB 22-2	2,4,6-trinitrophenol (0.44-2.2mM); 2,4- DNT	cometabolic	Soil samples from Nitroaromatic compound production sites, German	Behrend and Heesche-Wagner 1999
Ochrobactrum sp. B2	PNP (100mg/liter), methyl parathion	cometabolic	Soil, China	Qiu et al. 2007
Pseudomonas cepacia strain RKJ200	PNP, 0.5mM	cometabolic	Assam agricultural field	Prakash et al. 1996
Pseudomonas psudomallai ENB-10	PNP; 50mg/liter	cometabolic	Pharmaceutical industry wastewater, Pakistan	Rehman et al. 2007
Pseudomonas putida	PNP	cometabolic	Effluent sediment of the pesticide industry, Jalgaon, India	Kulkarni and Chaudhari 2006
Pseudomonas putida 2NP8	3-NP,	cometabolic	NR	Zhao and Ward 2001
Pseudomonas putida B2	3-Nitrophenol, 1mM	cometabolic	NR	Meulenberg et al. 1996
Pseudomonas putida DLL-E4	PNP and 4-nitrocatechol (0.5mM)	Catabolic (C, N source)	NR	Shen et al. 2010
Pseudomonas putida JS444	PNP	Catabolic	Activated sludge of California	Lei et al. 2005
Pseudomonas putida PNP1	PNP	cometabolic	El-Harrach River near Algiers	Löser et al. 1998
Pseudomonas sp. JHN	4-Chloro-3-nitrophenol	Catabolic	Wastewater, India	Arora et al. 2014a
Pseudomonas sp. PNP1	PNP; 100mg/liter	Catabolic	Municipal sludge, America	Heitkamp et al. 1990
Pseudomonas sp. *Bacillus* sp.	PNP, 15g/L	Catabolic	Parathion amended flooded soil.	Sudhakar-barik et al. 1976
Pseudomonas sp. BUR11	PNP, 200ppm	Catabolic	Agricultural soil, India,	Pailan and Saha 2015
Pseudomonas sp. WBC-3	PNP	Catabolic (C, N source)	NR	Zhang et al. 2009a
Pseudomonas sp. strain N26-8	2,4-Dinitrophenol 2,5-Dinitrophenol 2,6-Dinitrophenol (all utilize 0.5mM)	cometabolic	Mixed soil sample, Gottingen, The Federal Republic of Germany,	Bruhn et al. 1987
Ralstonia eutropha JMP 134 (DSMZ 4058)	3NP 0.5mM;2,4-dichloroPhenoxy-acetate, 2mM.	cometabolic	NR	Schenzle et al. 1997

Name of microorganism	Type of NPs & (conc. of degradation)	Nature of metabolism	Isolation site	Reference
Ralstonia eutropha JMP 134	2-chloro-5-nitrophenol; 0.46mM	Catabolic	NR	Schenzle et al. 1999
Rhodobacter capsulatus ElFI	2NP,3NP, PNP, 2,4DNP	cometabolic	NR	Blasco and Castillo 1992
Rhodococcus erythropolis strain HL PM-1	2,4,6-trinitrophenol, 2,4-dinitrophenol as sole source of Nitrogen	cometabolic	NR	Heiss et al. 2003
Rhodococcus erythropolis HL 24-2	2, 4-Dinitrophenol; Picric Acid; 0.5mM	cometabolic	Water from river Rhine, Germany	Lenke and Knackmuss 1992
Rhodococcus imtechensis RKJ300	PNP, 2,4-dinitrophenol	Catabolic (C source)	Pesticide contaminated soil of Punjab, India	Ghosh et al. 2010
Rhodococcus opacus SA0101	PNP	Catabolic	Soil of Japan	Kitagawa et al. 2004
Rhodococcus sp. PN1	PNP	Catabolic	Contaminated soil of Japan	Takeo et al. 2003
Rhodococcus opacus strain RB1	2,4-Dinitrophenol (0.5mM)	cometabolic	activated sludge, waste water plant in Alicante, Spain	Blasco et al. 1999
Rhodococcus wratislaviensis	PNP; 0.72mM	Catabolic	River sediment in Buenos Aires, Argentina	Gemini et al. 2005
Serratia sp. DS001	PNP, 0.3mM	Catabolic (C source)	Agricultural district of Anantapur district, Andhra Pradesh, India	Pakala et al. 2007
Sphingomonas sp. UG30	PNP and Pentachlorophenol	cometabolic	Agricultural site in Cambridge, Ontario, Canada	Alber et al. 2000
Sphingomonas sp. UG30; *Sphingomonas chlorophenolica* strain R2A; *Sphingomonas chlorophenolica* strain ATCC 39723	PNP; (0.31-1.10)mM	cometabolic	Fresh water sediment of Canada; contaminated soil of Canada	Leung et al. 1997
Burkholderia sp. KU-46.	2,4-dinitrophenol, 0.5mM	cometabolic	Agricultural soil contaminated pesticide in Japan	Iwaki et al. 2007
Arthrobacter sp. SJCon	2-Chloro-4-nitrophenol; 0.2mM	Catabolic	Pesticide contaminated soil of Punjab, India.	Arora and Jain 2011
Exiguobacterium sp. PMA (JQ182409)	4-chloro -2-nitrophenol; 0.5mM	Catabolic	soil from chemically conta - minated site- Gajraula, Uttar Pradesh, India	Arora et al. 2012
Bacillus sp. MW-1	4-chloro -2-nitrophenol; 0.3mM	cometabolic	Bay of Bengal, India	Arora and Jain 2011
Cupriavidus sp. strain CNP-8 (CCTCC M 2017546.)	2-chloro-4-nitrophenol; 0.3mM	cometabolic	soil of Yantai, Shandong, China	Min et al. 2018
Sphingomonas strain UG30	2,4-dinitrophenol, 150µM	cometabolic	PCP contaminated soil, Canada	Zablotowicz et al. 1999
Pseudomonas sp. JHN	4-chloro-2-nitrophenol; 0.2-0.6mM	cometabolic	Waste water	Arora and Bae 2014b
Burkholderia sp. SJ98	3-methyl-4-nitrophenol; 0.5mM	cometabolic	NR	Min et al. 2016
Burkholderia sp. strain SJ98	2-chloro-4-nitrophenol; PNP; 0.3mM	cometabolic	NR	Min et al. 2014
Burkholderia sp. strain RKJ 800	2-chloro-4-nitrophenol; 3-methyl-4-nitrophenol; PNP; 0.3mM	Catabolic	Pesticide contaminated soil, India	Arora and Jain 2012

Name of microorganism	Type of NPs & (conc. of degradation)	Nature of metabolism	Isolation site	Reference
Spirodela polyrrhiza	3-nitrophenol, 0.5mM	Catabolic	Water sample of Nigori river, Fuefuki River, Kamanashi River; Japan	Kristanti et al. 2012
Rhodococcus sp. CN6	PNP; 50mg/L	cometabolic	Effluentsediment of industry in Shandong province, China,	Zhang et al. 2009b
Pseudomonas sp. JHN	4-Chloro-3-nitrophenol; 0.4mM	Catabolic	Waste water, India	Arora et al. 2014a
Sphingobacterium sp. RB	PNP, 5mM	cometabolic	Rhizosphere soil of palm tree, Maharashtra, India	Samson et al. 2019
Fungi				
Phanerochaete chrysosporium (ATCC 34541)	PNP, 0.25mM	Catabolic	NR	Teramoto et al. 2004
Aspergillus niger VKM F-1119	3-methyl-4-nitrophenol; 25mg/L	cometabolic	NR	Kanaly et al. 2005
Algae				
Chlorella vulgaris; Chenochloris pyrenoidosa	PNP; 10mg/L	Catabolic	NR	Lima et al., 2003

Abbreviations: Conc., Concentration; NR, Data not reported.

2 Nitrophenols: Types, applications, and adverse effects

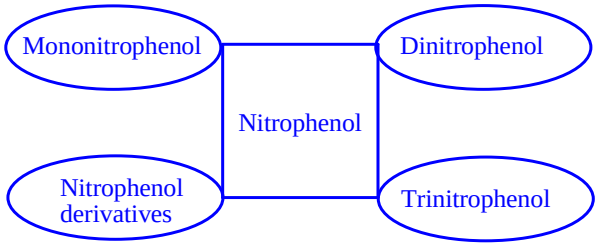
When one or more hydrogen atom(s) from the ring of phenol is replaced by one or more nitro groups, this structure is called NP. These compounds are generally water-soluble and moderately acidic. NPs are broadly classified into four subtypes, depending upon the number of nitro groups substituted (Figure 1a). These types are Mononitrophenol - (Example- PNP, 2NP, 3NP), Dinitrophenol - (Example- 2,4-DNP; 2,6-DNP; 2,5-DNP), Trinitrophenol - (Example- 2,4,6-trinitrophenol) and NP derivatives - (Example- 2-cl-5NP; 5-cl-2NP; 4-cl-2NP; 2-cl-4NP, 4,6-dinitro-2-methyl phenol; 2-cl-4,6-dinitrophenol; 2-amino-4-nitrophenol; 2-amino-4,6-dinitrophenol; 3-methyl-4-nitrophenol; 2-methyl-4-nitrophenol). Their structures are provided in Figure 1b.

NPs are commonly used organic xenobiotic compounds, having a variety of applications. Due to the occurrence of the highly active NO_2 (nitro) group, they are highly desirable chemicals in synthetic chemistry (Ju and Parales 2010) and their applications are listed in Table 2. However, extensive, widespread application of NP is of huge public concern due to its persistent, recalcitrant, hazardous nature and toxicity to non-target organisms. NPs like- 2,4-DNP, and PNP act as an uncoupler of mitochondrial oxidative phosphorylation and reduce ATP production. Again, some of them are water-soluble and mobile, contaminating drinking water sources (Samuel et al., 2014; Kuang et al., 2020). Available reports suggest that some NPs (e.g. PNP) have high toxicity and can threaten human health, through dysfunction of the liver, kidney, and other important physiological life processes, as has been documented for animal models (Wang et al. 2018; Kuang et al. 2020). Direct or indirect contact with these NPs either by inhalations or accidental ingestion may cause chronic toxicity (Wyman et al. 1992; Ju and parales 2010; Przybyla et al. 2021). Although some NPs were used in the health sector (di-nitrophenols were used in cataracts and among athletes for weight loss), due to their negative impact on health, these are no longer prescribed. Moreover, many NPs (like PNP and 2NP) are considered carcinogenic (Karim and Gupta 2001).

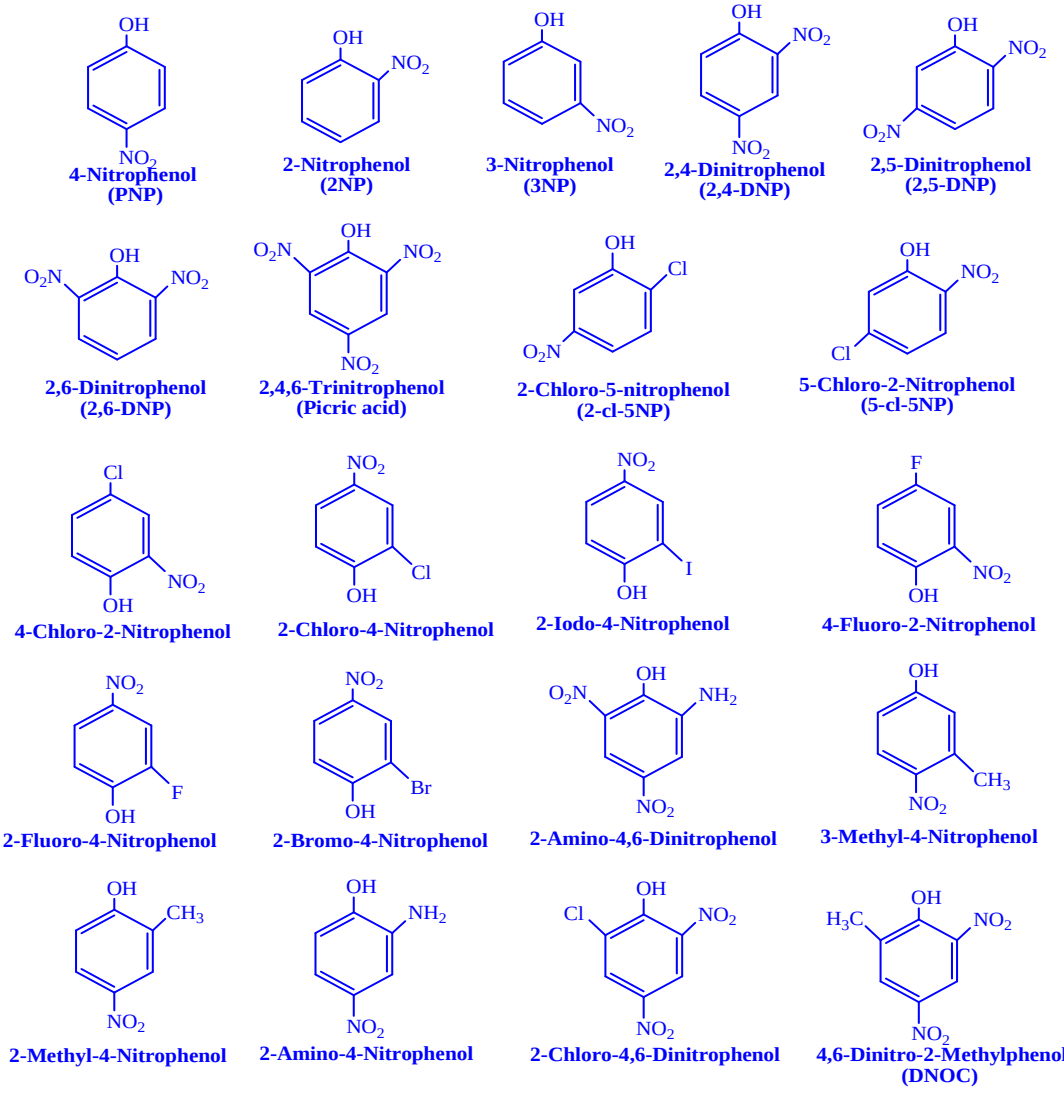
The Environmental Protection Agency (USEPA) has enlisted PNP, 2-NP, and 2, 4-dinitrophenol as priority pollutants because of their high toxicity and wide environmental distribution in the ecosystem (Karim and Gupta 2001; Zhang et al. 2022). USEPA recommended the restriction of these compound concentrations in natural water below <10ng/liter (Karim and Gupta 2001; She et al. 2005; Gemini et al. 2005). Some NPs may contaminate the environment during industrial or agricultural uses and their improper application and/ or storage practices by users (having no technical knowledge, especially in economically poor countries) have resulted in their pollution of the environment, especially soil and groundwater. PNP is toxic, and due to its extensive use and widespread abundance, it is believed to may have accumulated in the food chain (Herrera-Melián et al. 2012).

Although the ultimate impact and long-time fate of these toxic NPs released into the ecosystems remain known, studies indicated these inhibit the growth of microorganisms and are reported to destabilize ecosystems (for example, the sewage treatment plant as reported by Bruhn et al. 1987). NPs are toxic, and many are suspected to be mutagenic and carcinogenic (Wan et al. 2007).

These are harmful to the health of animals including humans and fauna. The LD_{50} (50% of lethal density) value and the half-life of some nitrophenols are provided in Table 3. From this information, it is deducible that most of the NPs may be considered as a potential threat to public health as well as ecosystems. Their presence in non-permissible limits in most ecosystems is of huge public concern and therefore suitable measures must be taken either to remove them completely or ensure their concentration below toxic levels. For microbial bioremediation, it is desirable to know how these NPs are degraded or hydrolyzed by microorganisms. The biodegradation pathways of NPs are discussed in the coming headings.



(a)



(b)

Figure 1a and 1b Classification of nitrophenol and structure of representative types

Table 2 Applications of different types of nitrophenol

Type of NP	Applications	References
Mononitrophenol	Paracetamol, a well-known analgesic and antipyretic, is prepared from PNP. Pesticides like parathion; methyl parathion is prepared by PNP. 2NP and 3NP are used for synthesis of fungicides, dyes, chemicals etc.	Arora et al. 2014b; Ebert et al., 2001
Dinitrophenol	Several herbicides, pesticides (like- 4,6-dinitro-o-cresol, Fluorodifen, Binapacryl) fungicides are prepared from dinitrophenols. 2,5-DNP is used as pH indicator. Dinoseb, DNOC are also used as herbicides. Azo dyes, pesticides, explosives are prepared. 2,4-DNP is used for wood preservatives, herbicides, pesticides.	Arora et al. 2014b; Bruhn et al. 1987
Trinitrophenols	2,4,6-trinitrophenol (picric acid) used as explosive during world war I and II; used for colouring for silk, leather, wool; synthesis of nitrofungin, dicapthon.	Arora et al. 2014a
Nitrophenol derivatives	2-cl-4NP is used for preparation of several pesticides like nitrofungin, dicapthon; Previously used for seed protection and leather conservation 2-A-4NP is used for acid dye like leather, nylon, silk, wool; used as mordant.	Arora et al. 2014b; Lang et al. 2001

Table3 LD_{50} and half-life of some nitrophenols

Compounds	LD_{50}	Half-life	References
PNP	21900µg/liter for daphnids; 8280µg/liter for bluegills; 7170µg/liter for mysid shrimp and 27100µg/liter for sheepshead minnow	In the top soil 1-3 days (aerobic); 14 days (anaerobic); In the sub soil 40 days (aerobic)	Epa 1980
2,4-Dinitrophenol	4090 µg/liter for daphnids; 620µg/liter for bluegills; 48505500 µg/liter for mysid shrimps and 5500µg/liter for herring embryo	28 days	Epa 1980; Przybyla et al. 2021
2,4,6-Trinitrophenol	84700µg/liter for daphnids; 167mg/liter for bluegills; 19.7mg/liter for mysid shrimp	13.4hour	Epa 1980; Wyman et al. 1992
Dinitrocresol/4,6-Dinitro-o-cresol (DNOC)	25-40 mg/kg for mice	153.6 hour	Brown and Chessin 1995

NR- data not reported.

3 Biodegradation of mono-nitrophenols and their responsible genes and proteins

3.1 PNP degradation

There were mainly two pathways of bacterial degradation of PNP viz. the first one is Hydroquinone pathway which was initially reported from Gram-negative bacteria, subsequently, many Gram-positive bacteria were also informed to follow this pathway, and the second pathway is nitrocatechol pathway which was initially known for Gram-positive bacteria, several Gram-negative bacteria also adopt this pathway.

Several bacteria like *Pseudomonas putida* JS444; *P. cepacia* strain RKJ200; *Arthrobacter protophormiae* strain RKJ100; *Rhodococcus opacus* SA0101; *Moraxella* sp.; *Arthrobacter aurescens* TW17 (Spain and Gibson 1991; Hanne et al. 1993; Prakash et al. 1996; Pandey et al. 2003; Kitagawa et al. 2004; Lei et al. 2005), etc. could degrade PNP via hydroquinone pathway. In this pathway, PNP was first transformed to 1,4 benzoquinone/ para benzoquinone via the enzyme monooxygenases, with the requirement of one mole of NADPH. In the following step, para benzoquinone was converted to hydroquinone by the enzyme reductase, and one mole of NADPH was required in this second step. In the subsequent step, the dioxygenase enzyme was responsible for the cleavage of hydroquinone to 4-hydroxymuconic semialdehyde (HMS). Next maleylacetate was produced from HMS by the enzyme Dehydrogenase, which was again degraded through β-ketoadipic acid and TCA cycle intermediates (Figure 2a)

Gram-positive, as well as Gram-negative bacteria, degraded PNP via the nitrocatechol pathway. These include- *Bacillus sphaericus* JS905; *Nocardia* sp. TW2 (Hanne et al. 1993; Kadiyala and Spain 1998). Few bacteria like *Pseudomonas* sp. 1-7, *Burkholderia* sp. SJ98; (Bhushan et al. 2000; Zhang et al. 2012) degraded PNP via both hydroquinone and nitro catechol pathways (figure 2a).

Jain et al. (1994) first time elaborated on the entire nitro catechol pathway of PNP degradation. In this pathway, PNP was first converted to 4-nitrocatechol (4NC) by the enzyme monooxygenase. In the next step, 4NC was converted to 1,2,4 benzenetriol by the enzyme monooxygenase. Here Oxygen was used and nitrite was released. In the next step maleyl acetate (MA) was produced by the enzyme dioxygenase. MA was further degraded via TCA cycle intermediates.

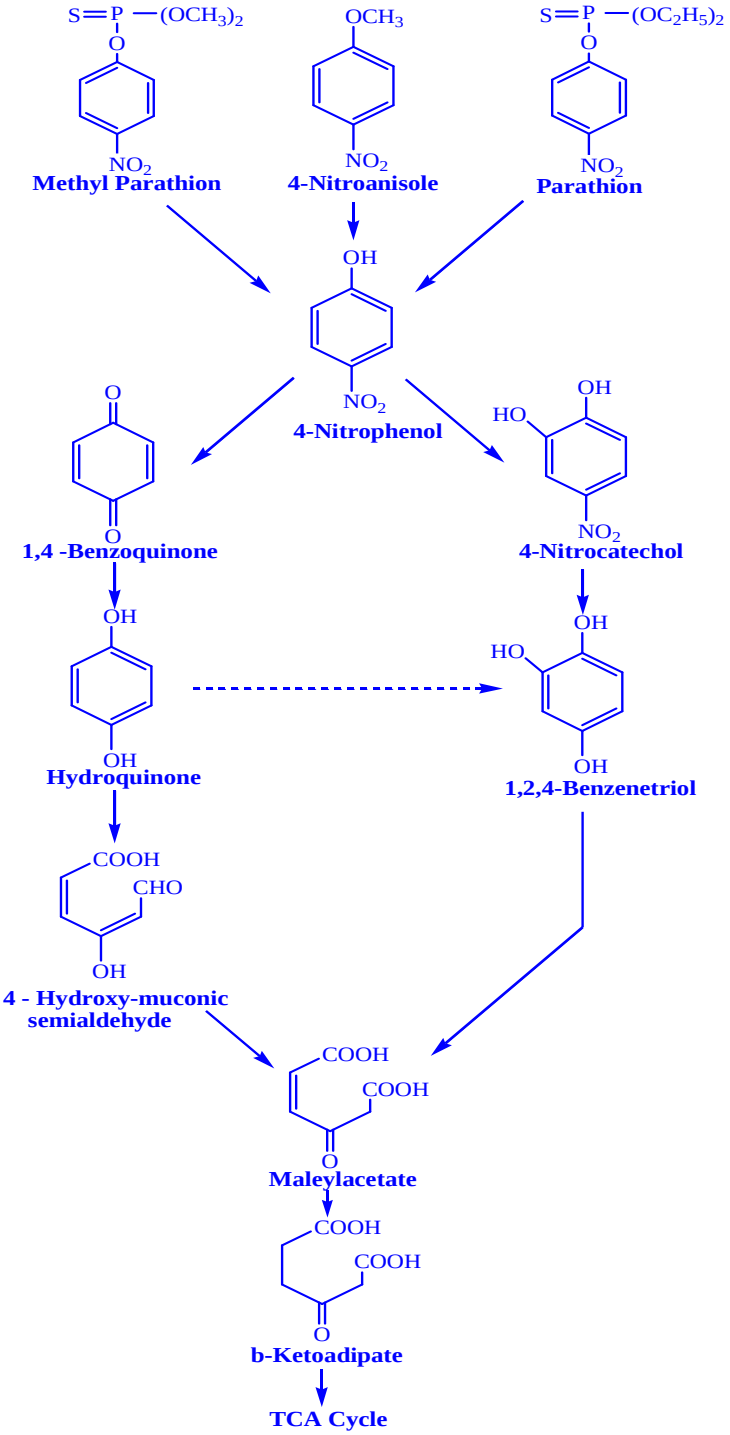


Figure 2a Biodegradation of PNP (from three different compounds) by hydroquinone pathway and nitrocatechol pathway

Prakash et al. (1996) demonstrated that approximately 50Kb transmissible plasmid was responsible for PNP degradation in *P. cepacia* strain RKJ 200. Further, Zhang et al. (2009a) characterized the enzymes i.e., monooxygenase, p-benzoquinone reductase, hydroquinone dioxygenase, 4-hydroxymuconic semialdehyde dehydrogenase, maleylacetate reductase, which catalyzed the transformation of PNP to β-ketoadipate in *Pseudomonas.* sp. strain WBC-3. Guo et al. (2021) demonstrated that a single component and two component monooxygenases were responsible for Gram negative and Gram positive bacterial PNP degradation, respectively.

Approximately 70Kb plasmid of the strain WBC-3, designated as pZWL0 was responsible for the degradation of parathion and PNP (Liu et al. 2005). Vikram et al. (2012) reported that a 41kb DNA fragment of *Burkholderia* sp. strain SJ98 contains multiple ORFs like *pnp*C, *pnp*D, *pnp*F, *pnp*E1, and *pnp*E2. Heterotetrameric protein complex *pnp*E1 and *pnp*E2 can transform hydroquinone to 4-hydroxymuconic semialdehyde. The Gram-positive bacteria *Rhodococcus opacus* SAO101 contain *npc*A, *npc*B, and *npc*C gene clusters for mineralization of PNP. Among these monooxygenases were encoded by the genes *npcA* and *npcB* while hydroxyquinole 1,2-dioxygenase was encoded by the *npcC* gene. When *npcA* and *npcC* genes were mutated, inhibition of growth was recorded under catabolic growth conditions (Kitagawa et al. 2004). A multicomponent enzyme, benzenetriol dioxygenase (*btd*) played a significant role in the PNP biodegradation of soil bacteria. This dioxygenase catalyzed the conversion of benzenetriol to maleylacetate (Paul et al. 2008). In the strain, *B. cepacia* AC1100, *tftH*, and *tftE* genes encoded the enzyme dioxygenase and maleylacetate reductase respectively responsible for the conversion of 1,2,4-Benzenetriol to 3-ketoadipae (Daubaras et al. 1996). The bacterium *Ralstonia pickettii* strain DTP0602 was reported to have the gene *hadC* encoded the enzyme 1,2-dioxygenase that transformed hydroxyquinol to maleylacetate (Hatta et al. 1999). Chauhan et al. (2010) demonstrated that within the genomic DNA of *Burkholderia* sp. SJ98, the *pnpC* gene-encoded Benzenetriol dioxygenase, and *pnpD* gene-encoded maleylacetate reductase. These pnpC, and pnpD genes converted Benzenetriol to beta-ketoadipate.

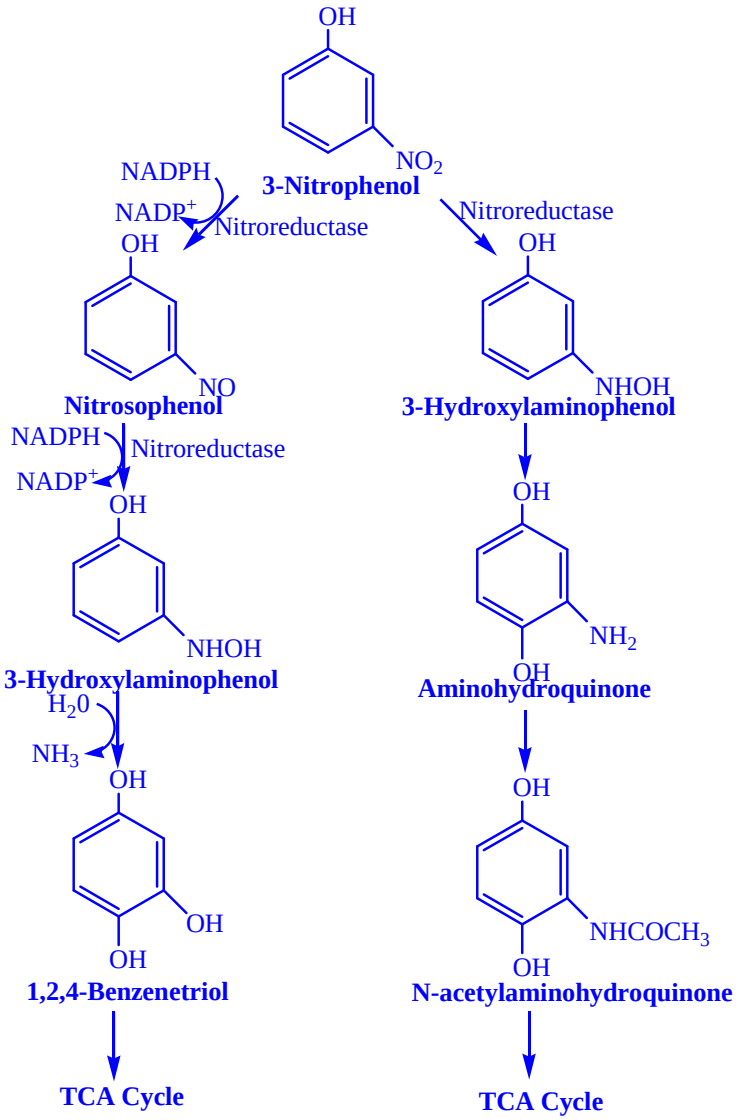


Figure 2b Biodegradation of 3-nitrophenol by the strain *Pseudomonas putida* strain B2 (left side) and *Ralstonia eutropha* strain JMP134 (right side)

Schäfer et al. (1996) reported that *Rhodococcus opacus* AS2 and *R. erythropolis* AS3 can demethylate 4-nitroanisole to PNP with the help of the enzyme oxygenase. After that, PNP entered into the TCA cycle via the Nitrocatechol pathway.

Liu et al. (2007) reported that the *Stenotrophomonas* sp. strain LZ-1 could utilize 4-chlorophenol and PNP co-metabolically and degrade those compounds via the hydroquinone pathway.

3.2 3NP degradation

Capability to degrade 3NP was reported for a few bacteria, in the literature, these includes *Spirodela polyrrhiza* (Kristanti et al. 2012), *P. putida* B2 (Meulenberg et al. 1996); *Cupriavidus nector* strain JMP 134 (Schenzle et al. 1997; 1999) (Figure 2b). Schenzle et al. (1997) first to report biodegradation of 3NP by *R. eutropha* JMP 134. Here, 3NP was first converted to 3-Hydroxylaminophenol by the enzyme nitro-reductase (MnpA) which was then transformed to amino hydroquinone by the enzyme mutase. It was then entered into the TCA cycle via N-acetylamino hydroxyquinone. A different pathway of 3NP biodegradation was reported in the case of *P. putida* strain B2. Whereas 3NP was first converted by the enzyme nitroreductase to nitrosophenol which was then cleaved to 3-Hydroxylamino phenol. Then it was converted to 1,2,4- Benzenetriol and ultimately entered into the TCA cycle.

The 3NP removal by anaerobic treatment is an effective method. She et al. (2005) reported that 60-80% of 3NP could be removed by anaerobic treatment co-metabolically. However, the detailed mechanism of anaerobic biodegradation is in scarcity.

Yin et al. (2010) proposed that in *Rhodococcus* JMP 134, mnp gene clusters played a significant role in the biodegradation of 3NP. MnpA encoded the nitroreductase enzyme to which flavin mononucleotide (FMN) was bound tightly, catalyzing the conversion of 3NP to 3-hydroxylaminophenol via nitrosophenol.

3.3 2NP degradation

Alcaligenes sp. NyZ215, *P. putida* B2 (Zeyer and Kearney 1984; Xiao et al. 2007) were reported to metabolize 2NP. In *Alcaligenes* sp. strain NyZ215, the ortho nitrophenol was first converted to 1,2-benzoquinone/ortho-benzoquinone (1,2-BQ) with the help of the enzyme monooxygenase. Next, 1,2-BQ was converted to catechol by the enzyme reductase, which was subsequently reduced to hexa-2,4-dienedioic acid/ muconic acid by the enzyme dioxygenase which was again degraded to β-Keto adipic acid. Then it was converted to succinic acid and acetyl CoA by TCA cycle intermediates (Figure 2c). In the case of *P. putida* strain B2 monooxygenase enzyme performed both steps.

Xiao et al. (2007) proposed that three genes were present in *Alcaligenes* sp. strain NyZ215, viz; *onpA*, *onp*B, and *onp*C which encoded important enzymes for the catabolism of 2NP. OnpA was encoded for the monooxygenase enzyme that catalyzed the alteration of 2NP to catechol; onpB was encoded for the reductase enzyme that converted benzoquinone to catechol; onpC was encoded for the dioxygenase enzyme that catalyzed the transformation of catechol to hexa-2,4-dienedioic acid. All three genes were transcribed from a single operon from genomic DNA.

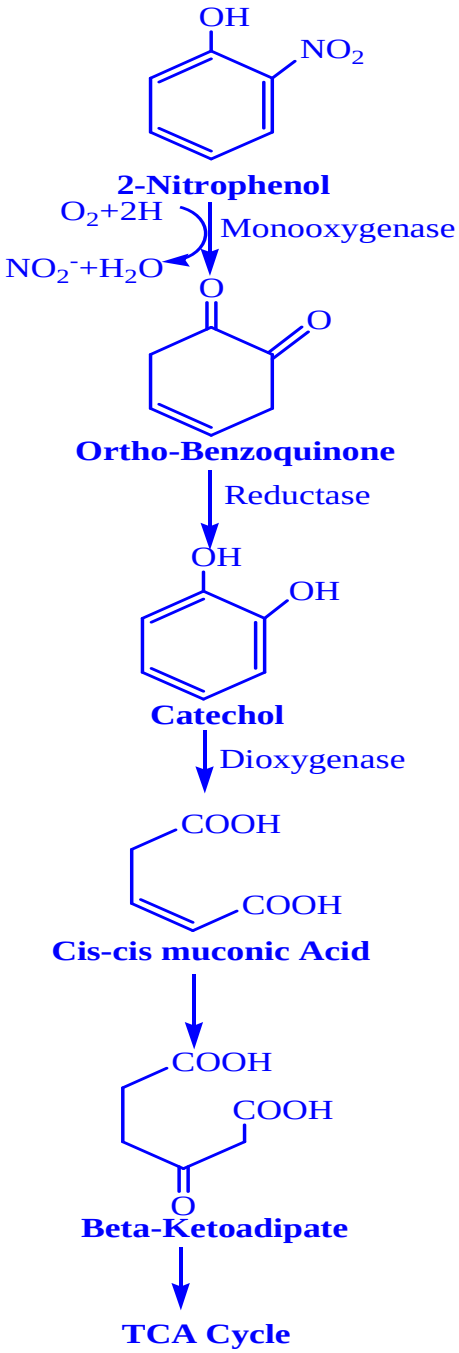


Figure 2c Biodegradation pathway of 2-Nitrophenol

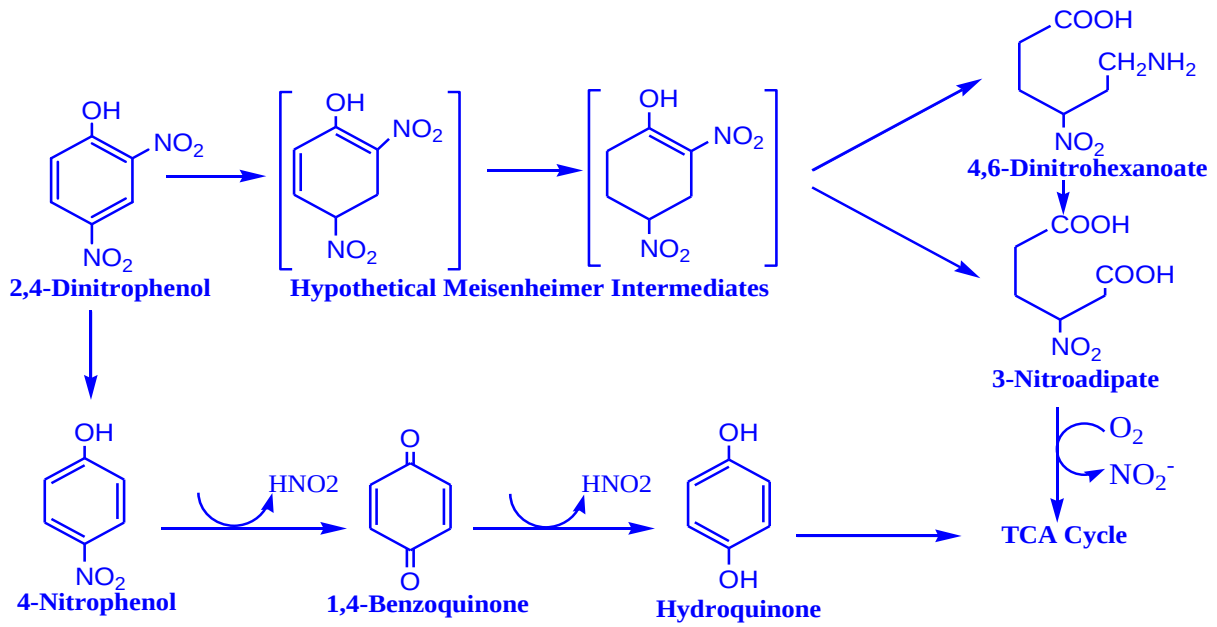


Figure 3a Biodegradation of 2,4-dinitrophenol

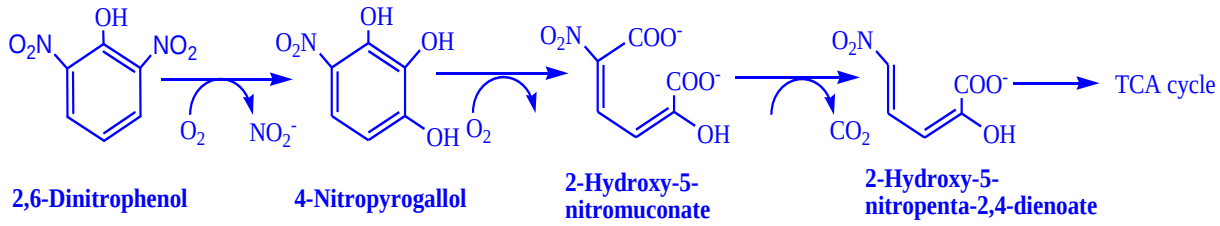


Figure 3b Biodegradation of 2,6-dinitrophenol

4 Biodegradation of dinitrophenol and their responsible genes and proteins

4.1 2,4-dinitrophenol degradation

Iwaki et al., (2007) first time reported that a soil-borne bacterium *Burkholderia* sp. strain KU-46 was capable of degrading 2,4-dinitrophenol cometabolically. This strain degraded 2,4-dinitrophenol via the intermediates of PNP, 1,4-BQ, and HQ. Two successive nitro groups were released during these biodegradation steps (Figure 3a).

Further, Blasco et al. (1999) reported that *R. opacus* strain RB1 mineralized 2,4 dinitrophenol cometabolically. During the mineralization process, this strain released two nitro groups as nitrite. The first phase was aromatic ring reduction by two successive hydride transfers; the second phase was the nitro group of ortho position released as nitrite and formation 3-nitroadipate and hydration of double bond may convert this intermediate to 4,6-Dinitrohexanoate. Ultimately 3-nitroadipate was metabolized with the simultaneous release of the nitro group in the form of nitrite ion (Figure 3a). Lenke et al. (1992) demonstrated that 2,4-dinitrophenol was cometabolically utilized by *R. erythropolis* strain HL 24-1 and HL-2. Here 4,6-Dinitrohexanoate acts as a minor dead-end product and nitrite were released in a significant amount. Heiss et al. (2003) proposed that *npdI* and npdG genes were responsible for the biodegradation of dinitrophenol in *Rhodococcus* sp.

4.2 2,6-dinitrophenol degradation

Bruhn et al. (1987) informed that the soil bacterium *Pseudomonas* sp. N-26-8, cometabolically utilizes 2,6-dinitrophenol. The strain degraded 2,6-dinitrophenol only under nitrogen limitation conditions in the presence of another carbon source. Though nitrite was released, no other intermediates could be recorded during this xenobiotic compound degradation. Ecker et al., (1992) reported that *Alcaligenes eutrophus* strain JMP134 could exploit 2,6-dinitrophenol catabolically. Concomitant nitrite release was recorded during biodegradation of 2,6-dinitrophenol. The parent compound was degraded by the enzyme dioxygenase to form 4-nitropyrogallol, which was converted to produce 2-hydroxy-5-nitromuconate. After that decarboxylation reaction proceeded to form 2-hydroxy-5-nitropenta-2,4-dienoate (Figure 3b).

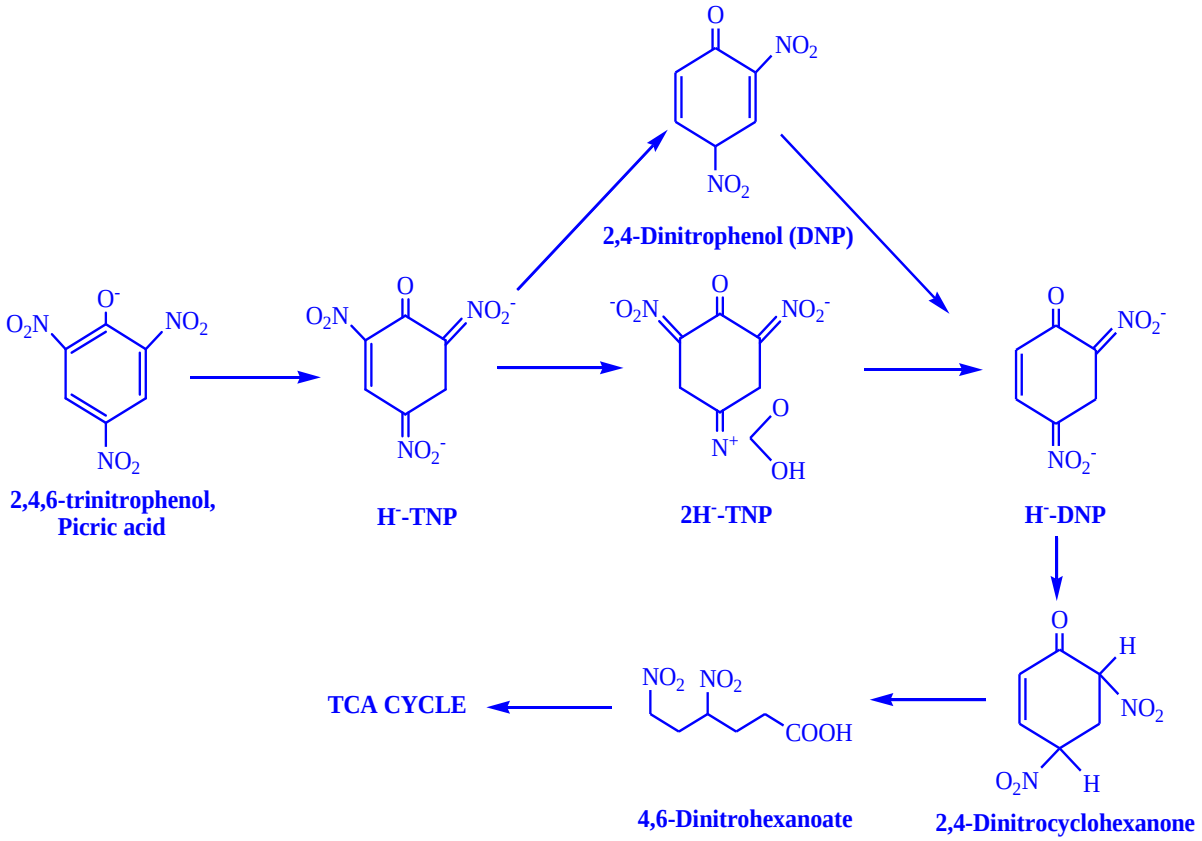


Figure 4 Biodegradation pathway of picric acid

5 Biodegradation of Trinitrophenol and their responsible genes and proteins

5.1 Picric acid degradation

Several microorganisms such as *Rhodococcus erythropolis* strain HL 24-1; *R. erythropolis* HL PM-1; *Rhodococcus* sp. NJUST16; *Nocardioiodes simplex* Nb; *N. simplex* FJ2-1A (Lenke and Knackmuss 1992; Rajan et al. 1996; Ebert et al. 1999; Heiss et al. 2003; Shen et al. 2009) utilized picric acid (2,4,6-Trinitrophenol or TNP) as a sole source of carbon. Picric acid biodegradation was investigated in the strain *Rhodococcus* sp. NJUST16. Initially, two hydrogenations occurred simultaneously in the TNP, converting it to the 2H-TNP (Figure 4). Nitrite was eliminated from the later compound to form hydride Meisenheimer complex, H^--DNP. Further hydrogenation of H^--DNP led to the form of 2,4-dinitrocyclehexanone, which then undergoes ring fission to form 4,6-dinitrohexanoate. The product 1,3,5-trinitriopentane, an analogue of 4,6-dinitrohexanoate, was obtained after acid treatment of culture fluid in the strain HL-2 (Lenke and Knackmuss 1992). Heiss et al. (2003) reported that reductase and hydride transferase II enzyme of strain HL PM-1, played a pivotal role in the conversion of 2,4,6-trinitrophenol and 2,4-dinitrophenol to their respective hydride Meisenheimer complexes.

6 Biodegradation of NP derivatives and their responsible genes and proteins

6.1 2-chloro-4-nitrophenol degradation

Multiple pathways were reported in the case of the biodegradation of 2-chloro-4-nitrophenol. This chlorinated nitroaromatic compound was degraded through the same metabolic pathway by *Burkholderia* sp. RKJ 800 and *R. imtechensis* RKJ 300 (Arora and Jain 2012; Ghosh et al. 2010). Here the nitro group was released with the help of the enzyme monooxygenase followed by cholo group which was released by the enzyme dehalogenase to form HQ. Ultimately dioxygenase enzyme converted it to 4-hydroxymuconic semialdehyde which entered into the TCA cycle (Figure 5a). Pandey et al. (2011) demonstrated the utilization of 2-cl-4NP by the strain *Burkholderia* sp. strain SJ98 which converted 2-cl-4NP to PNP with the help of the enzyme dehalogenase; after that PNP was degraded via the nitrocatechol pathway to enter into the TCA cycle. Arora and Jain (2011) demonstrated that *Arthrobacter nitrophenolicus* SjCon was able to degrade 2-cl-4NP via chlorohydroquinone and MA. CHQ dioxygenase enzyme was involved in the conversion of chlorohydroquinone to maleylacetate (Arora and Jain 2011).

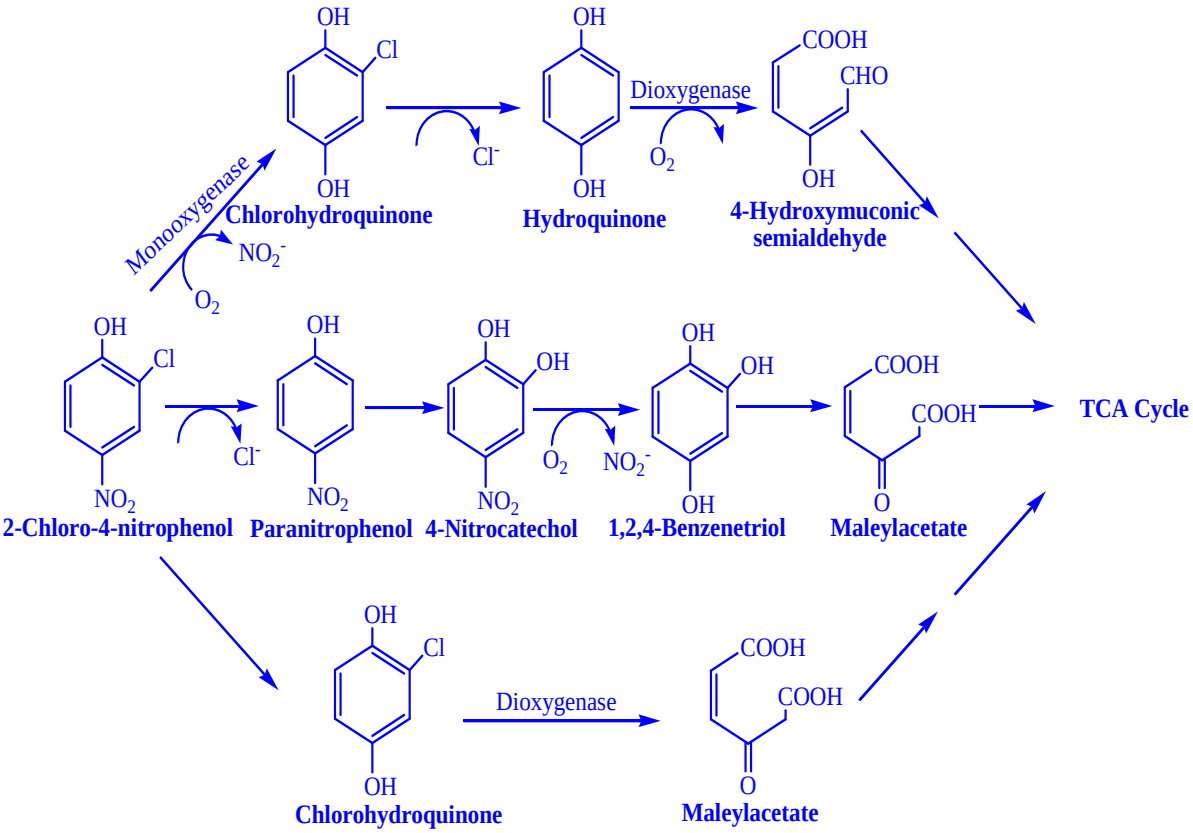


Figure 5a Biodegradation of 2-chloro-4-nitrophenol by *Rhodococcus imtechensis* strain RKJ 300 and *Burkholderia* sp. strain RKJ 800 (upper row); *Burkholderia* sp. strain SJ98 (middle row); *Arthrobacter nitrophenolicus* SiCon (lower row)

6.2 4-chloro-2-nitrophenol degradation

Arora et al. (2012) demonstrated that the strain *Exiguobacterium* sp. PMA was able to degrade 0.5mM (4-chloro-2-nitrophenol) 4-cl-2NP catabolically. Two important metabolites obtained in this degradation were 4-chloro-2-aminophenol and 2-aminophenol. At first reductase enzyme converted it into 4-chloro-2-aminophenol and then the dehalogenase enzyme released the chloro group from the para position of it and converted it into 2-aminophenol (2AP) (Figure 5b). Bruhn et al. (1988) prepared a transconjugant strain by transferring plasmid from either *Pseudomonas* sp. B13 or the bacterium *Alcaligenes eutrophus* JMP134 to the strain *Pseudomonas* sp. N31. The genetically modified bacterium *Pseudomonas* sp N31 was able to mineralize 0.5mM 4-chloro-2-nitrophenol co-metabolically. The parent compound was completely mineralized via the intermediate of 4-chlorocatechol. The soil and marine bacterium *Bacillus subtilis* RKJ 700 and *Bacillus* sp. strain MW-1 (Arora and Jain 2011; Arora 2012a) respectively decolorized and bio-transformed the highly toxic nitroaromatic compound into less poisonous 5-chloro-2-methylbenzoxazole via a same metabolic pathway. The chlorinated nitrophenol was biotransformation to 5-chloro-2-methylbenzoxazole through 4-chloro-2-aminophenol and 4-chloro-2-acetaminophenol. Beunink and Rehm (1990) investigated a mixed culture of bacteria *Alcaligenes* sp. TK-2 and *Enterbacter cloaceae* degraded the chlorinated nitrophenol to 4-chloro-2-aminophenol and then it was entered into the TCA cycle pathway. Arora et al. (2016) reported that *B. aryabhattai* strain PC-7 was capable of degrading 2mM 4-chloro-2-nitrophenol cometabolically. The strain bio transformed the parent compound into 5-chloro-2-methylbenzoxazole.

6.3 2-chloro-5-nitrophenol degradation

Schenzle et al. (1999) reported that the bacterium *Ralstonia eutropha* JMP 134 could exploit 2-chloro-5-nitrophenol (2-cl-5NP) and the parent compound was first converted to 2-chloro-5-hydroxylaminophenol by the enzyme reductase. The enzyme mutase in the next step converted it to 2-amino-5-chlorohydroquinone. In the third step, reductive dehalogenation of 2-Amino-5-chlorohydroquinone to amino hydroquinone was noticed (Figure 5c). Such a type of dehalogenation by aerobic bacteria is rarely observed in the aromatic ring. Schenzle et al. (1997) proposed that amino hydroquinone was further converted to N-acetyl-amino hydroquinone under anaerobic conditions.

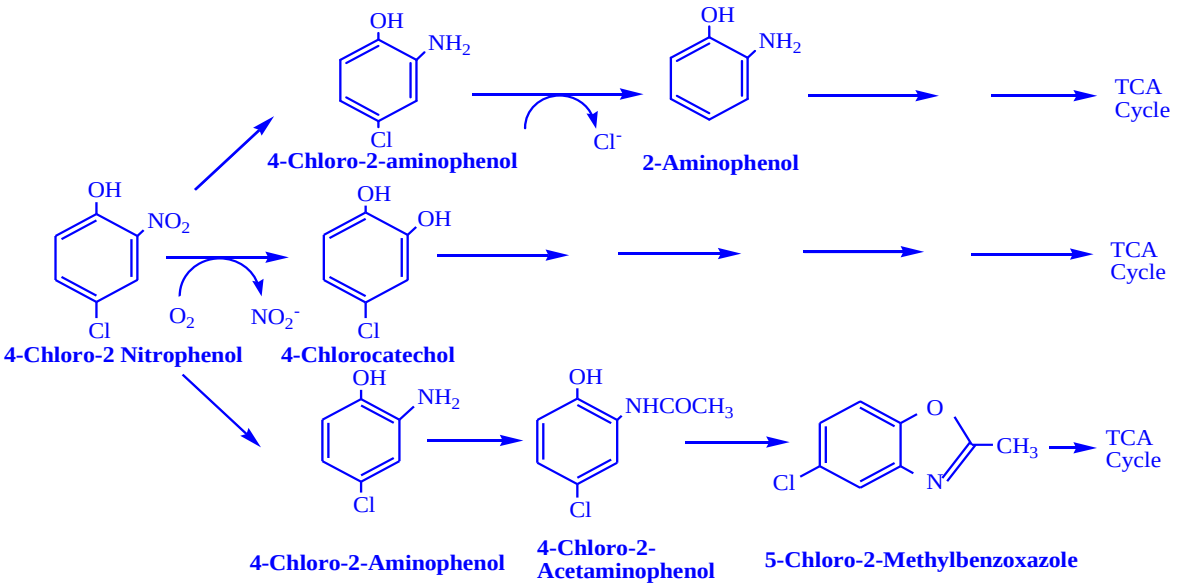


Figure 5b Biodegradation pathways of 4-chloro-2-nitrophenol by *Exiguobacterium* sp PMA (upper pathway); *Pseudomonas* sp. N31 (middle pathway); *Bacillus subtilis* strain RKJ 700 and *Bacillus* sp. strain MW-1 (lower pathway)

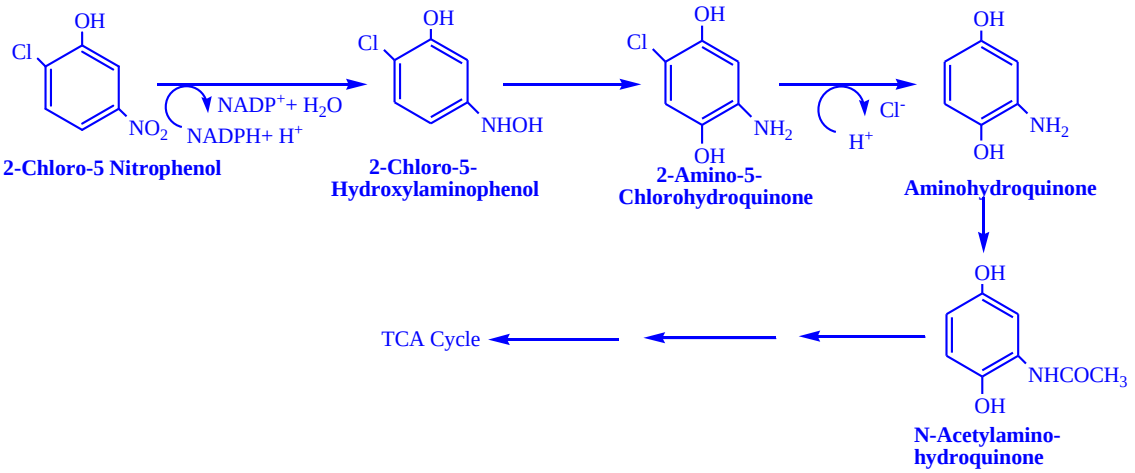


Figure 5c Biodegradation pathway of 2-chloro-5-nitrophenol

6.4 3-methyl-4-nitrophenol degradation

A survey of literature suggested that 3-methyl-4-nitrophenol (3M4NP) was reported to degrade by several bacterial strains like *Ralstonia* sp. SJ98; *Burkholderia* sp. FDS-1; *B.* sp. NF100 (Hayatsu et al. 2000; Bhushan et al. 2000; Zhang et al. 2006; Min et al. 2016) and fungal strain like *Aspergillus niger* VKM F-1119 (Kanaly et al. 2005). Bhushan et al. (2000) reported that the Gram-negative, chemotactic bacteria *Ralstonia* sp. SJ98 utilized 3M4NP as a sole source of carbon, energy, and nitrogen sources. The strain converted the methylated non-polar nitroaromatic compound to a highly polar compound catechol. Bacterial strain *Burkholderia* sp. NF100, *Burkholderia* sp. FDS-1 degraded the methylated nitroaromatic compound via methyl hydroquinone. Kanaly et al. (2005) reported that the soil fungi *Aspergillus niger* strain VKM F-1119 bio transformed 3M4NP to hydroxylated derivative like 2-methyl-1,4-benzenediol which would increase the water solubility of the target compound. Again, methylated nitroaromatic compounds may be transformed into carcinogenic compounds like 4-amino-3-methyl phenol which was further dimerized itself (Figure 5d). Min et al., (2016) demonstrated that the *pnpABA1CDEF* gene cluster was responsible for this methylated nitroaromatic compound degradation. In *Bacillus* sp. SJ98 (Min et al. 2016) pnp A gene-encoded monooxygenase enzyme, that catalized the conversion of 3M4NP to methyl-1,4-benzoquinone and nitrite was released during this conversion. The *Pnp B and PnpCD* genes were responsible for further transformation to 2-methyl-4-hydroxymuconic semi-aldehyde (Figure 5e).

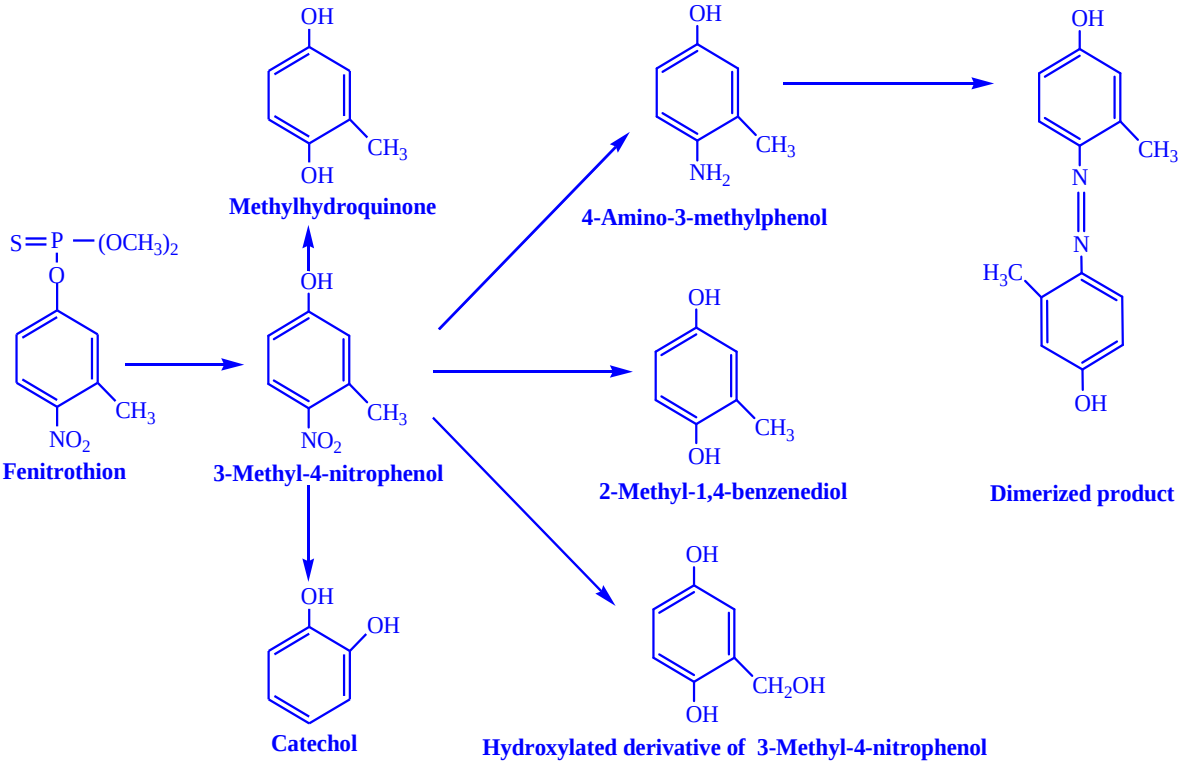


Figure 5d Biodegradation pathways of 3-methyl-4-nitrophenol

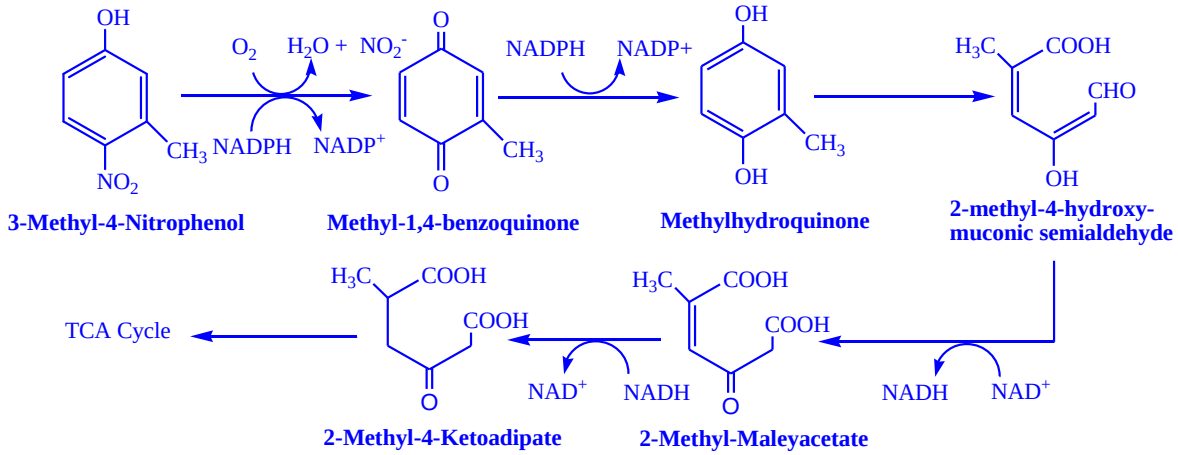


Figure 5e Biodegradation of 3-methy-4-nitrophenol

Hayatsu et al. (2000) reported that *Burkholderia* sp. NF100 harbored two plasmids pNF1 and pNF2; which were exclusively important for fenitrothion degradation. Loss of pNF1 and pNF2 from the strain NF100 destroyed methyl hydroquinone and fenitrothion hydrolyzing abilities respectively.

6.5 DNOC degradation

Several bacteria like *Rhizobium leguminosarum, R. trifolii, R. meliloti, Rhodococcus erythropolis* HL 24-1 (Hamdi and Tewfik 1970; Lenke and Knackmuss 1996), *Azotobacter* sp. and *Rhizobium* sp. (Wallnoefer et al. 1978) were reported to degrade 4,6-Dinitro-o-cresol (DNOC) (Figure 5f). In the strain *R. erythropolis* HL 24-1, DNOC was protonated by 2 hydrogen ions and subsequent protonation converted it to 4,6-Dinitro-2-methylhexanamine. After hydrolysis the later was converted to 4,6-Dinitro-2-methylhexanoate (Lenke and Knackmuss 1996). Wallnoefer et al. (1978) proposed that different *Azotobacter* and *Rhizobium* strains bio-transformed DNOC to 6-acetamido-2-methyl-4-nitrophenol.

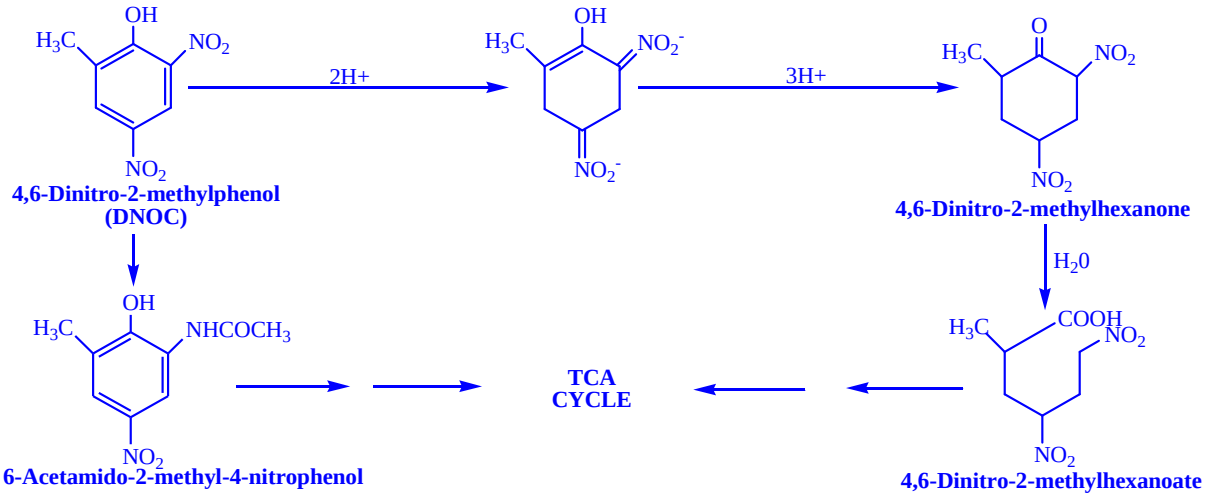


Figure 5f Biodegradation pathways of DNOC

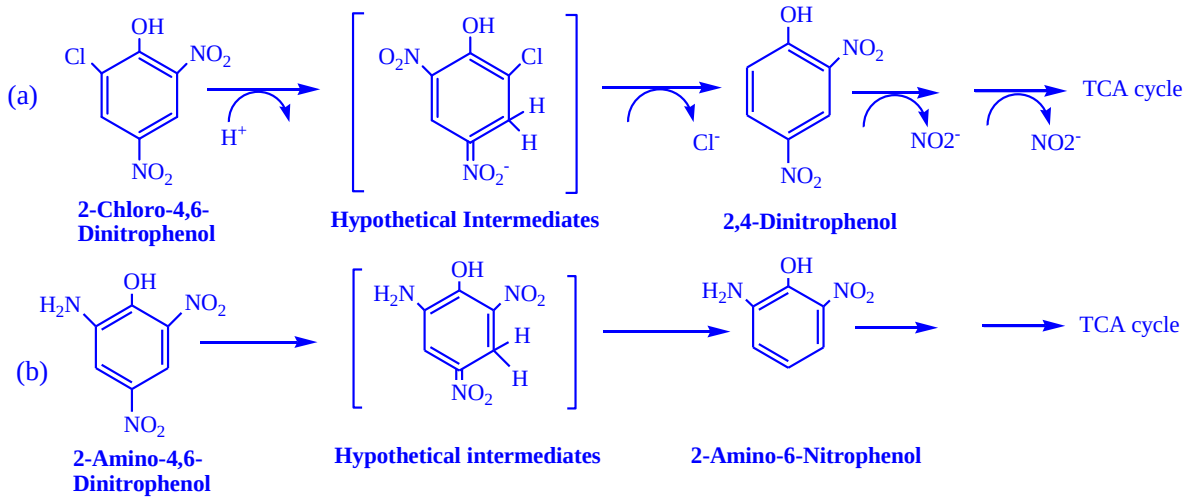


Figure 5g Degradation pathway of (a) 2-chloro-4,6-dinitrophenol and (b) 2-amino-4,6-dinitrophenol

6.6 2-amino-4,6-dinitrophenol and 2-chloro-4,6-dinitrophenol degradation

Lenke and Knackmuss (1996) proposed that the bacterium *R. erythropolis* strain HL 24-1 cometabolically transformed 2-amino-4,6-dinitrophenol to 2-amino-6-nitrophenol with the release of nitrite ion. Ammonia was absent in the culture fluid. They also proposed that during aerobic conversion of 2-Chloro-4,6-dinitrophenol, nitrite and chloride were liberated. This halo nitroaromatic compound was converted to 2,4-dinitrophenol and no other metabolite was obtained during conversion (Figure 5g).

7 Enzymes involved in biodegradation of nitrophenols

Nitrophenols are one of the xenobiotic compounds. NPs are generally recalcitrant due to reasons like substitution of the H group by other groups such as amino, carboxyl, nitro, and methoxy groups resulting in low electron density around the aromatic ring. As the nitro group has the electron-withdrawing capacity, so, it prevents oxidative degradation and electrophilic attack of oxygenase, that's why nitroaromatic compounds become resistant to aerobic degradation (Carbon-Halogen bond is more stable than the Carbon-Hydrogen bond and more energy is required for cleavage of such bonds). If the benzene ring contains more substituent groups, then this compound becomes more recalcitrance. Compounds that have a nitro group at the para position are supposed to be more recalcitrant than the other two positions (ortho or para) (Arora et al. 2012). Decreasing order of recalcitrant capacity of the NPs is Meta>Ortho>Para. Aliphatic hydrocarbons are more easily degraded than aromatic hydrocarbons. Aliphatic unsaturated hydrocarbons and branched aliphatic hydrocarbons show decreased biodegradation rate, and resist biodegradation. A list of enzymes reported to be involved in the biodegradation of nitrophenols are summarized in Table 4.

Table 4 Enzymes involved in biodegradation of Nitrophenols

Enzyme	Removal of	Insertion of	Role/ function	Reference
Monooxygenase	nitro group as nitrite ion	one oxygen atom added	PNP to Hydroquinone conversion	Kitagawa et al. 2004
Dioxygenase	nitro group as nitrite ion	insertion of two hydroxyl group	4-nitrocatechol to 1,2,4-benzenetriol conversion	Kitagawa et al. 2004
Meisenheimer Complex forming enzyme	nitro group as nitrite ion	hydride ion addition to the aromatic ring	Picric acid degradation	Rieger et al. 1999
Halogenase	halogen atom remove from halo nitrophenol	nothing	In the 2-cl-4NP degradation, hydroquinone to 4-hydroxy-muconic semialdehyde conversion	Pandey et al. 2011
Mutase	nothing	nothing	2-chloro-5-nitrophenol degradation;	Schenzle et al. 1999
Hydroxylaminolyase	nitro group as ammonia	nothing	3-nitrophenol degradation	Meulenberg et al. 1996; Haigler and Spain 1993

Table 5 Nitrophenol biodegradation by microorganism based whole cell immobilization technique

Immobilization substrate	Microorganism involved in biodegradation	Biodegradation	References
Ca-alginate	*Enterobacter cloacae; Alcaligenes* sp. TK-2	4-Chloro-2-nitrophenol	Beunik and Rehm 1990
k-carrageenan, alginate	*Moraxella* sp. G21	PNP	Errampalli et al. 1999
Diatomaceous earth	*Pseudomonas* sp. PNP1, PNP2, PNP3	PNP	Heitkamp et al. 1990
Zeolites	*Burkholderia cenocepacia* (JTLT00000000)	Methyl parathion, PNP	Fernandez-lopez et al. 2017
Agar	*Pseudomonas putida* C-11, BA-11	PNP	Ignatov et al. 2002
Agar	*Rhodococcus erythropolis* HL PM1	2,4 Dinitrophenol	Kitova et al. 2004
Polystyrene microplates	*Sphingomonas* sp. JK1	Methyl parathion	Kumar and D'Souza 2010
k-carrageenan	*Sphingomonas* sp. UG30	PNP	Alber et al. 2000

8 Bioremediation by immobilized cell technique

By the introduction of an immobilized cell system, impetuous bioremediation of NP compounds may be accomplished. Biological durability of cells together with plasmids increases in this technique. Such a technique protects the inoculated microorganisms from unfavorable conditions like minimization of conflict with indigenous microflora, the appearance of toxic substances, unsuitable pH, salt concentration, etc. Again, immobilized cells can degrade and withstand higher concentrations of xenobiotic compounds compared to the free cell system (Fernández-López et al. 2017). Various naturals (agarose, agar, clay, diatomaceous earth, alginate, k-carrageenan, dextran, zeolites, aubasidan, chitosan) and synthetic (polyvinyl alcohol, polyacrylamide) substrates are used to bewilder the limitations faced during bioremediation with free cell system (Mrozik and Piotrowska-Seget 2010; Fernández-López et al. 2017; Van Elsas and Heijnen 1990). One of the important drawbacks of the immobilized cell system technique is that oxygen transfer is restricted in the substrate matrix by molecular diffusion (Beunink and Rehm 1990). A reported list of microorganisms used for bioremediation by immobilized cell technique is summarized in Table 5.

9 Advantages and disadvantages of bioremediation

Bioremediation is a biological process wherein the controlled condition of organic pollutants is biologically degraded from contaminated sites. Nowadays, it has wide acceptance due to its low cost, eco-friendly, pollution-free, effective technique for the treatment of pollutant environment. Here microorganisms enzymatically utilize the pollutants and alter them into innocuous products. The purpose of bioremediation is not only to exclude environmental contaminants but also to regain environmental quality. In the soil environment, organic pollutants are mainly dissipated biologically. Various factors influence the complex phenomenon of bioremediation viz. pH, presence of oxygen, temperature, salt, nutrients; the presence of microbial population (inoculum size) to the pollutant site, activity, and density of introduced microorganism, nutrients, co-substrate, soil properties, microenvironment, etc. There are various bioremediation techniques such as land farming, phytoremediation, bioreactor, composting, enzyme-catalyzed bioremediation, and wastewater treatment. Again biostimulation, adaptation bioaugmentation, bacterial chemotaxis, and bioavailability of pollutants to the microorganism might enhance bioremediation rates (Mohan et al., 2006).

There are several reports available on the bioremediation of NP by single bacterial strains (Labana et al. 2005; Hong et al. 2007, Ghosh et al. 2010) and engineered strains (Xu et al. 2021), alongside reports are also available on bioremediation by bacterial consortia.

10 Bioremediation of nitrophenols

The *in-situ* and *ex-situ* represent two bioremediation processes for the bioremediation practice. The *in-situ* technique is the spoiling of pollutants at their original contaminated location. Kristanti et al. (2012) proposed that *Spirodela polyrrhiza* has the potential for rhizoremediation of 3-nitrophenol contaminated soil. Labana et al. (2005) investigated that *Arthrobacter protophormiae* RKJ100 can degrade PNP co-metabolically in soil microcosms. They reported that when cells were pre-exposed to PNP, it can degrade PNP more quickly than the uninduced cells; higher concentrations of pollutants act as poison for microorganisms; a rise in inoculum density significantly improves the mineralization efficiency of the pollutant. It was demonstrated by Labana et al. (2005) that the PNP bioremediation rate is inversely proportional to the increasing depth of soil. Barles et al. (1979) demonstrated that *P. stutzeri* is capable of decontaminating parathion-containing soil under *in situ* field condition. The bacterium *R. imtechensis* strain RKJ300 could degrade multiple NPs compounds such as PNP, 2,4-dinitrophenol, and 2-cl-4NP (Ghosh et al. 2010). They proved that the bioremediation rate is quicker in non-sterile soil as compared to sterile soil, thereby indicating the probable role of aboriginal microflora of the soil in NP biodegradation.

Ex-situ technique is the spoiling of pollutants elsewhere other than the original contaminated location like a bioreactor under controlled conditions. Donlon et al. (1996) observed that in the USAB reactor anaerobic granular sludge was able to transform PNP and 2,4-dinitrophenol to 4-aminophenol and 2,4-diaminophenol respectively; 2NP was completely degraded to methane via 2AP. Some notable demerits of bioremediation practices such as – the *ex-situ* technique is that, these are more expensive requiring the intervention of sophisticated equipment, than *in-situ* technique. Slow and low degradation rates were recorded in the case of the *in-situ* techniques, too long a time might be required to attain the desired reduction of the pollutant in the environment. Moreover, the strain survivability in the bio remedial site is another question, which needs to be addressed systematically using.

11 Future perspective black box of NP biodegradation study

Although, there are plenty of reports on the biodegradation of NPs by microorganisms, the search for novel microorganism continue from better catalytic perspectives. This is especially true because a major portion of microbial biodiversity is still unknown. Currently, the wealth of knowledge for biodegradation of NPs is based on aerobic microorganisms; ironically, the anaerobic microorganisms (especially prokaryotic ones) are dominants in the subsurface habitats of Earth. Technological barriers in the cultivation of obligate anaerobes and fewer exploration studies with anaerobic NPs degrading bacteria are other reasons. The study of anaerobic NPs degrading bacteria will lead to its better understanding in the future. As omics approaches have started to intervene in most the modern biological sciences, in the biodegradation study of NPs also omics approaches will help to decipher the molecular mechanism of adaptation of bacteria towards these toxic compounds and a better understanding of their fate and movement in the cell through integrated cell imaging approaches. There is a huge void as far as cell signaling mechanisms are concerned concerning NPs biodegradation activities. Future research will also aim to understand this signaling process which will help to design *in situ* bioremediation strategies in better ways. Another future aspect is cell imaging techniques. With current innovations and advancements, cell imaging techniques will evolve in the future that will benefit to realize the process of mineralization of NPs and track the movement of its hydrolytic metabolites in a living cellular milieu. Moreover, with integrated advanced computational systems, quantitative aspects of different cellular components and their probable interaction may also be anticipated/ speculated, in the future.

In the future, more promising strains will be targeted directly from the environmental samples through single cell genomics approaches followed by their omics studies to understand their potentiality. Synthetic biology-dependent metabolic engineering will be carried out to fish out the desirable genes and explore their expression possibilities in heterologous systems for better in situ application purposes. To have better effects on this, nanotechnology may also be incorporated.

Conclusion

The NPs are one of the most common chemical compounds in synthetic chemistry and are widely used in almost all industries, today. Due to their comprehensive use and applications, their presence in soil, water, and air at non-recommended levels has been considered as threat to ecosystems and all their living components (both microscopic and macroscopic, including humans). Therefore effective, eco-friendly, cost-effective approaches were considered for the decontamination of various habitats contaminated with NPs. With these aims, the initial focus was given to isolation, characterization, and identification of NPs degrading microorganisms. The latter were targeted because their enzymes have huge catalytic diversity as well as specificities. This was followed by the study of hydrolysis intermediates, and metabolites using analytical equipment. This added precision to the study and knowledge on pathways of biodegradation of NPs were

excavated. With these, the enzymology and genetic studies came that lead to the identification of specific genes encoding enzymes responsible for catalyzing individual steps of multi-step pathways of biodegradation. To develop bioprocess and engineering; these genetic elements were next fished out from the source organism and through recombinant DNA technology and genetic engineering were expressed in more efficient heterologous expression systems for better enzyme function leading to catalysis of the target NP compound and thus biodegradation. Very recently the five genes encoding PNP degradation function were chemically synthesized and through metabolic engineering was expressed in *E. coli* host. The latter was demonstrated to degrade PNP very efficiently under laboratory culture conditions. With the advent of NGS-based DNA sequencing technologies, many NPs degrading bacterial genome sequence were sequenced, to understand their overall genomic potential. These were followed by transcriptome and proteome studies to reveal expression products for understanding molecular mechanisms behind their adaptation during the biodegradation process. But these were all carried out at laboratory levels. Microcosm based studies were also conducted to understand the applicability of microbes mediated bioremediation process in the environmental habitats, both with free cells, immobilized cells and genetically engineered strains. Unfortunately, very few successful attempts have been carried out with environmental habitats for the removal of NPs (both in situ & ex-situ attempts). So, as far as the effectiveness of the bioremediation process for NP decontamination is concerned, we are far away. More explorative studies using efficient aerobic-anaerobic NP degrading bacterial consortium (or combination of microbes- plant systems) and advanced techniques including omics approaches and nanotechnologies may help towards developing better practicable bioremediation approaches, in the futures.

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Conflict of interest

Both the authors declare no conflict of interest in this study.

References

Alber, T., Cassidy, M., Zablotowicz, R., Trevors, J, & Lee, H. (2000). Degradation of p-nitrophenol and pentachlorophenol mixtures by *Sphingomonas* sp. UG30 in soil perfusion bioreactors. *Journal of Industrial Microbiology and Biotechnology*, *25*, 93-99.

Arora, P.K. (2012a). Decolorization of 4-chloro-2-nitrophenol by a soil bacterium, *Bacillus subtilis* RKJ 700. *PLoS One*, *7*, 52012.

Arora, P.K. (2012b). Metabolism of para-nitrophenol in Arthrobacter sp. SPG. *Journal of Environmental Science Management*, *3*, 52-57.

Arora, P.K., & Bae, H. (2014). Biotransformation and chemotaxis of 4-chloro-2-nitrophenol by *Pseudomonas* sp. JHN. *Microbial cell factories*, *13*, 1-6.

Arora, P.K., & Jain, R.K. (2011). Pathway for degradation of 2-chloro-4-nitrophenol in *Arthrobacter* sp. SJCon. *Current microbiology*, *63*, 568-573.

Arora, P.K., & Jain, R.K. (2012). Biotransformation of 4-chloro-2-nitrophenol into 5-chloro-2-methylbenzoxazole by a marine *Bacillus* sp. strain MW-1. *Biodegradation*, *23*, 325-331.

Arora, P.K., Sharma, A., Mehta, R., Shenoy, B.D., Srivastava, A., & Singh, V.P. (2012). Metabolism of 4-chloro-2-nitrophenol in a Gram-positive bacterium, *Exiguobacterium* sp. PMA. *Microbial Cell Factories*, *11*, 1-10.

Arora, P.K., Srivastava, A., & Singh, V.P. (2014a). Bacterial degradation of nitrophenols and their derivatives. *Journal of Hazardous Materials*, *266*, 42-59.

Arora, P.K., Srivastava, A., & Singh, V.P. (2014b) Degradation of 4-chloro-3-nitrophenol via a novel intermediate, 4-chlororesorcinol by *Pseudomonas* sp. JHN. *Scientific reports*, *4*, 1-6.

Arora, P.K., Srivastava, A., & Singh, V.P. (2016). Diversity of 4-chloro-2-nitrophenol-degrading bacteria in a wastewater sample. *Journal of Chemistry*, 2016.

Barles, R.W., Daughton, C.G., & Hsieh, D.P. (1979). Accelerated parathion degradation in soil inoculated with acclimated bacteria under field conditions. *Archives of Environmental Contamination and Toxicology*, *8*, 647-660.

Behrend, C., & Heesche-Wagner, K. (1999). Formation of hydride-Meisenheimer complexes of picric acid (2, 4, 6-trinitrophenol) and 2, 4-dinitrophenol during mineralization of picric acid by *Nocardioides* sp. strain CB 22-2. *Applied and Environmental Microbiology*, *65*, 1372-1377.

Beunink, J., & Rehm, H.J. (1990). Coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol by a co-immobilized mixed culture system. *Applied Microbiology and Biotechnology*, *34*, 108-115.

Bhushan, B., Chauhan, A., Samanta, S.K., & Jain, R.K. (2000). Kinetics of biodegradation of p-nitrophenol by different bacteria. *Biochemical and Biophysical Research Communications, 274*, 626-630.

Blasco, R., & Castillo, F. (1992). Light-dependent degradation of nitrophenols by the phototrophic bacterium *Rhodobacter capsulatus* E1F1. *Applied and environmental microbiology, 58*, 690-695.

Blasco, R., Moore, E., Wray, V., Pieper, D., Timmis, K., & Castillo, F. (1999). 3-Nitroadipate, a metabolic intermediate for mineralization of 2, 4-dinitrophenol by a new strain of a *Rhodococcus* species. *Journal of Bacteriology, 181*, 149-152.

Brown, M., & Chessin, R. (1995). Toxicological profile for dinitrocresols. In: Agency for Toxic CRC Press, Substances and Disease Registry's: Toxicological profiles, 1995, 1–143.

Bruhn, C., Bayly, R., & Knackmuss, H. (1988). The in vivo construction of 4-chloro-2-nitrophenol assimilatory bacteria. *Archives of microbiology, 150*, 171-177.

Bruhn, C., Lenke, H., & Knackmuss, H.J. (1987). Nitro substituted aromatic compounds as nitrogen source for bacteria. *Applied and Environmental Microbiology, 53*, 208-210.

Chauhan, A., Chakraborti, A.K., & Jain, R.K. (2000). Plasmid-encoded degradation of p-nitrophenol and 4-nitrocatechol by *Arthrobacter protophormiae*. *Biochemical and biophysical research communications, 270*, 733-740.

Chauhan, A., Pandey, G., Sharma, N.K., Paul, D., Pandey, J., & Jain, R.K. (2010). p-Nitrophenol degradation via 4-nitrocatechol in *Burkholderia* sp. SJ98 and cloning of some of the lower pathway genes. *Environmental Science & Technology, 44*, 3435-3441.

Chen, S., Geng, P., Xiao, Y., & Hu, M. (2012). Bioremediation of β-cypermethrin and 3-phenoxybenzaldehyde contaminated soils using Streptomyces aureus HP-S-01. *Applied Microbiology and Biotechnology, 94*, 505-515.

Cho, Y.G., Rhee, S.K., & Lee, S.T. (2000). Influence of phenol on biodegradation of p-nitrophenol by freely suspended and immobilized *Nocardioides* sp. NSP41. *Biodegradation, 11*, 21-28.

Daubaras, D.L., Saido, K., & Chakrabarty, A.M. (1996). Purification of hydroxyquinol 1, 2-dioxygenase and maleylacetate reductase: the lower pathway of 2, 4, 5-trichlorophenoxyacetic acid metabolism by *Burkholderia cepacia* AC1100. *Applied and Environmental Microbiology, 62*, 4276-4279.

Donlon, B., Razo-Flores, E., Lettinga, G., & Field, J. (1996). Continuous detoxification, transformation, and degradation of nitrophenols in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnology and Bioengineering, 51*, 439-449.

Ebert, S., Fischer, P., & Knackmuss, H. (2001). Converging catabolism of 2, 4, 6-trinitrophenol (picric acid) and 2, 4-dinitrophenol by *Nocardioides simplex* FJ2-1A. *Biodegradation, 12*, 367-376.

Ebert, S., Rieger, P.G., & Knackmuss, H. (1999). Function of coenzyme F420 in aerobic catabolism of 2, 4, 6-trinitrophenol and 2, 4-dinitrophenol by *Nocardioides simplex* FJ2-1A. *Journal of Bacteriology, 181*, 2669-2674.

Ecker, S., Widmann, T., Lenke, H., Dickel, O., Fischer, P., Bruhn, C., & Knackmuss, H. (1992). Catabolism of 2, 6-dinitrophenol by *Alcaligenes eutrophus* JMP 134 and JMP 222. *Archives of Microbiology, 158*, 149-154.

EPA. (1980). Ambient water quality criteria for nitrophenols. U.S Environmental Protection Agency, 440/5-80-032.

Errampalli, D., Tresse, O., Lee, H., & Trevors, J. (1999). Bacterial survival and mineralization of p-nitrophenol in soil by green fluorescent protein-marked *Moraxella* sp. G21 encapsulated cells. *FEMS microbiology ecology, 30*, 229-236.

Fernández-López, M. G., Popoca-Ursino, C., Sánchez-Salinas, E., Tinoco-Valencia, R., Folch-Mallol, J., Dantán-González, E., & Laura Ortiz-Hernández, M. (2017). Enhancing methyl parathion degradation by the immobilization of *Burkholderia* sp. isolated from agricultural soils. *Microbiology Open, 6*, 00507.

Gemini, V.L., Gallego, A., De Oliveira, V.M., Gomez, C., Manfio, G.P., & Korol, S.E. (2005). Biodegradation and detoxification of p-nitrophenol by *Rhodococcus wratislaviensis*. *International Biodeterioration & Biodegradation, 55*, 103-108.

Ghosh, A., Khurana, M., Chauhan, A., Takeo, M., Chakraborti, A.K., & Jain, R.K. (2010). Degradation of 4-nitrophenol, 2-chloro-4-nitrophenol, and 2, 4-dinitrophenol by *Rhodococcus imtechensis* strain RKJ300. *Environmental Science & Technology, 44*, 1069-1077.

Guo, Y., Li, D.F., Zheng, J., Xu, Y., & Zhou, N.Y. (2021). Single-Component and Two-Component para-Nitrophenol Monooxygenases: Structural Basis for Their Catalytic Difference. *Applied and Environmental Microbiology, 87*, 01171-21.

Haigler, B., & Spain, J. (1993). Biodegradation of 4-nitrotoluene by *Pseudomonas* sp. strain 4NT. *Applied and Environmental Microbiology, 59*, 2239-2243.

Hamdi, Y.A., & Tewfik, M.S. (1970). Degradation of 3, 5-dinitro-o-cresol by *Rhizobium*and*Azotobacter* spp. *Soil Biology and Biochemistry, 2*, 163-166.

Hanne, L., Kirk, L., Appel, S., Narayan, A., & Bains, K. (1993). Degradation and induction specificity in actinomycetes that degrade p-nitrophenol. *Applied and Environmental Microbiology*, *59*, 3505-3508.

Hatta, T., Nakano, O., Imai, N., Takizawa, N., & Kiyohara, H. (1999). Cloning and sequence analysis of hydroxyquinol 1, 2-dioxygenase gene in 2, 4, 6-trichlorophenol-degrading *Ralstonia pickettii* DTP0602 and characterization of its product. *Journal of Bioscience and Bioengineering*, *87*, 267-272.

Hayatsu, M., Hirano, M., & Tokuda, S. (2000). Involvement of two plasmids in fenitrothion degradation by *Burkholderia* sp. strain NF100. *Applied and Environmental Microbiology*, *66*, 1737-1740.

Heiss, G., Trachtmann, N., Abe, Y., Takeo, M., & Knackmuss, H.J. (2003). Homologous npdGI genes in 2, 4-dinitrophenol-and 4-nitrophenol-degrading *Rhodococcus* spp. *Applied and Environmental Microbiology*, *69*, 2748-2754.

Heitkamp, M., Camel, V., Reuter, T.J., & Adams, W. (1990). Biodegradation of p-nitrophenol in an aqueous waste stream by immobilized bacteria. *Applied and Environmental Microbiology*, *56*, 2967-2973.

Herrera-Melián, J., Martín-Rodríguez, A.J., Ortega-Méndez, A., Araña, J., Doña-Rodríguez, J., & Pérez-Peña, J. (2012). Degradation and detoxification of 4-nitrophenol by advanced oxidation technologies and bench-scale constructed wetlands. *Journal of Environmental Management*, *105*, 53-60.

Hess, T.F., Schmidt, S., Silverstein, J., & Howe, B. (1990). Supplemental substrate enhancement of 2, 4-dinitrophenol mineralization by a bacterial consortium. *Applied and Environmental Microbiology*, *56*, 1551-1558.

Hong, Q., Zhang, Z., Hong, Y., & Li, S. (2007). A microcosm study on bioremediation of fenitrothion-contaminated soil using *Burkholderia* sp. FDS-1. *International Biodeterioration & Biodegradation*, *59*, 55-61.

Ignatov, O., Guliy, O., Singirtsev, I., Shcherbakov, A., Makarov, O., & Ignatov, V. (2002). Effects of p-nitrophenol and organo phosphorous nitroaromatic insecticides on the respiratory activity of free and immobilized cells of strains C-11 and BA-11 of *Pseudomonas putida*. *Applied Biochemistry and Microbiology*, *38*, 240-246.

Iwaki, H., Muraki, T., Ishihara, S., Hasegawa, Y., Rankin, K., Sulea, T., Boyd, J., & Lau, P. (2007). Characterization of a pseudomonad 2-nitrobenzoate nitroreductase and its catabolic pathway-associated 2-hydroxylaminobenzoate mutase and a chemoreceptor involved in 2-nitrobenzoate chemotaxis. *Journal of Bacteriology*, *189*, 3502-3514.

Jain, R.K., Dreisbach, J.H., & Spain, J.C. (1994). Biodegradation of p-nitrophenol via 1, 2, 4-benzenetriol by an *Arthrobacter* sp. *Applied and Environmental Microbiology*, *60*, 3030-3032.

Ju, K., & Parales, R. (2010). Nitroaromatic compounds, from synthesis to biodegradation. *Microbiology and Molecular Biology Reviews*, *74*, 250-272.

Kadiyala, V., & Spain, J.C. (1998). A two-component monooxygenase catalyzes both the hydroxylation of p-nitrophenol and the oxidative release of nitrite from 4-nitrocatechol in *Bacillus sphaericus* JS905. *Applied and Environmental Microbiology*, *64*, 2479-2484.

Kanaly, R., Kim, I., & Hur, H. (2005). Biotransformation of 3-methyl-4-nitrophenol, a main product of the insecticide fenitrothion, by *Aspergillus niger*. *Journal of Agricultural and Food Chemistry*, *53*, 6426-6431.

Karim, K., & Gupta, S. (2001). Biotransformation of nitrophenols in upflow anaerobic sludge blanket reactors. *Bioresource Technology*, *80*, 179-186.

Kitagawa, W., Kimura, N., & Kamagata, Y. (2004). A novel p-nitrophenol degradation gene cluster from a gram-positive bacterium, *Rhodococcus opacus* SAO101. *Journal of bacteriology*, *186*, 4894-4902.

Kitova, A., Kuvichkina, T., Arinbasarova, A., & Reshetilov, A. (2004). Degradation of 2, 4-dinitrophenol by free and immobilized cells of *Rhodococcus erythropolis* HL PM-1. *Applied Biochemistry and Microbiology*, *40*, 258-261.

Kristanti, R.A., Kanbe, M., Toyama, T., Tanaka, Y., Tang, Y., Wu, X., & Mori, K. (2012). Accelerated biodegradation of nitrophenols in the rhizosphere of *Spirodela polyrrhiza*. *Journal of Environmental Sciences*, *24*, 800-807.

Kuang, S., Le, Q., Hu, J., Wang, Y., et al. (2020). Effects of p-nitrophenol on enzyme activity, histology, and gene expression in *Larimichthys crocea*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *228*, 108638.

Kulkarni, M., & Chaudhari, A. (2006). Biodegradation of p-nitrophenol by *P. putida*. *Bioresource Technology*, *97*, 982-988.

Kulkarni, M., & Chaudhari, A. (2007). Microbial remediation of nitro-aromatic compounds: an overview. *Journal of Environmental Management*, *85*, 496-512.

Kumar, J., & D'Souza, S.F. (2010). An optical microbial biosensor for detection of methyl parathion using *Sphingomonas* sp. immobilized on microplate as a reusable biocomponent. *Biosensors and Bioelectronics, 26,* 1292-1296.

Labana, S., Pandey, G., Paul, D., Sharma, N.K., Basu, A., & Jain, R.K. (2005). Pot and field studies on bioremediation of p-nitrophenol contaminated soil using *Arthrobacter protophormiae* RKJ100. *Environmental Science & Technology, 39,* 3330-3337.

Lang, M., Spiteller, P., Hellwig, V., & Steglich, W. (2001). Stephanosporin, a "Traceless" Precursor of 2-Chloro-4-nitrophenol in the Gasteromycete *Stephanospora caroticolor*. *Angewandte Chemie International Edition, 40,* 1704-1705.

Lei, Y., Mulchandani, A., & Chen, W. (2005). Improved degradation of organophosphorus nerve agents and p-nitrophenol by *Pseudomonas putida* JS444 with surface-expressed organophosphorus hydrolase. *Biotechnology Progress, 21,* 678-681.

Lenke, H., & Knackmuss, H. (1992). Initial hydrogenation during catabolism of picric acid by *Rhodococcus erythropolis* HL 24-2. *Applied and Environmental Microbiology, 58,* 2933-2937.

Lenke, H., & Knackmuss, H. (1996). Initial hydrogenation and extensive reduction of substituted 2, 4-dinitrophenols. *Applied and Environmental Microbiology, 62,* 784-790.

Lenke, H., Pieper, D., Bruhn, C., & Knackmuss, H. (1992). Degradation of 2, 4-dinitrophenol by two *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2. *Applied and Environmental Microbiology, 58,* 2928-2932.

Leung, K.T., Tresse, O., Errampalli, D., Lee, H., & Trevors, J.T. (1997). Mineralization of p-nitrophenol by pentachlorophenol-degrading *Sphingomonas* spp. *FEMS Microbiology Letters, 155,* 107-114.

Li, Y., Zhou, B., Li, W., Peng, X., Zhang, J., & Yan, Y. (2008). Mineralization of p-nitrophenol by a new isolate *Arthrobacter* sp. Y1. *Journal of Environmental Science and Health Part B, 43,* 692-697.

Lima, S., Castro, P., & Morais, R. (2003). Biodegradation of p-nitrophenol by microalgae. *Journal of Applied Phycology, 15,* 137-142.

Liu, H., Zhang, J.J., Wang, S.J., Zhang, X.E., & Zhou, N.Y. (2005). Plasmid-borne catabolism of methyl parathion and p-nitrophenol in *Pseudomonas* sp. strain WBC-3. *Biochemical and Biophysical Research Communications, 334,* 1107-1114.

Liu, Z., Yang, C., & Qiao, C. (2007). Biodegradation of p-nitrophenol and 4-chlorophenol by *Stenotrophomonas* sp. *FEMS Microbiology Letters, 277,* 150-156.

Löser, C., Oubelli, M.A., & Hertel, T. (1998). Growth kinetics of the 4-nitrophenol degrading strain *Pseudomonas putida* PNP1. *Acta Biotechnologica, 18,* 29-41.

Meulenberg, R., Pepi, M., & de Bont, J.A. (1996). Degradation of 3-nitrophenol by *Pseudomonas putida* B2 occurs via 1, 2, 4-benzenetriol. *Biodegradation, 7,* 303-311.

Min, J., Lu, Y., Hu, X., & Zhou, N.Y. (2016). Biochemical characterization of 3-methyl-4-nitrophenol degradation in *Burkholderia* sp. strain SJ98. *Frontiers in Microbiology, 7,* 791.

Min, J., Wang, J., Chen, W., & Hu, X. (2018). Biodegradation of 2-chloro-4-nitrophenol via a hydroxyquinol pathway by a Gram-negative bacterium, *Cupriavidus* sp. strain CNP-8. *AMB Express, 8,* 1-11.

Min, J., Zhang, J.J., & Zhou, N.Y. (2014). The gene cluster for para-nitrophenol catabolism is responsible for 2-chloro-4-nitrophenol degradation in *Burkholderia* sp. strain SJ98. *Applied and Environmental Microbiology, 80,* 6212-6222.

Mohan, S., Kisa, T., Ohkuma, T., Kanaly, R.A., & Shimizu, Y. (2006). Bioremediation technologies for treatment of PAH-contaminated soil and strategies to enhance process efficiency. *Reviews in Environmental Science and Biotechnology, 5,* 347-374.

Mrozik, A., & Piotrowska-Seget, Z. (2010). Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. *Microbiological Research, 165,* 363-375.

Nielsen, M.B., Kjeldsen, K.U., & Ingvorsen, K. (2011). Description of *Citricoccus nitrophenolicus* sp. nov., a *para*-nitrophenol degrading actinobacterium isolated from a wastewater treatment plant and emended description of the genus *Citricoccus* Altenburger et al. 2002. *Antonie van Leeuwenhoek, 99,* 489-499.

Ningthoujam, D. (2005). Isolation and Identification of a *Brevibacterium linens* strain degrading p-nitrophenol. *African Journal of Biotechnology, 4,* 256-257.

Pailan, S., & Saha, P. (2015). Chemotaxis and degradation of organophosphate compound by a novel moderately thermo-halo tolerant *Pseudomonas* sp. strain BUR11: evidence for possible existence of two pathways for degradation. *Peer J, 3,* 1378.

Pakala, S.B., Gorla, P., Pinjari, A.B., Krovidi, R.K., et al. (2007). Biodegradation of methyl parathion and p-nitrophenol: evidence for the presence of a p-nitrophenol 2-hydroxylase in a Gram-negative *Serratia* sp. strain DS001. *Applied Microbiology and Biotechnology, 73,* 1452-1462.

Pandey, G., Paul, D., & Jain, R.K. (2003). Branching of o-nitrobenzoate degradation pathway in *Arthrobacter protophormiae* RKJ100: identification of new intermediates. *FEMS Microbiology Letters, 229,* 231-236.

Pandey, J., Heipieper, H., Chauhan, A., Arora, P.K., et al. (2011). Reductive dehalogenation mediated initiation of aerobic degradation of 2-chloro-4-nitrophenol (2C4NP) by *Burkholderia* sp. strain SJ98. *Applied Microbiology and Biotechnology, 92,* 597-607.

Paul, D., Rastogi, N., Krauss, U., Schlomann, M., et al. (2008). Diversity of 'benzenetriol dioxygenase' involved in p-nitrophenol degradation in soil bacteria. *Indian Journal of Microbiology, 48,* 279-286.

Prakash, D., Chauhan, A., & Jain, R.K. (1996). Plasmid-Encoded Degradation of p-Nitrophenol by *Pseudomonas cepacia*. *Biochemical and Biophysical Research Communications, 224,* 375-381.

Przybyla, J., Carlson-Lynch, H., Klotzbach, J.M., & Crisman, J.S. (2021). Toxicological profile for dinitrophenols. U.S. *Environmental Protection Agency* (EPA). PP:1-211.

Qiu, X., Wu, P., Zhang, H., Li, M., & Yan, Z. (2009). Isolation and characterization of *Arthrobacter* sp. HY2 capable of degrading a high concentration of p-nitrophenol. *Bioresource Technology, 100,* 5243-5248.

Qiu, X., Zhong, Q., Li, M., Bai, W., & Li, B. (2007). Biodegradation of p-nitrophenol by methyl parathion-degrading *Ochrobactrum* sp. B2. *International Biodeterioration and Biodegradation, 59,* 297-301.

Rajan, J., Valli, K., Perkins, R.E., Sariaslani, F.S., et al. (1996). Mineralization of 2, 4, 6-trinitrophenol (picric acid): characterization and phylogenetic identification of microbial strains. *Journal of Industrial Microbiology and Biotechnology, 16,* 319-324.

Rehman, A., Raza, Z., Afzal, M., & Khalid, Z. (2007). Kinetics of p-nitrophenol degradation by *Pseudomonas pseudomallei* wild and mutant strains. *Journal of Environmental Science and Health, Part A, 42,* 1147-1154.

Rieger, P.G., Sinnwell, V., Preub, A., Francke, W., & Knackmuss, H. (1999). Hydride-Meisenheimer complex formation and protonation as key reactions of 2, 4, 6-trinitrophenol biodegradation by *Rhodococcus erythropolis*. *Journal of Bacteriology, 181,* 1189-1195.

Samson, R., Bodade, R., Zinjarde, S., & Kutty, R. (2019). A novel *Sphingobacterium* sp. RB, a rhizosphere isolate degrading para-nitrophenol with substrate specificity towards nitrotoluenes and nitroanilines. *FEMS Microbiology Letters, 366,* 168.

Samuel, M., Sivaramakrishna, A., & Mehta, A. (2014). Bioremediation of p-Nitrophenol by *Pseudomonas putida* 1274 strain. *Journal of Environmental Health Science and Engineering, 12,* 1-8.

Schäfer, A., Harms, H., & Zehnder, A.J. (1996). Biodegradation of 4-nitroanisole by two *Rhodococcus* spp. *Biodegradation, 7,* 249-255.

Schenzle, A., Lenke, H., Fischer, P., Williams, P.A., & Knackmuss, H. (1997). Catabolism of 3-nitrophenol by *Ralstonia eutropha* JMP 134. *Applied and Environmental Microbiology, 63,* 1421-1427.

Schenzle, A., Lenke, H., Spain, J.C., & Knackmuss, H.J. (1999). Chemoselective nitro group reduction and reductive dechlorination initiate degradation of 2-chloro-5-nitrophenol by *Ralstonia eutropha* JMP134. *Applied and Environmental Microbiology, 65,* 2317-2323.

Schleifer, K. (2004). Microbial diversity: facts, problems and prospects. *Systematic and applied microbiology, 27,* 3.

Sethunathan, N., & Yoshida, T. (1973). A *Flavobacterium* sp. that degrades diazinon and parathion. *Canadian Journal of Microbiology, 19,* 873-875.

She, Z., Gao, M., Jin, C., Chen, Y., & Yu, J. (2005). Toxicity and biodegradation of 2, 4-dinitrophenol and 3-nitrophenol in anaerobic systems. *Process Biochemistry, 40,* 3017-3024.

Shen, J., Zhang, J., Zuo, Y., Wang, L., et al. (2009). Biodegradation of 2, 4, 6-trinitrophenol by *Rhodococcus* sp. isolated from a picric acid-contaminated soil. *Journal of Hazardous Materials, 163,* 1199-1206.

Shen, W., Liu, W., Zhang, J., Tao, J., Deng, H., Cao, H., & Cui, Z. (2010). Cloning and characterization of a gene cluster involved in the catabolism of p-nitrophenol from *Pseudomonas putida* DLL-E4. *Bioresource Technology, 101,* 7516-7522.

Siddaramappa, R., Rajaram, K.P., & Sethunathan, N. (1973). Degradation of parathion by bacteria isolated from flooded soil. *Applied microbiology, 26,* 846-849.

Spain, J. (1995). Biodegradation of nitroaromatic compounds. *Annual Review of Microbiology, 49,* 523-555.

Spain, J., & Gibson, D. (1991). Pathway for biodegradation of p-nitrophenol in a *Moraxella* sp. *Applied and Environmental Microbiology, 57,* 812-819.

Sudhakar-Barik, Siddaramappa, R., & Sethunathan, N. (1976). Metabolism of nitrophenols by bacteria isolated from parathion-amended flooded soil. *Antonie van Leeuwenhoek, 42,* 461-470.

Takeo, M., Yasukawa, T., Abe, Y., Niihara, S., Maeda, Y., & Negoro, S. (2003). Cloning and characterization of a 4-nitrophenol hydroxylase gene cluster from *Rhodococcus* sp. PN1. *Journal of Bioscience and Bioengineering, 95,* 139-145.

Teramoto, H., Tanaka, H., & Wariishi, H. (2004). Degradation of 4-nitrophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Applied Microbiology and Biotechnology, 66,* 312-317.

Van Elsas, J., & Heijnen, C. (1990). Methods for the introduction of bacteria into soil: a review. *Biology and Fertility of Soils, 10,* 127-133.

Vikram, S., Pandey, J., Bhalla, N., Pandey, G., Ghosh, A., Khan, F., Jain, R.K., & Raghava, G.P. (2012). Branching of the p-nitrophenol (PNP) degradation pathway in *Burkholderia* sp. strain SJ98: evidences from genetic characterization of PNP gene cluster. *AMB Express, 2,* 1-10.

Wallnoefer, P.R., Ziegler, W., Engelhardt, G., & Rothmeier, H. (1978). Transformation of dinitrophenol-herbicides by *Azotobacter* sp. *Chemosphere, 7,* 967-972.

Wan N, Gu J, & Yan Y (2007) Degradation of p-nitrophenol by *Achromobacter xylosoxidans* Ns isolated from wetland sediment. *International Biodeterioration and Biodegradation, 59,* 90-96.

Wang, J., Ren, L., Jia, Y., Ruth, N., Shi, Y., Qiao, C., & Yan, Y. (2016). Degradation characteristics and metabolic pathway of 4-nitrophenol by a halotolerant bacterium *Arthrobacter* sp. CN2. *Toxicological and Environmental Chemistry, 98,* 226-240.

Wang, X., Xing, D., Mei, X., Liu, B., & Ren, N. (2018). Glucose and applied voltage accelerated p-nitrophenol reduction in biocathode of bioelectrochemical systems. *Frontiers in Microbiology, 9,* 580.

Wyman, J., Serve, M., Hobson, D., Lee, L., & Uddin, D. (1992). Acute toxicity, distribution, and metabolism of 2, 4, 6-trinitrophenol (picric acid) in Fischer 344 rats. *Journal of Toxicology and Environmental Health Part A Current Issues, 37,* 313-327.

Xiao, Y., Zhang, J., Liu, H., & Zhou, N. (2007). Molecular characterization of a novel ortho-nitrophenol catabolic gene cluster in *Alcaligenes* sp. strain NyZ215. *Journal of Bacteriology, 189,* 6587-6593.

Xu, J., Wang, B., Zhang, W.H., Zhang, F.J., et al. (2021). Biodegradation of p-nitrophenol by engineered strain. *AMB Express, 11,*1-8.

Yin, Y., Xiao, Y., Liu, H., Hao, F., Rayner, S., Tang, H., & Zhou, N. (2010). Characterization of catabolic meta-nitrophenol nitroreductase from *Cupriavidus necator* JMP134. *Applied Microbiology and Biotechnology, 87,* 2077-2085.

Zablotowicz, R., Leung, K., Alber, T., Cassidy, M., et al. (1999). Degradation of 2, 4-dinitrophenol and selected nitroaromatic compounds by *Sphingomonas* sp. UG30. *Canadian Journal of Microbiology, 45,* 840-848.

Zeyer, J., & Kearney, P. (1984). Degradation of o-nitrophenol and m-nitrophenol by a *Pseudomonas putida*. *Journal of Agricultural and Food Chemistry, 32,* 238-242.

Zhang, J., Liu, H., Xiao, Y., Zhang, X., & Zhou, N. (2009a). Identification and characterization of catabolic para-nitrophenol 4-monooxygenase and para-benzoquinone reductase from *Pseudomonas* sp. strain WBC-3. *Journal of Bacteriology, 191,* 2703-2710.

Zhang, J., Sun, Z., Li, Y., Peng, X., Li, W., & Yan, Y. (2009b). Biodegradation of p-nitrophenol by *Rhodococcus* sp. CN6 with high cell surface hydrophobicity. *Journal of Hazardous Materials, 163,* 723-728.

Zhang, J., Zhang, H., Chen, L., Fan, X., & Yang, Y. (2022). Optimization of PNP Degradation by UV-Activated Granular Activated Carbon Supported Nano-Zero-Valent-Iron-Cobalt Activated Persulfate by Response Surface Method. *International Journal of Environmental Research and Public Health, 19,* 8169.

Zhang, S., Sun, W., Xu, L., Zheng, X., et al. (2012). Identification of the para-nitrophenol catabolic pathway, and characterization of three enzymes involved in the hydroquinone pathway, in *Pseudomonas* sp. 1-7. *BMC microbiology, 12,* 1-11.

Zhang, Z., Hong, Q., Xu, J., Zhang, X., & Li, S. (2006). Isolation of fenitrothion-degrading strain *Burkholderia* sp. FDS-1 and cloning of mpd gene. *Biodegradation, 17,* 275-283.

Zhao, J., & Ward, O. (2001). Substrate selectivity of a 3-nitrophenol-induced metabolic system in *Pseudomonas putida* 2NP8 transforming nitroaromatic compounds into ammonia under aerobic conditions. *Applied and Environmental Microbiology, 67,* 1388-1391.

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Effect of Titanium, Silver and Zinc Nanoparticles on Microalgae in the Aquatic Environment

Arularasi Thenarasu[1], Chai Mee Kin[5], Wong Ling Shing[2*], Sinouvassane Djearamane[3], Chinnasamy Thirunavukkarasu[4]

[1]Universiti Tenaga Nasional, Jalan Ikram-Uniten, 43000 Kajang, Selangor, Malaysia
[2]Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN, Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia
[3]Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, 31900 Kampar, Perak, Malaysia
[4]Department of Biochemistry and Molecular Biology, Pondicherry University, 605014 Puducherry, India
[5]Institute of Sustainable Energy, Universiti Tenaga Nasional, Jalan Ikram-Uniten, 43000 Kajang, Selangor, Malaysia

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ABSTRACT

Metallic nanoparticles (MNPs) are commonly incorporated in products found in households, industries, and agriculture. The presence of MNPs in the aquatic environment causes damage to living organisms and pollutes the water body rendering it harmful for human consumption. Several studies have been made on the toxicity of MNPs toward microalgae. Most of these studies reported changes in the cellular structure, growth rate, pigments, proteins, and enzymatic activity of microalgae. This review paper focuses on the toxic effects of titanium, zinc, and silver nanoparticles on microalgae in the aquatic environment. A better understanding of the behavior of MNPs in the ecosystem will allow scientists to produce environmentally safe MNPs.

* Corresponding author

E-mail: lingshing.wong@newinti.edu.my (Wong Ling Shing)

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1 Introduction

Nanoparticles (NPs), with a size range of fewer than 100 nanometers (Strambeanu et al. 2014) can be classified into different categories based on their shape, size, chemical or physical properties (Khan et al. 2017). These nanoparticles are released into the environment, causing serious environmental implications (Wang et al. 2019). Metallic nanoparticles (MNPs) are nano-sized metals that are known to cause inconsiderable damage to the organisms in aquatic habitats (Krysanov et al. 2010). Water must be easily accessible, sufficient, and clean for the consumption of living beings (Hunter et al. 2010). The MNPs bring a potential threat to aquatic organisms as these pollutants cause bioaccumulation due to their size and unique properties (Kahlon et al. 2018). Contaminants in water are a threat to the aquatic ecosystem and will indirectly affect human health (Madhav et al. 2020). The unprecedented use of NPs leads to harmful effects on soil and water when NPs make their way into the environment. The NPs enter the environment during the production, usage, and disposal of the products made of NPs (Bundschuh et al. 2018). There are an abundance of previous studies evidence the potential of engineered nanoparticles to harm aquatic organisms if present in enough high concentrations (Liang et al. 2020). The most common MNPs that are easily found in water bodies are titanium dioxide, zinc oxide, and silver nanoparticles (Wang et al. 2019).

There are difficulties in the development of a highly reliable method to observe the effect of MNPs on the environment. Frequently, hefty and costly equipment such as aerosal-mass spectrometry systems delayed detection through inductively coupled plasma atomic emission spectroscopy, size-exclusion chromatography, microfiltration, field-flow fractionation, and capillary electrophoresis are required to detect the presence of contaminants in the water body. These detection methods are impractical for detection at the location of the exposure due to the high cost, time-consuming, and requisition of an expert to carry out the testing (Lenaghan et al. 2013).

Primary producers, especially photosynthetic microorganisms such as algae, are efficient bioindicators of NPs toxicity due to their high bioaccumulation ability, sensitivity, rapid growth phase, ease of culturing, and observation at the cellular level (Wang et al. 2019). These microbes are often used in environmental studies because of their sensitivity and the ability to accumulate pollutants (Gadzała-Kopciuch et al. 2004).

This review paper is aimed at analyzing the previously published studies from 2010 to 2020 on the toxicity effects of three MNPs namely titanium dioxide (TiO_2), zinc oxide (ZnO), and silver (Ag) nanoparticles towards the growth, photosynthetic fluorescence, and cell structure of the microalgae.

2 Effect of Titanium Nanoparticle Exposure on Microalgae

Titanium dioxide nanoparticles (TiO_2NPs) are present in three forms such as rutile, anatase, and brookite (Cho et al. 2013). TiO_2NPs are used to remove ethylene from the air for the longer shelf life of fruits, vegetables, and cut flowers, and are also used for the production of toothpaste and paints to give them opaque features (Frazer 2001). Cho et al. (2013) further stated that TiO_2 increases its transparency when the particle size decreases. The smaller TiO_2 particles possess higher UV-blocking properties and prevent microbial growth which is useful for food storage applications (Frazer 2001). They are also used as a potent photocatalyst to break down nearly any organic compound (Frazer 2001). Due to the restricted toxicity, biocompatibility, and inertness, TiO_2 is identified as “the environmental white knight”. The food and Drug Administration (FDA) approved it as a food additive in 1996 (Shah et al. 2017). Karakoti et al. (2006) reported that TiO_2NPs are more reactive due to their physiochemical properties of larger surface area and smaller size.

Iswarya et al.(2015) measured the toxic effects of anatase and rutile forms of TiO_2NPs using *Chlorella* sp. at three concentrations from 0.25 to 1 mg/L under UV radiation for 72 hours. Authors deduced that the cell viability and chlorophyll content were reduced to a great extent in the TiO_2NPs treated cells. It was reported that the rutile form had a significant effect on the reduction of chlorophyll content as compared to the anatase form. The size of NPs correlates to reactive oxygen species (ROS) produced per surface area. The ROS was also observed to have a direct relationship with the decline in the chlorophyll in the NPs treated cell. All cells were observed under a transmission electron microscope to detect the impairment in the cell membrane and nucleus of the microalgae due to the exposure of NPs. Anatase treated cells showed altered morphology, cellular uptake of NPs, and damaged cell membrane while the rutile treatment resulted in altered structure and caused damage to chloroplast and internal organelle. The authors inferred that the different crystalline structures of NPs can cause different impacts on the cell.

In another study by Chen et al. (2012), the harmful effects of TiO_2NPs on *Chlamydomonas reinhardtii* were investigated. The nanoparticle used was a combination of anatase and rutile nanoparticles. The microalgae were cultured in SE medium (Bristol Medium and Soil–water supernatant), followed by exposure to four varying concentrations from 0 to 100 mg/L of TiO_2NPs for 8 hours to 72 hours. Upon exposure, the cells were then observed. The authors found that the TiO_2NPs affected the photosynthetic activity and cell growth. After 8 hours of exposure, carotenoid, chlorophyll *b*, and malondialdehyde (MDA) content increased. However, after 8 hours, the MDA content decreased to a very low amount at 72 hours. The observation indicated that when

Table 1 Toxic effect of TiO_2 nanoparticles on microalgae

Microalgae	Effects	Reference
Dunaliella tertiolecta	Inhibition of cell growth	Manzo et al. 2013
Phaeodactylum tricornutum	Inhibition of protein synthesis (reduction in soluble protein)	Deng et al. 2017
Raphidocelis subcapitata	Increased lipid peroxidation of the cell membrane leads to deformation of the membrane structure	Ozkaleli and Erdem 2018
Chlorella vulgaris	Deformation of the cell wall and irregular morphology	Xia et al. 2018
Chaetoceros gracilis	Increase in polyunsaturated and monounsaturated fatty acids, and a decrease in saturated fatty acids	Baharlooeian et al. 2021

Table 2 Toxic Effects of ZnO nanoparticles on microalgae

Microalgae	Effects	Reference
Pseudokirchneriella subcapitata	Inhibition of cell growth	Aruoja et al. 2009
Chlorella vulgaris	Morphological changes and cell wall damage	Ji et al.2011; Suman et al. 2015
Thalassiosira pseudonana *Chaetoceros gracilis* *Phaeodactylum tricornutum*	Decrease in cell division rates	Peng et al. 2011
Coelastrella terrestris	Cell organelle damage, cell wall breakage, and cytoplasm shrinkage	Sendra et al. 2017
Chlorella sp.	Increased lipid production ability, chlorophyll pigmentation, carotenoid, and starch accumulation	Kaliamurthi et al. 2019
Chlorella vulgaris	Decrease in the chlorophyll content, algal biomass, and cell viability. Cell rupture and aggregation were observed in treated cells	Djearamane et al. 2019a, b
Haematococcus pluvialis	Induced oxidative stress through an increase in reactive oxygen species and lipid peroxidation levels.	Djearamane et al. 2020

the concentrations of TiO_2NPs increased, more cells were disrupted with decreased chlorophyll content and degradation of organelles. The toxic effects of TiO_2NPs on other microalgae are listed in Table 1.

3 Effect of Zinc Oxide Nanoparticle Exposure on Microalgae

Zinc oxide nanoparticle (ZnO NP) is the world's third largest manufactured NPs with 550 tonnes produced yearly (Piccinno et al. 2012). ZnO NPs are known to have high exciton binding energy of 60 meV, piezoelectric and pyroelectric properties, a wide band gap of 3.37ev, and a wurtzite structure lacking the centre of symmetry (Sendra et al. 2017). Zinc is important for the human body's metabolism as it's an essential trace element needed for the activation of numerous metabolic enzymes. This element is also important for protein and nucleic acid synthesis, hematopoiesis, and neurogenesis. ZnO NP can be easily absorbed by the human body and thus used as a food additive. The FDA graded ZnO as a "GRAS" (generally recognized as safe) substance (Jiang et al. 2018).

The authors of a project studied the toxicity effects of different sized and shaped ZnONPs, bulk ZnO, and Zn^{2+} towards freshwater microalgae, *Raphidocelis subcapitata*. Spherical and rod-shaped ZnO NPs of varying sizes and lengths were used for toxicity testing. The microalgae were exposed to various concentrations of each type of ZnO NP from 0.01 to 0.7 mg/L. After 96 hours, it was observed that the highest concentration of ZnO NP completely inhibited the growth of microalgae. The size of spherical-shaped particles did not show any effect on the toxicity. However, the toxicity decreased as the surface area of rod-shaped particles increased. In terms of toxicity toward *R. subcapitata*, spherical ZnO NPs were more damaging than rod ZnO NPs. The exposure of the highest concentration of ZnO nanorods caused 30% cell death, while 50% cell death was detected by treating with nanospherical ZnO on *R. subcapitata* (Samei et al. 2018).

In another study by Manzo et al. (2013), the toxic effects of ZnO NPs and bulk ZnO on green microalgae *Dunaliella tertiolecta* were investigated. The growth rate was measured daily and the study inferred that the nanosized ZnO caused higher toxicity than the bulk ZnO. The increased toxicity of ZnO NPs directly correlates to the physicochemical properties of the nano state compared with the bulk ZnO. The toxic effects of ZnONPs on other microalgae are summarized in Table 2.

4 Effect of Silver Nanoparticle Exposure on Microalgae

Silver nanoparticles (AgNPs) are leading as solutions to the issues of food security and diseases. This nanoparticle is also useful in

Table 3 Toxic effects of Ag nanoparticles on microalgae

Algae Species	Effects	Reference
Pseudokirchneriella subcapitata; Chlamydomonas reinhardtii	Reduced chlorophyll content and cell growth inhibition	Wang and Wang 2014; Wang et al. 2016
Dunaliella tertiolecta; Chlorella vulgaris	Deterioration of photosynthetic system; *D. tertiolecta* showed a strong decrease in fluorescence indicating more toxicity than *C. vulgaris*	Oukarroum et al. 2012
Thalassiosira weissflogii	Reduction in cell growth, photosynthesis and chlorophyll production; showed secretion of polysaccharide-rich Algal exopolymeric substances (EPS) for Ag+ detoxification	Miao et al. 2009

solar energy applications. The antimicrobial properties and low toxicity toward mammalian cells allowed the vast usage of AgNPs in many household products such as shampoo, soap, toothpaste, and biocidal coatings.

The AgNPs are potentially released into the environment through improper disposal as there are at least 250 products in which these nanoparticles were used and these vary from medical devices, electronic devices, and clothes to disinfectants. Over 2500 tonnes of AgNPs were produced in the United States every year where 80 tonnes were disposed of in surface waters and 150 tonnes in sewage sludge (Tripathi et al. 2017).

Silver nanoparticles were used to control infections in ancient times due to their physicochemical features and biological peculiarities. AgNPs are found to kill the bacteria present in the wound exudates. Thus, FDA approved the use of these MNPs for treating wound infections (Burdușel et al. 2018).

Nam and An (2019) tested the effects of AgNPs, nanowires (AgNWs), and nanoplates (AgPLs) treated with polyvinyl pyrrolidone on the growth and photosynthetic activity of *Chlorococcumin fusionum*. The test solutions were diluted with Bold's Basal Medium (BBM) and were placed into a microplate in triplicates. Algae in the exponential phase were inoculated in each well and incubated. Chlorophyll fluorescence from the algal cells was measured at excitation at 420 nm and emission at 671 nm. The authors reported that the toxicity of AgNPs in *C. infusionum* decreased in the order of AgPLs, AgNWs, and AgNPs based on the inhibition of photosynthesis and growth. The mechanisms for the toxicity of silver nanomaterials depend on the different structures that contain many atoms that allow for more contact surface due to the high surface area of the nanoparticle. This further allowed for a conclusion that the toxicity of nanoparticles is dependent on the diameter and shape.

Li et al. (2015) demonstrated the effect of AgNPs on *Euglena gracilis*, a green alga with a pellicle by showing a decrease in the photosynthetic yield as the concentration of AgNPs increased. The cells showed an irregular round morphology indicating an algal stress response induced by AgNPs. The toxic effects of AgNPs on other microalgae are stated in Table 3.

Conclusion

Upon reviewing studies on the effect of nanoparticle exposures on microalgal growth as above, it raises concerns that the properties, size, and shape of a MNP may influence the adsorption rate of the MNPs into the microalgal cells. As microalgal cells can accumulate these toxicants into their cells, the different sizes and shapes of nanoparticles are crucial to determine the toxicity level of the said nanoparticles in microalgal cells and thus in the environment. More studies should be done on the relationship between the morphological character of MNPs and the toxicity level of the MNPs in the aquatic environment. Apart from that, the properties of MNPs may influence different behaviors in different types of aquatic habitats wherein volume of water, presence of other toxicants, a combination of more than one MNPs, and concentrations of MNP also play a role in determining the pollution level of this environment. Smaller nanoparticles have a higher surface area that provides an attachment site for the interaction with the cellular components and causes cellular damage. More studies and investigations are needed to study the relationship between the toxicity of nanoparticles to microalgal cells and the fate of nanoparticles such as biosorption, uptake, and bioaccumulation in these cells. It is also crucial to determine if the type, shape, and size of the nanoparticle influence the toxicity level of the nanoparticle in the aquatic environment. Scientists will be able to design environmentally safe MNPs through a better understanding of MNP behaviors in biologically active environments.

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Conflict of Interest

The author would like to declare that there is no conflict of interest.

References

Aruoja, V., Dubourguier, H. C., Kasemets, K., & Kahru, A. (2009). Toxicity of nanoparticles of CuO, ZnO and TiO2 to

microalgae *Pseudokirchneriella subcapitata*. *Science of the Total Environment*, *407*(4), 1461–1468. https://doi.org/10.1016/j.scitotenv.2008.10.053

Baharlooeian, M., Kerdgari, M., & Shimada, Y. (2021). Ecotoxicological effects of TiO2 nanoparticulates and bulk Ti on microalgae *Chaetoceros muelleri*. *Environmental Technology & Innovation*, *23*, 101720. https://doi.org/10.1016/j.eti.2021.101720

Bundschuh, M., Filser, J., Lüderwald, S., McKee, M. S., et al. (2018). Nanoparticles in the environment: Where do we come from, where do we go to? *Environmental Sciences Europe*, *30*(1), 6. https://doi.org/10.1186/s12302-018-0132-6

Burdușel, A.C., Gherasim, O., Grumezescu, A. M., Mogoantă, L., et al. (2018). Biomedical applications of Silver nanoparticles: An up-to-date overview. *Nanomaterials*, *8*(9), 681. https://doi.org/10.3390/nano8090681

Chen, L., Zhou, L., Liu, Y., Deng, S., et al. (2012). Toxicological effects of nanometer titanium dioxide (nano-TiO2) on *Chlamydomonas reinhardtii*. *Ecotoxicology and Environmental Safety*, *84*, 155–162.https://doi.org/10.1016/j.ecoenv.2012.07.019

Cho, W. S., Kang, B. C., Lee, J. K., Jeong, J. et al. (2013). Comparative absorption, distribution, and excretion of titanium dioxide and zinc oxide nanoparticles after repeated oral administration. *Particle and Fibre Toxicology*, *10*, 9. https://doi.org/10.1186/1743-8977-10-9

Deng, X. Y., Cheng, J., Hu, X. L., Wang, L., et al. (2017). Biological effects of TiO2 and CeO2 nanoparticles on the growth, photosynthetic activity, and cellular components of a marine diatom *Phaeodactylum tricornutum*. *Science of the Total Environment*, *575*, 87–96. https://doi.org/10.1016/j.scitotenv.2016.10.003

Djearamane, S., Wong, L. S., Lim, Y. M., & Lee, P. F. (2019a). Short-Term cytotoxicity of Zinc oxide nanoparticles on *Chlorella vulgaris*. *Sains Malaysiana*, *48*(1), 69–73.

Djearamane, S., Wong, L. S., Yang, M. L., & Poh, F. L. (2019b). Cytotoxic effects of zinc oxide nanoparticles on *Chlorella Vulgaris*. *Pollution Research*, *38*(2), 479–484.

Djearamane, S., Wong, L. S., Yang, M. L., & Poh, F. L. (2020). Oxidative stress effects of zinc oxide nanoparticles on fresh water microalga *Haematococcus pluvialis*. *Ecology, Environment and Conservation*, *26*(2), 663–668.

Frazer, L. (2001). Titanium dioxide: Environmental white knight? *Environmental Health Perspectives*, *109*(4), A174–A177.

Gadzała-Kopciuch, R., Berecka, B., Bartoszewicz, J., & Buszewski, B. (2004). Some considerations about bioindicators in environmental monitoring. *Polish Journal of Environmental Studies*, *13*(5), 453–462.

Hunter, P. R., MacDonald, A. M., & Carter, R. C. (2010). Water Supply and Health. *PLoS Medicine*, *7*(11), e1000361. https://doi.org/10.1371/journal.pmed.1000361

Iswarya, V., Bhuvaneshwari, M., Alex, S. A., Iyer, S., et al. (2015). Combined toxicity of two crystalline phases (anatase and rutile) of Titania nanoparticles towards freshwater microalgae: *Chlorella* sp. *Aquatic Toxicology*, *161*, 154–169.

Ji, J., Long, Z., & Lin, D. (2011). Toxicity of oxide nanoparticles to the green algae *Chlorella* sp. *Chemical Engineering Journal*, *170*(2), 525–530. https://doi.org/10.1016/j.cej.2010.11.026

Jiang, J., Pi, J., & Cai, J. (2018). The advancing of zinc oxide nanoparticles for biomedical applications. *Bioinorganic Chemistry and Applications*, *2018*, 1–18. https://doi.org/10.1155/2018/1062562

Kahlon, S. K., Sharma, G., Julka, J. M., Kumar, A., Sharma, S., & Stadler, F. J. (2018). Impact of heavy metals and nanoparticles on aquatic biota. *Environmental Chemistry Letters*, *16*(3), 919–946. https://doi.org/10.1007/s10311-018-0737-4

Kaliamurthi, S., Selvaraj, G., Cakmak, Z. E., Korkmaz, A. D., & Cakmak, T. (2019). The relationship between Chlorella sp. and zinc oxide nanoparticles: Changes in biochemical, oxygen evolution, and lipid production ability. *Process Biochemistry*, *85*, 43–50. https://doi.org/10.1016/j.procbio.2019.06.005

Karakoti, A. S., Hench, L. L., & Seal, S. (2006). The potential toxicity of nanomaterials—The role of surfaces. *Journal of the Minerals, Metals & Materials Society*, *58*, 77–82. https://doi.org/10.1007/s11837-006-0147-0

Khan, I., Saeed, K., & Khan, I. (2017). Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry*, *12*(7), 908–931. https://doi.org/10.1016/j.arabjc.2017.05.011

Krysanov, E., Pavlov, D., Demidova, T., & Dgebuadze, Y. (2010). Effect of nanoparticles on aquatic organisms. *Biology Bulletin*, *37*(4), 406–412. https://doi.org/10.1134/s1062359010040114

Lenaghan, S. C., Li, Y., Zhang, H., Burris, J. N., et al. (2013). Monitoring the environmental impact of TiO_2 nanoparticles using a plant-based sensor network. *IEEE Transactions on Nanotechnology*, *12*(2), 182–189. https://doi.org/10.1109/tnano.2013.2242089

Li, X., Schirmer, K., Bernard, L., Sigg, L., Pillai, S., & Behra, R. (2015). Silver nanoparticle toxicity and association with the alga

Euglena gracilis. *Environmental Science: Nano*, *2*(6), 594–602. https://doi.org/10.1039/c5en00093a

Liang, S. X. T., Wong, L. S., Dhanapal, A. C. T. A., & Djearamane, S. (2020). Toxicity of Metals and Metallic Nanoparticles on Nutritional Properties of Microalgae. *Water, Air, & Soil Pollution*, *231*(2). https://doi.org/10.1007/s11270-020-4413-5

Madhav, S., Ahamad, A., Singh, A. K., Kushawaha, J., et al. (2020). Water pollutants: Sources and impact on the environment and human health. *Sensors in Water Pollutants Monitoring: Role of Material*, 43–62. https://doi.org/10.1007/978-981-15-0671-0_4

Manzo, S., Miglietta, M. L., Rametta, G., Buono, S., & Di Francia, G. (2013). Toxic effects of ZnO nanoparticles towards marine algae *Dunaliella tertiolecta*. *Science of the Total Environment*, *445*, 371–376.

Miao, A.J., Schwehr, K.A., Xu, C., Zhang, S.J., et al. (2009). The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. *Environmental Pollution*,*157* (11), 3034-3041

Nam, S.H., & An, Y.J. (2019). Size- and shape-dependent toxicity of silver nanomaterials in green alga *Chlorococcuminfusionum*. *Ecotoxicology and Environmental Safety*, *168*, 388–393. https://doi.org/10.1016/j.ecoenv.2018.10.082

Oukarroum, A., Bras, S., Perreault, F., & Popovic, R. (2012). Inhibitory effects of silver nanoparticles in two green algae, Chlorella vulgaris and *Dunaliella tertiolecta*. *Ecotoxicology and Environmental Safety*, *78*, 80-85.

Ozkaleli, M., & Erdem, A. (2018). Biotoxicity of TiO2 Nanoparticles on *Raphidocelis subcapitata* Microalgae Exemplified by Membrane Deformation. *International Journal of Environmental Research and Public Health*, *15*(3), 416. https://doi.org/10.3390/ijerph15030416

Peng, X., Palma, S., Fisher, N. S., & Wong, S. S. (2011). Effect of morphology of ZnO nanostructures on their toxicity to marine algae. *Aquatic Toxicology*, *102*(3-4), 186–196. https://doi.org/10.1016/j.aquatox.2011.01.014

Piccinno, F., Gottschalk, F., Seeger, S., & Nowack, B. (2012). Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *Journal of Nanoparticle Research*, *14*. https://doi.org/10.1007/s11051-012-1109-9

Samei, M., Sarrafzadeh, M.H., & Faramarzi, M. A. (2018). The impact of morphology and size of zinc oxide nanoparticles on its toxicity to the freshwater microalga, *Raphidocelis subcapitata. Environmental Science and Pollution Research*, *26*, 2409–2420. https://doi.org/10.1007/s11356-018-3787-z

Sendra, M., Moreno-Garrido, I., Yeste, M. P., Gatica, J. M., & Blasco, J. (2017). Toxicity of TiO2, in nanoparticle or bulk form to freshwater and marine microalgae under visible light and UV-A radiation. *Environmental Pollution*, *227*, 39–48. https://doi.org/ 10.1016/j.envpol.2017.04.053

Shah, S. N. A., Shah, Z., Hussain, M., & Khan, M. (2017). Hazardous Effects of Titanium Dioxide Nanoparticles in Ecosystem. *Bioinorganic Chemistry and Applications*, *2017*, 1–12. https://doi.org/10.1155/2017/4101735

Strambeanu, N., Demetrovici, L., Dragos, D., & Lungu, M. (2014). Nanoparticles: Definition, Classification and General Physical Properties. *Nanoparticles' Promises and Risks*, 3–8. https://doi.org/10.1007/978-3-319-11728-7_1

Suman, T. Y., Radhika Rajasree, S. R., & Kirubagaran, R. (2015). Evaluation of zinc oxide nanoparticles toxicity on marine algae *chlorella vulgaris* through flow cytometric, cytotoxicity and oxidative stress analysis. *Ecotoxicology and Environmental Safety*, *113*, 23–30. https://doi.org/10.1016/j.ecoenv.2014.11.015

Tripathi, D. K., Tripathi, A., Shweta, Singh, S., et al. (2017). Uptake, accumulation and toxicity of silver nanoparticle in autotrophic plants, and heterotrophic microbes: A concentric review. *Frontiers in Microbiology*, *8*. https://doi.org/https:// doi.org/10.3389/fmicb.2017.00007

Wang, F., Guan, W., Xu, L., Ding, Z., et al. (2019). Effects of nanoparticles on algae: Adsorption, distribution, ecotoxicity and fate. *Applied Sciences*, *9*(8), 1534. https://doi.org/10.3390/app9081534

Wang, J., & Wang, W. (2014). Significance of physicochemical and uptake kinetics in controlling the toxicity of metallic nanomaterials to aquatic organisms. *Journal of Zhejiang University SCIENCE A*, *15*, 573–592. https://doi.org/10.1631/jzus.a1400109

Wang, S., Lv, J., Ma, J., & Zhang, S. (2016). Cellular internalization and intracellular biotransformation of silver nanoparticles in *Chlamydomonas reinhardtii*. *Nanotoxicology*, *10*(8), 1129–1135. https://doi.org/10.1080/17435390.2016.1179809

Xia, B., Sui, Q., Sun, X., Han, Q., et al. (2018). Ocean acidification increases the toxic effects of TiO2 nanoparticles on the marine microalga *Chlorella vulgaris*. *Journal of Hazardous Materials*, *346*, 1–9. https://doi.org/10.1016/j.jhazmat.2017.12.017

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Synthesis and Characterization of Magnesium Doped Ferric Sulphate Nanoparticles (Mg-Fe_2SO_3 NPs) for Agriculture Applications

Karthikkumar Dhanabalan[1], Divya Balasubramanian[2], Ranjithkumar Rajamani[1*], Chandar Shekar Bellan[2], Ling Shing Wong[3], Sinouvassane Djearamane[4*]

[1]Viyen Biotech LLP, Coimbatore, Tamil Nadu - 641031, India
[2]Department of Physics, Kongunadu Arts and Science College, Coimbatore, 641 029, India
[3]Life Science Division, Faculty of Health and Life Sciences, INTI International University, Nilai, 71800, Malaysia
[4]Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Kampar, 31900, Malaysia

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XRD

FESEM

Seed germination

Cowpea seed

Vigna unguiculata

ABSTRACT

The present study aimed to synthesize the magnesium doped ferric sulphate nanoparticles (Mg-Fe_2SO_3 NPs) and investigate their seed germination efficacy. Mg-Fe_2SO_3 NPs were prepared by a simple and cost-effective method and subjected to characterization. The X-ray Diffraction (XRD) spectrum revealed the crystalline nature of Mg-Fe_2SO_3 NPs with an average crystallite size of 36.41 nm. The field emission scanning electron microscope (FESEM) image displayed the agglomeration of Mg-Fe_2SO_3 NPs with the shape of the grains appeared like starfish which has limbs grown from a common cluster. The energy dispersive X-ray spectroscopy (EDS) demonstrated the existence of C (10.5%), O (49.14%), Fe (26.67%), Mg (0.78%) and S (13.35%) elements in Mg-Fe_2SO_3 NPs. It also revealed the absence of impurities in the synthesized NPs. Through Fourier transform infrared spectroscopy (FTIR), Mg-Fe_2SO_3 NPs showed the characteristic peaks at 615.29cm^{-1}, 1130.29cm^{-1}, 1400.32 cm^{-1}and 1633.71cm^{-1} which corresponded to Fe-O, C-N, O-H and N-H vibration respectively. Further, the seed germination study revealed that the Mg-Fe_2SO_3 NPs treatment caused a significant increase in seedling growth of cowpea (*Vigna unguiculata*) seeds compared to the untreated samples.

* Corresponding author
E-mail: biotechranjith@gmail.com (Ranjith Kumar Rajamani);
sinouvassane@utar.edu.my (Sinouvassane Djearamane)

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1 Introduction

Presently, the agriculture sector has been fronting extensive challenges like unpredictable climate change (due to different types of pollution), utilization of several harmful chemicals in agricultural land, and toxic industry outlets (Pouratashi and Iravani 2012; Fraceto et al. 2016; Sun 2019; Mittal et al. 2020; Yilin et al. 2020). The United Nations Project reports that, in the year 2030 the world population will become 8.5 billion, which gives an alert of food demand in the future (Mittal et al. 2020). Nano-size materials display different physicochemical properties such as chemicals, optical, physical, and biomedical characteristics when compared to bulk particles, because of the quantum properties of the materials with large surface area (Thakkar et al. 2010; Jeevanandam et al. 2018; Khan et al. 2019). These new characteristics of nano-size particles allow us to focus on unique output in different sectors such as biomedical, electronics, solar cell, water purification, agriculture, etc., (Yata et al. 2018; Zhu and Zhou 2019; Mittal et al. 2020). Nanomaterials, at the cutting-edge technology of nanoscience, are combined into a diversity of commercial goods due to their formidable properties including antimicrobial, antiviral, anticancer, growth promotor, etc., compared to their bulk materials. Hence, the novel and emerging characteristics of these nano-size particles have gained more attention in the agronomics sector. With the assistance of nano-size particles, the current agriculture practice can be transformed into precision cultivation with the existing resources to meet the food demands (Somenath et al. 2020; Weitao et al. 2020).

Multiple shapes of nano-size particles, including nanotubes, nanowires, and nanoclays possess unique physicochemical, magnetic, and electrical characteristics with unique applications in different sectors (Imada et al. 2016; Kumbhakar et al. 2014; Yaqoob et al. 2020). Fertilizers act as key molecules in the agriculture system and are necessary to improve crop production, but on the other side, the chemical fertilizers disturb the soil mineral balance and decrease the fertility of the soil. Currently, nanomaterials play a vital role in the agriculture system to create sustainable farming and increase soil fertility (An and Zhong 2019; Kumari and Singh 2020). Several factors such as size, charge, and shape are responsible for the interaction of nanomaterials with plants. Previously, different metals and metal oxides nanomaterials such as aluminum (Al), Cerium (Ce), Molybdenum (Mo), carbon (C), Iron (Fe), Titanium (Ti), Magnesium (Mg), Carbon (C), Gold (Au), Zinc (Zn), Silicon (Si), Copper (Cu), Silver (Ag), etc., were used in agriculture applications (Partila 2019; Sadak 2019; Anthony et al. 2020; Fuad et al. 2021). These nano-size particles are introduced into the agronomics sector to enhance seed treatments, and soil and foliar applications (Deshpande et al. 2017; Khan et al. 2019).

Magnesium (Mg) is a crucial micro component for all the crops to utilize photo energy for the photosynthesis process, as Mg is the central atom of the chlorophyll molecule, responsible for energy utilization. In general, Mg also plays significant responsibility in triggering enzymes, which are involved in photosynthesis, respiration, and nucleic acid synthesis. Seed germination is the first step (seed into the plant) and the most sensitive stage with great significance for improving crop yield and quality. Owing to this, the present study aimed to synthesize and characterize the magnesium doped ferric sulphate nanoparticles (Mg-Fe_2SO_3 NPs) to investigate the effect of these nanoparticles on the germination property of cowpea (*Vigna unguiculata*). It is reported that the deficiency of Mg and Fe in plant systems resulted in a decrease in the production of chlorophyll (Balakrishnan et al. 2000). This study was planned to synthesize the nanoparticles with the help of Mg and Fe for agriculture applications through green synthesis approach for getting additional benefit of incorporating phytochemicals into the nanoparticles for the better yield. To the best of our knowledge, this is the first report of utilizing the leaves extract of *Aegle marmelosby* in synthesizing Mg doped Fe_2SO_3 NPs. The *A. Marmelosby* contains bioactive molecules in various parts of the plants especially the leaves contain different types of phytochemicals (Pathirana et al. 2020). The leaves extract of *A. Marmelosby* was used as an effective reduction agent in the green synthesis of Mg doped Fe_2SO_3 NPs for seed germination applications.

2 Materials and Methods

2.1 Materials and preparation of plant extract

Ferric chloride (Fe_2Cl_3), sodium sulphide (Na_2S), and magnesium chloride ($MgCl_2$) were obtained from Himedia, Mumbai, India. The leaves of *A. marmelosby* (Vilvam) were collected from the local area nearby the college campus and authenticated by a plant taxonomist. The collected leaves were washed with distilled water thoroughly and these leaves were dried for 10 days at room temperature. To prepare aqueous leaves extract of *A. marmelosby*, about 20g of leaves (find powder form) was well mixed with 100mL of sterile water, and the reaction vessel was kept in a soxhlet extractor system to obtain the secondary phytochemicals from *A. marmelosby*. Obtained aqueous leaf extract was used for the synthesis of Mg doped Fe_2SO_3NPs.

2.2 Preparation of Mg doped Fe_2SO_3 NPs

Mg doped Fe_2SO_3 nanoparticles were synthesized via green synthesis by adding Fe_2Cl_3, Na_2S, and $MgCl_2$ to the plant extract *A. marmelosby*. Initially, about 5mL of plant extract was added to 20mL deionized water and kept in stirred condition for 15 minutes using a magnetic stirrer. About 6.48g of Fe_2Cl_3 was mixed in 40mL of deionized water and this was added drop by drop into the

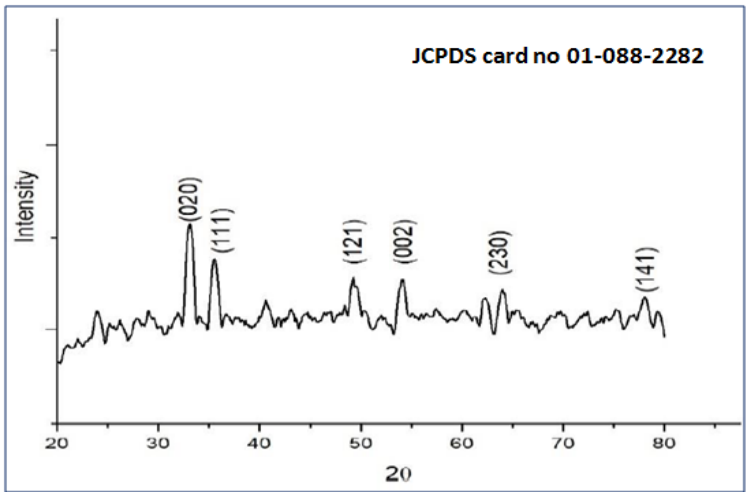

Figure 1 XRD spectrum of Mg doped Fe_2SO_3 NPs

prepared extract solution for 15 minutes. Then Na_2S (1.56g) was added slowly into the reaction mixture under stirred conditions. Finally, $MgCl_2$ solution dissolved in 40mL of deionized H_2O was added into a reaction vessel and continuously stirred for 1 hour. After that, the obtained reaction mixture was centrifuged for 30 minutes at 5000 rpm, then the obtained pellet was washed with distilled water followed by calcined at 600°C for 4 hours.

2.3 Characterization of Mg doped Fe_2SO_3 NPs

The crystallite peaks of prepared Mg doped Fe_2SO_3 NPs were analyzed by using the X-Ray diffraction **(**XRD) spectrum (PANalytical-X-pertPro) at the range of 2θ =20°- 80°. The surface topology and the elemental composition of the prepared Mg doped Fe_2SO_3 NPs were studied by scanning electron microscopy (TESCAN MIRA-3 attached with EDS spectrum). The function groups of the prepared NPs were identified using Fourier transform infra-red (FTIR) spectra (FTIR00585, Perkin-Elmer).

2.4 Effect of Mg doped Fe_2SO_3 NPs on Cowpea seed germination

In 10 mL of distilled water, about 0.005 g of Mg doped Fe_2SO_3 nanomaterial was dissolved and used as germination stock solution. Collected seeds were surface sterilized by ethanol to remove unwanted bacteria. In a petri dish, the filter paper was used instead of soil and collected seeds were placed. One of the petri dishes that contain seeds received only water (2mL/day) served as a control for the experiment and other petri dishes with seeds received Mg doped Fe_2SO_3 NPs solution (2mL/day). These petri dishes were placed under visible light conditions. The whole experiment was set at lab scale level and the seedling growth of nanoparticles treated and untreated seeds were measured and demonstrated after 5 days. The obtained results were statistically analyzed.

3 Results and Discussion

3.1 XRD study

The structure of the Mg doped Fe_2SO_3 NPs was examined by XRD and measurements were carried out over the diffraction angle 2θ = 10°- 90°. The XRD results showed diffraction peaks at 33.05°, 35.53°, and 49.41° which correspond to (020), (111), and (121) orientation planes, respectively, indicating orthorhombic crystallinity (JCPDS card no. 01-088-2282) of the synthesized nanoparticles (Figure 1).

The crystallite size of the Mg doped Fe_2SO_3 nanoparticles was estimated using Scherrer's formula (Ning et al. 2020; Madhan et al. 2021).

$$D = \frac{K\lambda}{\beta \cos\theta}$$

Where D = crystallite size, K = constant (shape), λ = X-ray wavelength, β = Full Width Half Maximum [refection located at 2θ] and θ = angle of reflection

The average crystallite size of the prepared nanoparticles was estimated to be around 36.41 nm. The observed peaks confirmed the predominately crystalline nature of the magnesium doped ferric sulphate nanoparticles (Yu et al. 2011).

3.2 FESEM analysis

The surface topology of the prepared Mg doped Fe_2SO_3 NPs was studied through FESEM analysis. The FESEM image showed the agglomeration of Mg doped Fe_2SO_3 NPs with the shape of grains looking like starfish which have limbs grown from a common cluster (Figure 2).

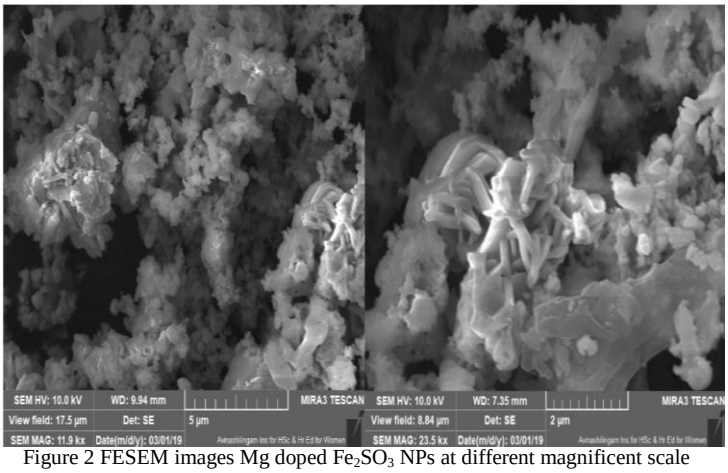

Figure 2 FESEM images Mg doped Fe_2SO_3 NPs at different magnificent scale

The different sizes and shapes of the nanomaterials certainly influence the multiple fields of biological applications like clinical, agricultural, and food technology because nanomaterials possess unique chemical and physical properties due to their surface area and nanoscale size. The size of the nanomaterials can influence the physiochemical properties of the substance, which led to novel applications (Ibrahim et al. 2019). Pradeev et al. (2018) described the nanoscale particles with hexagonal crystalline structure of ZnO NPs and Mg doped ZnO NPs around 30-110 nm through the precipitation method. In addition, they reported that the grain size of the final particles inside the ZnO matrix was increased with a high concentration of Mg ions doped ZnO NPs. In this study, the FESEM images of prepared Mg doped Fe_2SO_3 NPs showed that the larger particles cause the particles to aggregate on their surfaces.

3.3 EDAX analysis

EDAX is an analytical technique and standard method for identifying chemical characterization or elemental composition using a very small quantity of samples. The elemental analysis of the prepared Mg doped Fe_2SO_3 NPs was carried out by EDAX and confirmed that the prepared sample was free from impurities as the peaks showed the existence of only C, O, Fe, Mg and S elements (Figure 3) (Pradeev et al.2018).

3.4 Fourier Transformation Infrared Spectroscopy (FTIR)

The prepared Mg doped Fe_2SO_3 NPs was subjected to FTIR spectrum to investigate the vibration of the functional molecules through infrared absorption spectrum. Figure 4 displays the FTIR spectrum of Mg doped Fe_2SO_3 NPs.

The FTIR spectrum of prepared Mg doped Fe_2SO_3 NPs showed characteristic peaks at 3142.04 cm^{-1}, 1633.71 cm^{-1}, 1400.32 cm^{-1}, 1130.29 cm^{-1} and 615.29 cm^{-1}. The band appeared at a low frequency of 615.29 cm^{-1} corresponds to the stretching vibration of Fe-O bonding (Bharathi et al. 2019; Piyush et al. 2021; Win et al. 2021). The obtained band at 1633.71 cm^{-1} corresponds to the vibration mode of N-H bending and the band vibration at 1130.29 cm^{-1} was due to the vibration of C-N stretching. These vibration modes of N-H and C-N indicated amines and aromatic amine groups. The characteristic band at 1400.32 cm^{-1} was assigned to the vibration mode of C-F stretching of fluoro compounds. The strong peak at 3142.04 cm^{-1} was assigned to the vibration of the hydroxyl group due to the O-H vibration of stretching (Kumuth and Alias 2006; Pradeep et al. 2008; Anam et al. 2018; Lesiak et al. 2019).

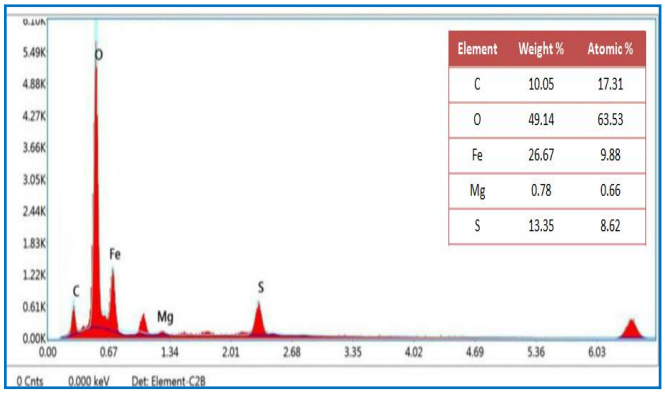

Figure 3 EDAX spectrum of Mg doped Fe_2SO_3 NPs

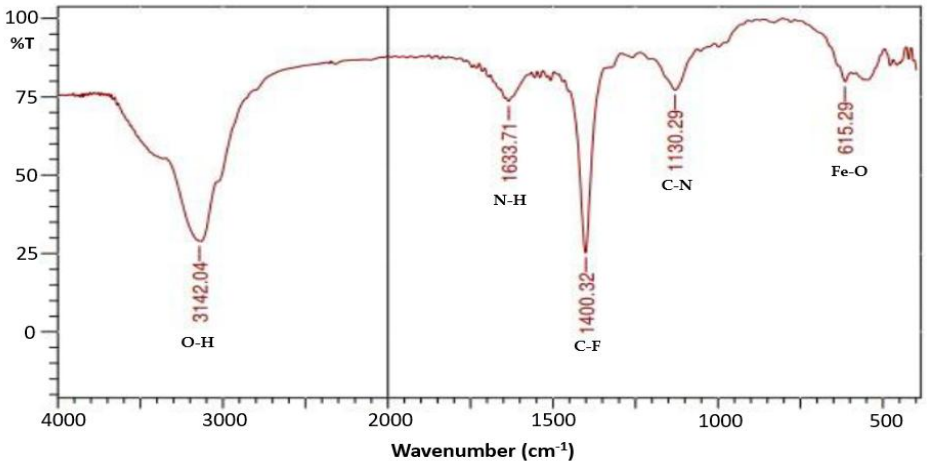

Figure 4 FTIR spectrum of Mg doped Fe_2SO_3 NPs

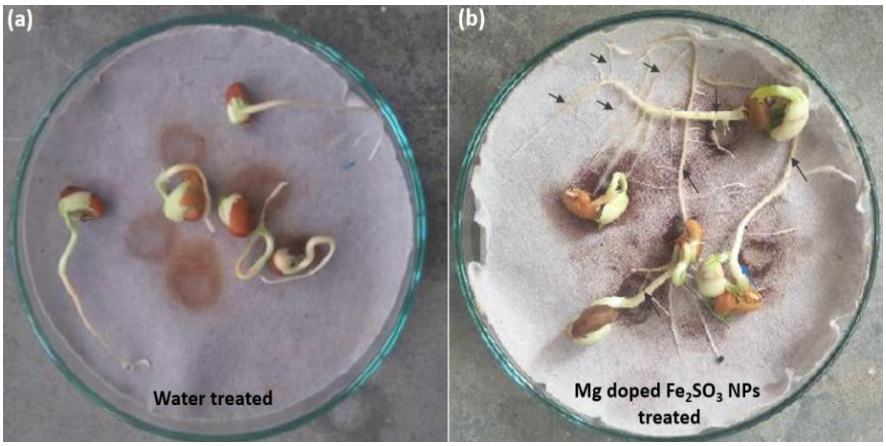

Figure 5 Effect of Mg doped Fe_2SO_3 NPs on cowpea seed germination

Table 1 Effect of Mg doped Fe_2SO_3 NPs on cowpea seed germination

Parameters	Cowpea seed treated with water (cm)	Cowpea seed treated Mg doped Fe_2SO_3 NPs (cm)
Shoot length	2	4.5
Root length	2.5	6
Leaves length	Nil	1

3.5 Mg doped Fe_2SO_3 NPs on Cowpea Seed Germination

The data presented in Table 1 shows the effect of the Mg doped Fe_2SO_3 NPs treatment of @ 2 mL/day on cowpea seeds in comparison with double distilled water treated as the control at day 5. At the end of day 5, of the experimental period, the shoot and root lengths were measured for both the treatment and control. The water treated seeds showed a maximum shoot length of around 2 cm, and a root length of around 2.5 cm, whereas, the nanoparticles treated seeds showed a maximum shoot and root length of 4.5 cm and 6 cm, respectively. In addition, the nanoparticles treated seeds showed 2 leaves with a length of 1 cm, while no leaf growth was noticed in the control (Figure 5). The results indicate that the treatment of Mg doped Fe_2SO_3 NPs has brought a significant effect on the seed germination process.

Currently, many industries focus on applying nano-size particles to the agriculture sector, with applications such as nanopesticides and nanofertilizers to increase the productivity and health of the agriculture crops (Prasad et al. 2017). Yi et al. (2016) first time studied the effect of Fe_2O_3 nanocubes, Fe_2O_3 long nanorods, and Fe_2O_3 short nanorods on the germination of rice and noted that these nanomaterials promoted shoots growth and stimulated roots elongation at all concentrations. According to the recent report by

Asma et al. (2019), AgNPs also have a significant effect on the various growth parameters such as height of the plant, yields of the crop, fresh biomass, dry biomass, and number of roots and shoot in *Solanum lycopersicum*. Likewise, in the present study, the prepared Mg doped Fe_2SO_3 NPs showed a substantial seedling growth promotor activity.

Conclusion

The present study has successfully synthesized and characterized the Mg doped Fe_2SO_3 NPs using the aqueous leaves extract of *A. marmelosby*. The synthesized nanoparticles exhibited an average crystallinity of 36.41nm according to XRD spectrum analysis. The shape of grains looked like starfish which has limbs grown from a common cluster as appeared under FESEM analysis. The elemental composition of the prepared nanomaterial by EDAX analysis indicated that the formulated nanomaterial was free from impurities as it was composed of only C, O, Fe, Mg, and S elements. The FTIR spectrum of the nanomaterial showed the stretching vibration of Fe-O bonding, N-H bending, C-N was stretching, the C-F stretching and O-H stretching. Further, the seed germination study demonstrated a significant seedling growth promotor activity of the synthesized Mg doped Fe_2SO_3 NPs on cowpea seeds, indicating the potential of Mg doped Fe_2SO_3 NPs to be utilized as a plant growth promotor in the agriculture industry.

Conflicts of interest

The authors affirm that they do not have any conflict of interest**.**

References

An, Y., & Zhong, C. (2019). Impacts of Silver Nanoparticles on Plants: A Focus on the Phytotoxicity and Underlying Mechanism. *International Journal of Molecular Sciences*, *20*,1003, 1-21, https://doi.org/10.3390/ijms20051003

Anam, A., Abad, A., Mohd, A., & Shamsuzzaman. A. (2018). Microwave-assisted MgO NP catalyzed one-pot multicomponent synthesis of polysubstituted steroidal pyridines. *New Journal of chemistry*, *42*, 184-197.

Anthony, C., Kyle, J., Christina, M., Anne, A., & David, W. B. (2020). A Review of Metal and Metal-Oxide Nanoparticle Coating Technologies to Inhibit Agglomeration and Increase Bioactivity for Agricultural Applications. *Agronomy*, *10*(7), 1018, 1-20, https://doi.org/10.3390/agronomy10071018

Asma, N., Crispin, H., & Mudassar, I. (2019). Impact of AgNPs on Seed Germination and Seedling Growth: A Focus Study on Its Antibacterial Potential against *Clavibacter michiganensis* subsp. *michiganensis* Infection in *Solanum lycopersicum*. *Journal of Nanomaterials*, *2019*,6316094, 1-13, https://doi.org/10.1155/2019/6316094

Balakrishnan, K., Rajendran, C., & Kulandaivelu, G. (2000). Differential responses of iron, magnesium, and zinc deficiency on pigment composition, nutrient content, and photosynthetic activity in tropical fruit crops. *Photosynthetica*, *38*(3), 477-479

Bharathi, D., Ranjithkumar, R., Vasantharaj, S., Chander Shekar, B., & Bhuvaneshwari, V. (2019). Synthesis and characterization of chitosan/iron oxide nanocomposite for biomedical applications. *International Journal of Biological Macromolecules*, *132*, 880-887

Deshpande, P., Dapkekar, A., Oak, M. D., Paknikar, K. M., & Rajwade, J. M. (2017). Zinc complexed chitosan/TPP nanoparticles: A promising micronutrient nanocarrier suited for foliar application. *Carbohydrate polymers*, *165*, 394-401

Fraceto, L. F., Grillo, R., de Medeiros, G. A., Scognamiglio, V., et al. (2016). Nanotechnology in Agriculture: Which Innovation Potential Does It Have? *Frontiers in Environmental Science*, *4*,1-5. doi: 10.3389/fenvs.2016.00020

Fuad, A., Khawla, A., Jamila, A. A., & Saleh, A. (2021). A review on metal-based nanoparticles and their toxicity to beneficial soil bacteria and fungi. *Ecotoxicology and Environmental Safety*, *213*, 112027, 1-17, https://doi.org/10.1016/j.ecoenv.2021.112027

Ibrahim, K., Khalid, S., & Idrees, K. (2019). Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry*, *12*, 908-931

Imada, K., Sakai, S., Kajihara, H., Tanaka, S., & Ito, S. (2016). Magnesium oxide nanoparticles induce systemic resistance in tomato against bacterial wilt disease. *Plant Pathology*, *65*, 551-560

Jeevanandam, J., Barhoum, A., Chan, Y. S., Dufresne, A., & Danquah, M. K. (2018). Review on nanoparticles and nanostructured materials: History, sources, toxicity and regulations. *Beilstein Journal of Nanotechnology*, *9*, 1050-1074

Khan, I., Saeed, K., & Khan, I. (2019). Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry*, *12*, 908-931

Kumari, R., & Singh, D. P. (2020). Nano-biofertilizer: An Emerging Eco-friendly Approach for Sustainable Agriculture. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *90*, 733-741, https://doi.org/10.1007/s40011-019-01133-6

Kumbhakar, P., Ray, S. S., & Stepanov, A. L. (2014). Optical properties of nanoparticles and nanocomposites. *Journal of*

Nanomaterial, *2014*, 181365. 1-2,https://doi.org/10.1155/ 2014/181365

Kumutha, K., & Alias, Y. (2006). FTIR spectra of plasticized grafted natural rubber-$LiCF_3SO_3$ electrolytes. *Spectrochimica Acta Part A,64*, 442-447

Lesiak, B., Rangam, N., Jiricek, P., Gordeev, I., et al. (2019). Surface Study of Fe_3O_4 Nanoparticles Functionalized with Biocompatible Adsorbed Molecules. *Frontiers in Chemistry*, *7*:642, 1-16, https://doi.org/10.3389/fchem.2019.00642

Madhan, G., Begam, A. A., Varsha, L. V., Ranjithkumar, R., & Bharathi, D. (2021). Facile synthesis and characterization of chitosan/zinc oxide nanocomposite for enhanced antibacterial and photocatalytic activity. *International Journal of Biological Macromolecules*, *190*, 259-269

Mittal, D., Kaur, G., Singh, P., Yadav, K., & Ali, S.A. (2020). Nanoparticle-Based Sustainable Agriculture and Food Science: Recent Advances and Future Outlook. *Frontiers in Nanotechnology*, *2* (579954), 1-38

Ning, P., Liu, C. C., Wang, Y. J., Li, X. Z., et al. (2020). Facile synthesis, antibacterial mechanisms and cytocompatibility of Ag-$MnFe_2O_4$ magnetic nanoparticles. *Ceramic International*, *46*, 20150-20115

Partila, A. M. (2019). Bioproduction of Silver Nanoparticles and Its Potential Applications in Agriculture. In: D. Panpatte, Y. Jhala (eds) *Nanotechnology for Agriculture. Springer, Singapore*. https://doi.org/10.1007/978-981-32-9370-0_2

Pathirana, C. K., Madhujith, T., & Eeswara, J. (2020). Bael (*Aegle marmelos L*. Corrêa), a Medicinal Tree with Immense Economic Potentials. *Advances in Agriculture*, *2020*, 88140148, 1-13, https://doi.org/10.1155/2020/8814018

Piyush, G.K., Senthilkumar, P., Tamilarasi, G., Ranjithkumar. R., et al. (2021). Synthesis and Characterization of Novel Fe_3O_4/PVA/Egg-shell Hybrid nanocomposite for photodegradation and antibacterial activity. *Journal of Composite Science*, *5*(267), 1-9, https://doi.org/ 10.3390/jcs5100267

Pouratashi, M., & Iravani, H. (2012). Farmers' knowledge of integrated pest management and learning style preferences: implications for information delivery. *International Journal Pest Management*, *58*, 347-353, doi: 10.1080/09670874.2012.724468

Pradeep, A., Priyadharsini, G., & Chandrasekaran, G. (2008). Sol-gel route of synthesis of nanoparticles of $MgFe_2O_4$ and XRD, FTIR and VSM study. *Journal of Magnetism and Magnetic Materials*, *320*, 2774-2779

Pradeev R. K., Sadaiyandi, K., Kennedy, A., Suresh, S., et al. (2018). Influence of Mg Doping on ZnO Nanoparticles for Enhanced Photocatalytic Evaluation and Antibacterial Analysis. *Nanoscale Research Letters*, *13:229*, 1-13, https://doi.org/10.1186/s11671-018-2643-x

Prasad, R., Bhattacharyya, A., & Nguyen, Q. D. (2017). Nanotechnology in Sustainable Agriculture: Recent Developments, Challenges, and Perspectives. *Frontiers in Microbiology*, *8*:1014, 1-13, https://doi.org/10.3389/fmicb.2017.01014

Sadak, M. S. (2019). Impact of silver nanoparticles on plant growth, some biochemical aspects, and yield of fenugreek plant (*Trigonellafoenum graecum*). *Bulletin National Research Cent*re *43:38*, 1-6, https://doi.org/10.1186/s42269-019-0077-y

Somenath, D., Arpan. M., Gereraj, S., &Vipin, K. S. (2020). Overview of nanomaterials synthesis methods, characterization techniques and effect on seed germination. *Nano-Materials as Photocatalysts for Degradation of Environmental Pollutants*, *2020*, 371-401

Sun, H. (2019). Grand Challenges in Environmental Nanotechnology. *Frontiers in Nanotechnology*, *1*(2), 1-3, doi:10.3389/fnano.2019.00002

Thakkar, K. N., Mhatre, S. S., & Parikh, R. Y. (2010). Biological synthesis of metallic nanoparticles. *Nanomedicine Nanotechnology Biology Medicine*, *6*, 257-262

Weitao, L., Aurang, Z., Jiapan, L., Jiani, W., et al. (2020). Interactions of metal-based nanoparticles (MBNPs) and metal-oxide nanoparticles (MONPs) with crop plants: a critical review of research progress and prospects. *Environmental Reviews*, *28*(3), 294-310

Win, T. T., Khan, S., Bo, B., Shah, Z., & Pengcheng, F. (2021). Green synthesis and characterization of Fe_3O_4 nanoparticles using Chlorella-K01 extract for potential enhancement of plant growth stimulating and antifungal activity. *Scientific Reports*, *11*:21996, 1-11, https://doi.org/10.1038/s41598-021-01538-2

Yaqoob, A. A., Parveen, T., Umar, K., & Mohamad, M. N. (2020). Role of nanomaterials in the treatment of wastewater: a review. *Water*, *12*(2), 495, 1-30, doi: https://doi.org/10. 3390/w12020495

Yata, V. K., Tiwari, B. C., & Ahmad, I. (2018). Nanoscience in food and agriculture: Research, industries and patents. *Environmental Chemistry Letter*,*16*, 79-84

Yi, H., Zetian, Z., Yukui, R., Jing, Y. R., et al. (2016). Effect of Different Nanoparticles on Seed Germination and Seedling Growth in Rice, *2nd Annual International Conference on Advanced Material Engineering* (AME 2016), 166-173

Yilin, Z., Jiajun, Y., Astrid, A., Xiaoyu, G., et al. (2020). Temperature- and pH-Responsive Star Polymers as Nanocarriers with Potential for in Vivo Agrochemical Delivery. *ACS Nano*, *14*(9), 10954-10965

Yu, W. T., Chieh-Chao, Y., Ming-Hang, Y., Chum-Sam, H., et al. (2011). Preparation and characterization of p-type Fe_2O_3 pellets from Mg doping in pure oxygen atmosphere at high temperatures. *Journal of the Taiwan Institute of Chemical Engineers*, *42*, 669-673

Zhu, D., & Zhou, Q. (2019). Action and mechanism of semiconductor photocatalysis on degradation of organic pollutants in water treatment: A review. *Environmental Nanotechnology Monitoring Management*, *12*, 100255, 1-11

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Hyperspectral signatures and reflectance models related to the ripening index in four grape varieties

Héctor Flores-Breceda[1], Alejandro Isabel Luna-Maldonado[1*], María del Carmen Ojeda-Zacarías[1], Humberto Rodríguez-Fuentes[1], Juan Antonio Vidales-Contreras[1], Juan Arredondo-Valdez[1], Beatriz Adriana Rodríguez-Romero[1], Marina Burgaya-Ribell[2]

[1]Universidad Autónoma de Nuevo León, Facultad de Agronomía, Departamento de Ingeniería Agrícola y de los Alimentos, Francisco Villa S/N, Ex-Hacienda El Canadá, General Escobedo, Nuevo León, 66050, México.
[2]Universitat Autònoma de Barcelona, Facultat de Veterinària.Edifici V, Travessera dels Turons,08193 Cerdanyola del Vallès, Barcelona, España

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KEYWORDS

°Brix

Color

Hyperspectral Signature

Ripening Index

pH

Texture

ABSTRACT

The preference for the consumption of red wine in Mexico is increasing because its components derived from the grape are attributed to health benefits. The quality of wine depends mostly on the vineyard conditions. The objective of this study was able to differentiate the physicochemical composition in the harvest stage of four varieties of red grapes that are used in the production of wine to relate their maturation with those of their hyperspectral signatures. Various parameters including pH, total soluble solids, color, weight, and morphology were determined from the bunches of grapes. Concerning the maturity index, it was observed that the grapes with the highest degree of maturity were Shiraz and Merlot at harvest time. The pH of grape juice is a measure of active acidity; the texture is considered a quick and inexpensive technique. The hyperspectral signatures reflectances versus color, total soluble solids, morphology, weight, texture, and pH for each grape variety was best fitted with Gaussian curves of order 8 to Cabernet sauvignon and Merlot, 7 to Malbec, and 5 to Shiraz with R^2 above 0.99.

* Corresponding author
E-mail: alejandro.lunaml@uanl.edu.mx (A. I. Luna-Maldonado)

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1 Introduction

The practice of viticulture developed in the Middle East in 8000 BC through the cultivation of wild grapes (Gur et al. 2021), later the grape variety *Vitis vinifera* was found, which originated in Europe and till now more than ten thousand white and red grapes varieties were recognized throughout the world (Lumbreras 2003). The development of grapevine plants starts from the shoots (a portion of stems) and after the fourth year, the crop starts giving a constant annual production of grapes, which can ensure their use for industrialization in winemaking (Gattullo et al. 2020). The importance of grapes production has been given slowly in the world and it is currently known that countries such as Italy, France, the United States, Germany, and China, are some of the largest grapes producers and consumers (Fernandez and Meraz Ruiz 2022).

One of the considerations to evaluate the quality of the wine is influenced by intrinsic and extrinsic elements, where the intrinsic factors such as flavor, color, acidity, and level of alcohol are contemplated while the extrinsic factors such as brand, price, year of production, country of origin, grape variety, labeling, tradition, awards, and recommendation are important (Ruso et al. 2021). Concerning the price of wine, these factors influenced the globalization of wine because it allows more wines to circulate throughout the world, increasing the competition for this product, and causing competition between the old wine producers and the countries considered new producers (Moscovici et al. 2022). As per the sale registered in 2007, the most consumed wine in Mexico is red wine, its consumption reached 61.4%, this was followed by white wine with 27% (Andrade et al. 2011). The wine has complex components which are associated with the grapes varieties and are released during the fermentation process (Sun et al. 2020). Further, the vine has antioxidant properties which coupled with the resveratrol, naturally improves blood circulation (Shaito et al. 2020) and reduces low-density lipoprotein (LDL) cholesterol (Merchant Martí 2017).

In general, grapes skin, pulp, and seed are used in vine making, and among these polyphenolic compounds are mainly found in the skin on the epidermal cells and seed (Hornedo-Ortega et al. 2020). Wine also has various phenolic compounds including cinnamic acids, tyrosine, phenolic acid derivatives, stilbenes, and flavonoids which are responsible for the antioxidant properties of the wine (Zeb 2020).

Traditional criteria to determine the ripening index in grape varieties are skin color, softening, titratable acidity, the concentration of soluble solids, and the availability of volatile compounds (Shahab et al. 2020). A physical property considered relevant in the food industry for evaluating the external quality of grapes is color (Peppi et al. 2006; Pisciotta et al. 2020). Zouid et al. (2010) studied the evolution of the mechanical properties of Cabernet Franc grapes, during their maturation, belonging to three vineyards from different regions. The rheological tests (compression and puncture) on the grapes were made to analyze changes in their maturity versus their ability to extract anthocyanins from the skin. They concluded that grapes with higher skin breaking forces produce extracts with a higher total content of anthocyanins. Further, according to Wang et al. (2020) aroma, firmness, and berry shape are three important quality traits that are perceived for table grape berries. On the other matter, throughout the development of the berry, the transverse and longitudinal diameters gradually increase, tending to certain regularity at the end of its maturation (Zhang et al. 2021).

Recently, an emerging technique that integrates conventional imaging and spectroscopy is hyperspectral imaging (HSI) to obtain a spatial image and wavelengths of objects (Grajeda et al. 2015). HSI is a non-destructive technique, in the case of fruits, it has taken greater use because fruits are not damaged during analysis and can carry out a greater amount of analysis in less time or during its maturation in real-time (Scalisi and O'Connell 2021). The use of hyperspectral images in agriculture is serving to detect problems of fruit and leaf damage in real-time (Grajeda et al. 2018). A hyperspectral image is composed of a series of sub-images, which represent the intensity distribution in each spectral band (Jia et al. 2020; Lavadiya et al. 2022).

When some fruit is exposed to light, the reflected radiation can be measured and recorded with a reflectance spectrum, which is related to the chemical composition of the fruit (Baiano et al. 2012). For the management of the information of its components, multivariate analysis, and machine learning have proven to be very efficient methodologies for the prediction of the oenological parameters of the grape berries (Melo-Pinto et al. 2022). The objective of this work is to predict the ripening index of the main grape varieties used for red wine production in Mexico using hyperspectral signatures.

2 Materials and methods

2.1 Grape sampling

The vineyard of the Agricultural Production Research Center of the UANL (Figure 1) is divided into four plots of approximately one hectare and had an arrangement per plot of 37 columns and 60 rows of vine plants, in which different varieties were grown of grapes.

The samples of red grape varieties were collected from the Cabernet Sauvignon, Shiraz, Melot, and Malbec vineyards. For sample collection, 10 plants from each of the red grape varieties were selected and from each plant, a bunch of 2.5 kg per plant was

Figure 1 Distribution of grape varieties in the plots of the CIPA-UANL vineyard

taken for analysis. From each selected bunch, six grapes were selected from the different positions of the bunch (upper, intermediate, and lower part) and used to carry out the physicochemical analyzes (morphology, weight, color, pH, texture, °Brix), as well as the acquisition and processing of hyperspectral images that were taken of the entire grape bunch.

2.2 Hyperspectral imaging system

To obtain the hyperspectral images, a system composed of a Pike F-210B camera and a V10E spectrograph that takes images with a resolution of 1392 X 1040 pixels, with a spectral range of 400 to 1000 nm with intervals of 2.8 nm and a 30 µm slot, integrated into a support structure with LED lighting and a conveyor belt with a variable frequency drive motor was used.

2.3 Software used for Hyperspectral image analysis

For the acquisition and analysis of the images, programs were developed on the Matlab R2020 platform and for image processing, the HyperTools V3 software (Graphical user interface for the analysis of hyperspectral images) was used. In addition, Matlab R2020 was used to do the curve fitting of the hyperspectral signatures.

2.4 Color measurement

For the measurement of the color parameters of the grapes (L*, a*, b*, C*, H*) the SPEC portable equipment was used. The color is measured directly on the skin of the grape.

2.5 Total soluble solids measurement

Benelli et al. (2020) measured soluble solids in grapes in their different stages of maturation using the manual refractometer in the field. Total soluble solids were measured in grape juice concentrate using a manual refractometer (Atago model, Tokyo, Japan), which has a measurement range of 0 to 33 ° Brix. The juice of the grape was extracted and poured into the prism of the refractometer, it was closed with the daylight plate and the total soluble solids in ° Brix were read after light.

2.6 Morphology, weight, and texture

One of the widely applied techniques in the food industry is the evaluation of mechanical and physical characteristics by texture analysis (Zulkifli et al. 2020). The thickness and hardness of the grape skin are indices that reflect the extraction potential of anthocyanins and the dehydration kinetics (Corona et al. 2020). Grape firmness was measured by puncture measured in Newtons and measured with a TAxT2i Texture Analyzer (Stable MicroSystems, Surrey, UK). The morphology of the grape was obtained using a vernier and the weight was measured with the help of a pomegranate scale.

2.7 pH measurement

A digital potentiometer (HANNA; HI99163, Woonsoket RI, USA) was used to measure the pH. For this, the skin and pulp of the grape were ground with the help of a porcelain crucible, filtered, and obtained juice was used for the estimation of pH.

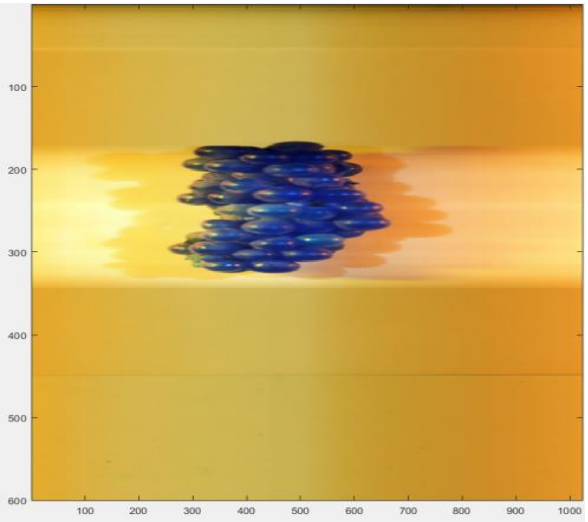

Figure 2 Composite image of grapes bunches

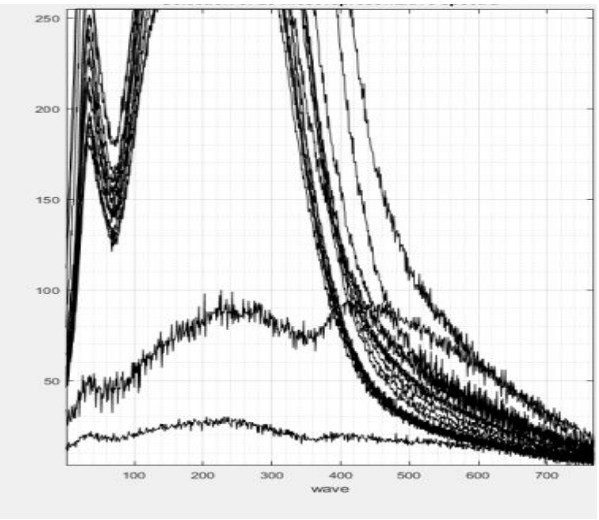

Figure 3 Selection of the 20 most representative spectra of grapes bunches

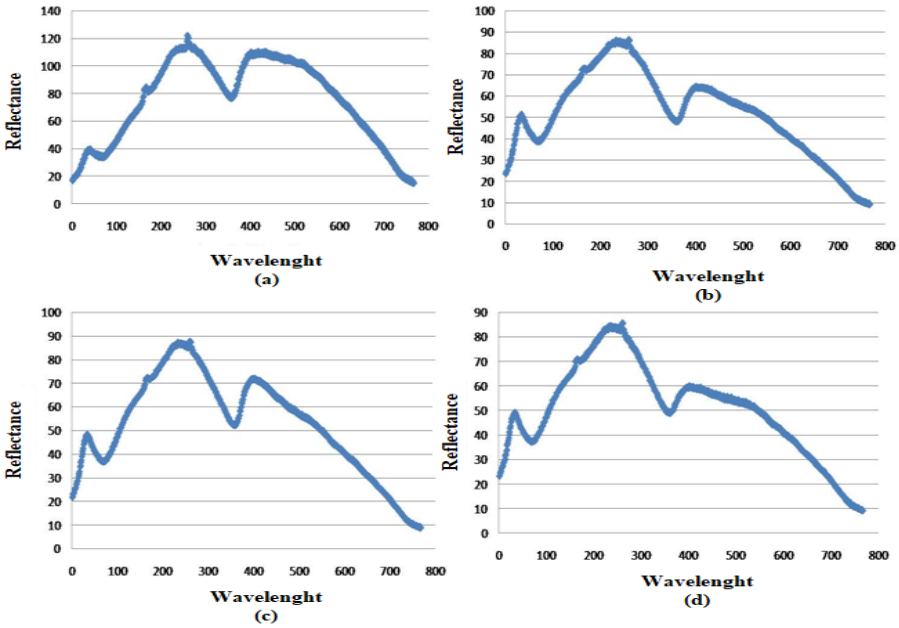

Figure 4 Hyperspectral signatures of the selected grape varieties (a) Cabernet Sauvignon, (b) Malbec, (c) Merlot, (d) Shiraz

3 Results and discussion

3.1 Hyperspectral imaging system

The images of the selected four varieties were acquired with a program developed in Matlab (Figure 2) and subsequently post-processed with HyperTools V3 (Figures 3 and 4). For this, the grapes bunches were placed on a conveyor belt at 30 cm away from the hyperspectral camera and the images were taken (Figure 2). From obtaining the possible spectral signatures, the most representative curves of the grapes bunch were selected by the program (Figure 3), and by averaging the representative curves, the hyperspectral signatures of the four grape varieties were obtained (Figure 4).

The hyperspectral signatures refer to the specific radiation profile emitted by the grapes varieties (Table 1). From these signatures, the area under the curve was calculated to ensure that a reliable result has been obtained and to avoid behaviors of the same curves. Among the studied species, the highest area under the curve was reported for cabernet sauvignon while the lowest value was reported for the Shiraz.

Table 1 Area under the curve of the hyperspectral signature

Grape Variety	Area under curve
Cabernet sauvignon	57,784.91
Malbec	39,195.16
Merlot	40,574.22
Shiraz	38,772.03

The adjustment of each of the hyperspectral signatures of the grape varieties was carried out, and the following models were developed for all four varieties:

3.1.1 Cabernet Sauvignon

$$\text{Reflectance}(X) = 57.92^{-(((X-271.8)/66.95))^8} + 271.8^{-(((X-397.8)/41.89))^7} + 0.5231^{-(((X-472.9)/3.112))^6} + 20.49^{-(((X-33.83)/30.25))^5} + 15.5^{-(((X-322.8)/28.1))^4} + 88.59^{-(((X-461.4)/113.5))^3} + 76.3^{-(((X-190)/124.2))^2} + 57.44^{-(((X-615.4)/126.6))} \quad (1)$$

The general fit model was Gaussian of order 8 with an error sum of squares of 1631, R squared of 0.9977, adjusted R squared of 0.9976 and root mean square error (RMSE) of 1.481.

3.1.2 Malbec

$$\text{Reflectance}(X) = 816^{-(((X-191.3)/91.59))^7} + 899.1^{-(((X-192.3)/95.64))^6} + 34.49^{-(((X-30.22)/30.52))^5} + 6.521^{-(((X-319.9)/31.65))^4} + 12.49^{-(((X-397.5)/28.97))^3} + 15.42^{-(((X-431.1)/63.14))^2} + 50.39^{-(((X-512.6)/196.2))} \quad (2)$$

The general fit model was Gaussian of order 7 with an error sum of squares of 591.7, R^2 was 0.998, adjusted R^2 was 0.9979 and RMSE was 0.89.

3.1.3 Merlot

$$\text{Reflectance}(X) = 2.608^{-(((X-259.4)/7.771))^8} + 6.721^{-(((X-233.2)/25.53))^7} + 18.39^{-(((X-326.8)/35.41))^6} + 20.41^{-(((X-140)/97.91))^5} + 19.20^{-(((X-360.8)/21.4))^4} + 80.03^{-(((X-301.2)/203.7))^3} + 32.58^{-(((X-577.1)/160.7))^2} + 26.41^{-(((X-31.29)/25.53))} \quad (3)$$

The general fit model was Gaussian of order 8 with an error sum of squares of 405.8, R^2 was 0.9988, adjusted R^2 was 0.9987 and RMSE was 0.7385.

3.1.4 Shiraz

$$\text{Reflectance}(X) = 5.997^{-(((X-242.6)/30.87))^5} + 73.03^{-(((X-220)/181.4))^4} + 15.99^{-(((X-348.3)/36.48))^3} + 22.58^{-(((X-31.45)/22.42))^2} + 47.99^{-(((X-520)/196.9))} \quad (4)$$

The general fit model was Gaussian of order 5 with an error sum of squares of 659.2, R squared of 0.9976, adjusted R squared of 0.9975, and RMSE of 0.9357.

3.2 Grape skin color parameters

The data obtained from the color analysis on the skin of freshly harvested four grapes varieties and the results based on the CIE L*a*b* and L*C*H* color spaces are presented in Table 2. Except for Malbec, rest three grape varieties have similar values. Suca Colana et al. (2019) established a relationship between the RGB and L*a*b models of grape skin color and this was based on the increased concentration of soluble solids and a regression with the total acidity parameter.

3.3 Total soluble solids

The total soluble solids, which is a measure of the potential alcohol content of a wine before it is manufactured was represented in Table 3. The highest °Bx (21.10) was reported for the Merlot while the lowest value was reported for the Cabernet Sauvignon (18.23). According to Perrot et al. (2015) established that the optimum measure of sugar content for the grape harvest is between 21 to 23 °Bx, from our results obtained, only the Merlot variety was at its optimum harvest point.

Table 3 Total soluble solids values of four grape varieties

Grape varieties	(°Brix)
Cabernet Sauvignon	18.23
Malbec	19.67
Merlot	21.10
Shiraz	20.62

3.4 Morphology, weight, and texture

The morphological parameters and weight of the grape are important factors in its commercial value, as well as an index of its

Table 2 Color parameters of four grape varieties

Grapes varieties	Grapes color				
	a	b	C	L	H
Cabernet Sauvignon	2.17	-1.13	2.63	31.26	331.57
Malbec	1.67	-1.37	2.23	31.87	324.12
Merlot	2.97	-1.30	3.33	29.17	338.95
Shiraz	2.52	0.48	3.02	29.33	319.07

quality (Table 4). Among the tested four grapes varieties, the Malbec variety had the highest grapes diameter and weight, rest three varieties have almost similar values. While in the case of puncture value, the highest value was reported for the Merlot, and this value was followed by Cabernet Sauvignon, Shiraz, and Malbec. The texture expressed as grape firmness is an attribute related to its quality and is a desirable characteristic for good storage (Table 5). Xu et al. (2022) suggested that grapes juice pH and fruit firmness are directly related to grape quality and price and these two could be effectively predicted using hyperspectral images.

3.5 Grape juice pH

The pH value is an important factor in the wine quality because it can influence the various factors of the wine, such as the level of oxidation, color, and flavor, among others (Table 6). Fernandes et al. (2015) establish that the average pH value of grape juice must be close to 3.6, therefore, the results of the current study are in agreement with the findings of Fernandes et al. (2015), and all four grapes varieties had values similar to the standard value. From these results, it can be concluded that selected grapes varieties are suitable for the optimum production of red wine.

3.6 Ripening index

The ripening index provides important information to determine the right time to harvest the grapes.

This index was calculated as the product of pH squared multiplied by the total soluble solids expressed in ° Brix (Table 7). All four varieties had ripening indexes in the range from 200 to 300 which was similar to the standard value proposed by Adsule (2014).

Conclusions

Results of the study suggested that the pH values of all four grape varieties are optimal at their harvest and suitable for the production of more oxidized wine with less color. The indexes of maturation and the soluble solids for the Merlot and Shiraz varieties are observed to have a direct relationship with their degree of maturity because in the field they were the varieties that were harvested first. The texture is a low-cost and fast application analytical technique that can be favorably applied to wine production, for grape monitoring. The fit models of the spectral signatures were of the Gaussian type of order 8, 7, 5 and their R^2 was greater than 0.99, which indicates that there is a fit of the curve to a known equation that can be related to the results obtained from the measurements of four grape varieties.

Table 4 Morphology and weight of the four grape varieties

Grapes varieties	Grapes diameters		Weight (g)
	Axis X (mm)	Axis Y (mm)	
Cabernet Sauvignon	11.35	11.13	0.92
Malbec	14.42	14.80	1.97
Merlot	12.40	12.50	1.25
Shiraz	13.47	13.40	1.46

Table 5 Puncture values for the four grape varieties

Grape varieties	Puncture (N)
Cabernet Sauvignon	4.08
Malbec	2.56
Merlot	4.54
Shiraz	2.62

Table 6 pH values of the four grape varieties

Grape varieties	pH
Cabernet Sauvignon	3.45
Malbec	3.43
Merlot	3.60
Shiraz	3.78

Table 7 Ripening index of the fourth grape varieties

Grape varieties	Ripening index
Cabernet Sauvignon	216.97
Malbec	230.70
Merlot	273.51
Shiraz	293.80

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References

Adsule, G. D. (2014). Manual of good agricultural practices for quality wine production. National Research Center for Grapes, New Delhí, India, Pp. 125.

Andrade, J. G. R., Moreno, O. C. M., Quiñones, R. V., & Martínez, J. A. V. (2011). Aproximaciones al turismo enológico y sus estrategias de mercadotecnia en México. *Gestión turística*, 16, 137-155.

Benelli, A., Cevoli, C., & Fabbri, A. (2020). In-field Vis/NIR hyperspectral imaging to measure soluble solids content of wine grape berries during ripening. In *2020 IEEE International Workshop on Metrology for Agriculture and Forestry (MetroAgriFor)* (pp. 99-103). IEEE.

Baiano, A., Terracone, C., Peri, G., & Romaniello, R. (2012). Application of hyperspectral imaging for prediction of physico-chemical and sensory characteristics of table grapes. *Computers and Electronics in Agriculture, 87*, 142-151.

Corona, O., Planeta, D., Bambina, P., Giacosa, S., et al. (2020). Influence of different dehydration levels on volatile profiles, phenolic contents and skin hardness of alkaline pre-treated grapes cv Muscat of alexandria (*Vitis vinifera* L.). *Foods*, *9*(5), 666.

Fernandes, A. M., Franco, C., Mendes-Ferreira, A., Mendes-Faia, A., da Costa, P. L., & Melo-Pinto, P. (2015). Brix, pH and anthocyanin content determination in whole Port wine grape berries by hyperspectral imaging and neural networks. *Computers and Electronics in Agriculture, 115*, 88-96.

Fernández, M. J., & Meraz Ruiz, L. (2022). Etiqueta como estrategia de compra. Vinos ganadores de concurso internacional. *RIVAR* (Santiago), *9*(25), 230-245.

Gattullo, C. E., Mezzapesa, G. N., Stellacci, A. M., Ferrara, G., et al. (2020). Cover crop for a sustainable viticulture: Effects on soil properties and table grape production. *Agronomy*, *10*(9), 1334.

Grajeda-Gonzalez, F., Contreras-Salazar, E. A., & Luna-Maldonado, A. I. (2015) Sistema de Procesamiento de Imágenes para Obtener los Parámetros del Color en Frutos de dos Variedades de Tomate. *Academia Journals*, Pp. 1156-116.

Grajeda-González, U. F. , Luna-Maldonado, A. I. , Rodriguez-Fuentes, H., Vidales-Contreras, J. A. , Contreras-Salazar, E. A. , & Flores-Breceda, H. (2018). Models Fitting to Pattern Recognition in Hyperspectral Images. In A. I. L. Maldonado, H. R. Fuentes, & J. A. V. Contreras (Eds.), Hyperspectral Imaging in Agriculture, Food and Environment. IntechOpen. https://doi.org/10.5772/intechopen.73159.

Gur, L., Reuveni, M., Cohen, Y., Cadle-Davidson, L., Kisselstein, B., Ovadia, S., & Frenkel, O. (2021). Population structure of *Erysiphe necator* on domesticated and wild vines in the Middle East raises questions on the origin of the grapevine powdery mildew pathogen. *Environmental Microbiology*, *23*(10), 6019-6037.

Hornedo-Ortega, R., González-Centeno, M. R. , Chira, K., Jourdes, M., & Teissedre, P. (2020). Phenolic Compounds of Grapes and Wines: Key Compounds and Implications in Sensory Perception. In F. Cosme, F. M. Nunes, & L. Filipe-Ribeiro (Eds.), Chemistry and Biochemistry of Winemaking, Wine Stabilization and Aging. IntechOpen. https://doi.org/10.5772/intechopen.93127.

Jia, B., Wang, W., Ni, X., Lawrence, K. C., Zhuang, H., Yoon, S. C., & Gao, Z. (2020). Essential processing methods of hyperspectral images of agricultural and food products. *Chemometrics and Intelligent Laboratory Systems*, *198*, 103936.

Lavadiya, D. N., Sajid, H. U., Yellavajjala, R. K., & Sun, X. (2022). Hyperspectral imaging for the elimination of visual ambiguity in corrosion detection and identification of corrosion sources. *Structural Health Monitoring*, *21*(4), 1678-1693..

Lumbreras, E. L. (2003). Sobre las formas naturalizadas de" *Vitis vinífera L.*" en la Comunidad Valenciana, I. Especies. *Flora Montiberica*, *23*, 46-82.

Melo-Pinto, P., Gomes, V., Fernandes, A., & Mendes-Ferreira, A. (2022). Wine grape ripeness assessment using Hyperspectral imaging. Retrieved from https://www.infowine.com/en/technical_articles/wine_grape_ripeness_assessment_using_hyperspectral_imaging_sc_19341.htm.

Moscovici, D., Gow, J., Ugaglia, A. A., Rezwanul, R., Valenzuela, L., & Mihailescu, R. (2022). Consumer preferences for organic wine-Global analysis of people and place. *Journal of Cleaner Production*, 133215.

Peppi, M. C., Fidelibus, M. W., & Dokoozlian, N. (2006). Abscisic Acid Application Timing and Concentration Affect Firmness, Pigmentation, and Color ofFlame Seedless' Grapes. *HortScience*, *41*(6), 1440-1445.

Perrot, N., Baudrit, C., Brousset, J. M., Abbal, P., et al. (2015). A decision support system coupling fuzzy logic and probabilistic graphical approaches for the agri-food industry: prediction of grape berry maturity. *PloS one*, *10*(7), e0134373.

Pisciotta, A., Planeta, D., Giacosa, S., Paissoni, M. A., Di Lorenzo, R., & Rolle, L. (2020). Quality of grapes grown inside paper bags in Mediterranean area. *Agronomy*, *10*(6), 792.

Ruso, J., Filipović, J., Maričić, M., & Spasojević-Brkić, V. (2021). Quality perception and willingness to pay: The case of red wine with health-beneficial effects. *Italian Journal of Food Science*, *33*(2), 1-12.

Scalisi, A., & O'Connell, M. G. (2021). Application of Visible/NIR spectroscopy for the estimation of soluble solids, dry matter and flesh firmness in stone fruits. *Journal of the Science of Food and Agriculture*, *101*(5), 2100-2107.

Shahab, M., Roberto, S. R., Ahmed, S., Colombo, R. C., Silvestre, J. P., Koyama, R., & de Souza, R. T. (2020). Relationship between

anthocyanins and skin color of table grapes treated with abscisic acid at different stages of berry ripening. *Scientia Horticulturae*, *259*, 108859.

Shaito, A., Posadino, A. M., Younes, N., Hasan, H., et al. (2020). Potential adverse effects of resveratrol: A literature review. *International Journal of Molecular Sciences*, *21*(6), 2084.

Suca-Colana, C., Vilca-Curo, R. & Cotacallapa-Sucapuca, M. (2019). Grape maturity (*Vitis vinifera*) negra criolla, moscatel and quebranta: Analysis of the berry color on the sugar content and total acidity. *Agroindustrial Science*, *9*(2), 109-113.

Sun, L., Li, S., Jiang, J., Tang, X., et al. (2020). New quantitative trait locus (QTLs) and candidate genes associated with the grape berry color trait identified based on a high-density genetic map. *BMC Plant Biology*, *20*(1), 1-13.

Wang, H., Yan, A., Sun, L., Zhang, G., Wang, X., Ren, J., & Xu, H. (2020). Novel stable QTLs identification for berry quality traits based on high-density genetic linkage map construction in table grape. *BMC plantbiology*, *20*(1), 1-15.

Xu, M., Sun, J., Yao, K., Cai, Q., Shen, J., Tian, Y., & Zhou, X. (2022). Developing deep learning based regression approaches for prediction of firmness and pH in Kyoho grape using Vis/NIR hyperspectral imaging. *Infrared Physics & Technology*, *120*, 104003.

Zeb, A. (2020). Concept, mechanism, and applications of phenolic antioxidants in foods. *Journal of Food Biochemistry*, *44*(9), e13394.

Zhang, C., Fan, X., Liu, C., & Fang, J. (2021). Anatomical berry characteristics during the development of grape berries with different shapes. *Horticultural Plant Journal*, *7*(4), 295-306..

Zouid, I., Siret, R., Mehinagic, E., Maury, C., Chevalier, M., & Jourjon, F. (2010). Evolution of grape berries during ripening: Investigations into the links between their mechanical properties and the extractability of their skin anthocyanins. *OENO One*, *44*(2), 87-99.

Zulkifli, N., Hashim, N., Harith, H. H., & Shukery, M. F. M. (2020). Finite element modelling for fruit stress analysis-A review. *Trends in Food Science & Technology*, *97*, 29-37.

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Growth and development of transgenic peanut (*Arachis hypogaea*) lines containing chitinase 42 kDa gene from *Trichoderma asperellum* SH16

Phung Thi Bich Hoa[1,2*], Hoang Lan Phuong[2], Nguyen Thi Trang[2], Nguyen Thi Thanh Tuyen[2], Huynh Kim Vu[2], Truong Thi Hieu Thao[1], Nguyen Hoang Tue[2], Nguyen Xuan Huy[3*]

[1]Faculty of Biology, University of Education, Hue University, 34 Le Loi, Hue 530000, Vietnam
[2]Faculty of Biology, University of Sciences, Hue University, 77 Nguyen Hue, Hue 530000, Vietnam
[3]Department of Science, Technology and International Relations, Hue University, 03 Le Loi, Hue 530000, Vietnam

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Yield

ABSTRACT

Peanut (*Arachis hypogaea* L.) is vulnerable to many diseases. Vietnam and other regions where peanut is widely cultivated have a high threat of fungal and other plant diseases. Various fungicides are available to control the fungal disease but these have various harmful effects on the natural flora, fauna, and environment. Transgenic peanut lines which possess antifungal activity provide a possible solution in managing fungal diseases apart from the traditional resistance and fungicide usage. Therefore, this study evaluated the probable growth and development of chitinase transgenic peanut lines against *Sclerotium rolfsii*, a pathogen that causes "southern blight" in plants, under greenhouse conditions. This study provided evidence that through *Agrobacterium itumefaciens* mediated transformation, 42 kDa chitinase genes from *Trichoderma asperellum*, which is under the regulation of 35S promoter, were successfully incorporated into the peanut's (*A. hypogaea* L.) genome and expressed in their plants. This evidence also demonstrated that transgenic peanut lines were suitable for growing and developing in the greenhouse. Further, it was reported that transgenic peanut lines took approximately 133 to 145 days from planting to maturity. These results also revealed that various growth characteristics of transgenic peanut lines having two synthetic genes (*syncod Chi42-2* i.e. S2-2, S2-4, S2-6, and *syncod Chi42-1* i.e. S1-1, S1-2, S1-3) were greater than that from the wild-type *Chi42* (WT-1, WT-2, and WT-3). In addition, yield-related parameters including the number of mature pods, 100 pods weight and 100 seeds weight for

* Corresponding author
E-mail: ptbhoa@hueuni.edu.vn (Phung Thi Bich Hoa);
nguyenxuanhuy@hueuni.edu.vn (Nguyen Xuan Huy)

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all the transgenic peanut lines were higher than that of the non-transformed plant. Among the transgenic lines, line S2-4 exhibited significantly higher growth and yield than the other transgenic lines. These results demonstrated that 42 kDa chitinase genes overexpressing peanut lines could be a candidate for improvement against plants to phytopathogenic fungus *S. rolfsii* and high yield.

1 Introduction

Peanut (*Arachis hypogaea* L.) is an annual leguminous plant that is cultivated in many countries around the world. In Vietnam, it is one of the most crucial oil seed crops with a total cultivating area of 177.043 hectares and a productivity of 0.44 million tons in 2019 (FAO 2020). Despite its values, peanut cultivation is hampered by many pathogens. The most harmful soil-borne pathogen of groundnut is root-and stem-rot caused by *Sclerotium rolfsii*. Further, S. *rolfsii* is difficult to control as a result of its wide variety of hosts (Javaid et al. 2021; Sharf et al. 2021) and persistent sclerotia (Kumar et al. 2012). This fungus is mostly associated with the stem and pod rot of peanuts and might cause 10 – 25% pod yield losses which sometimes reached up to 80% (Mehan et al. 1994). Currently, only a few resistant cultivars are commercially available (Branch and Brenneman 1999; 2009; Woodward et al. 2008). Control of stem rot disease mostly relies on cultural practices and fungicide treatment. However, cultural practices are not always effective due to the wide range of pathogens. Besides, fungicides are often too expensive for local groundnut farmers in Vietnam.

Nowadays, with the advancement of agricultural biotechnology, scientists are developing more and more new transgenic crop plants having desired qualities such as higher yield, resistance to insects, phytopathogenic fungi, and diseases. By using these technologies, the different origins derived chitinase genes have been successfully transformed into different types of plants such as rice (Lin et al. 1995), tobacco (Zhu et al. 1994), cucumber (Kishimotoiet al. 2002), Italian ryegrass I (Takahashi et al. 2005), banana (Sreeramanan et al. 2009), cotton (Ganesan et al. 2009), and peanut I (Chu et al. 2008, 2013) and developed the ability of fungal resistance in these crops. Though various attempts were made to enhance the fungal resistance in groundnut by utilizing tobacco chitinase (Rohini and Rao 2001), barley oxalate oxidase (Livingstone et al. 2005), mustard of defensin (Anuradha et al. 2008) and β-1,3-glucanase from tobacco (Sundaresha et al. 2009), there are currently no reports regarding the usage of 42 kDa chitinase genes from *Trichoderma asperellum* SH16, except for those published by Loc et al. (2022), Hoa et al. (2022a) and Tue et al. (2022). The antifungal activity of peanut-containing 42 kDa chitinase genes (Hoa et al. 2022b) and two genes *(syncod Chi42-1 and syncod Chi42-2)* were codons optimized for expression in the plant from the *Chi42* gene (Luong et al. 2021) were reported in these two types of research. Therefore, this study aimed to evaluate the growth and development rate under greenhouse conditions of the three previously mentioned transgenic peanut lines. The transgenic plants with an increase in chitinase activity could become a valuable source of biocontrol genes against plant-pathogenic fungi.

2 Materials and Methods

2.1 Plant Materials

Nine chitinase transgenic peanut lines were used as test materials in the present study. Peanut varieties having the plant expression vector pMYV719 harboring three genes (*Chi42*, *syncodChi42-1*, and *syncodChi42-2*) expressing i42 kDa chitinase were used in this study I (Loc et al. 2022).

2.2 Greenhouse experiments

Nine transgenic peanut lines containing *Chi42* (WT-1, WT-2, WT-3), *syncodChi42-1* (S1-1, S1-2, S1-3), and *syncodChi42-2* (S2-2, S2-4, S2-6) transgenic gene and one non-transgenic control (NC) were planted in greenhouse conditions at Institute of Bioactive Compounds and Department of Biotechnology, University of Sciences, Hue University, Hue, Vietnam. Tissue-cultured peanuts have enough stems, leaves, roots, and height (6 – 8 cm) and are grown under greenhouse conditions. Cultured plants were gently removed from the culture tubes and carefully washed in the medium using sterile distilled water. Transferred the rooted shoots to pots filled with a mixture of antisepticized - soil: sand: and vermiculite (1:1:1) and immediately enclosed in polythene bags to retain a high moisture content (85%) at 25°C in a growth cabinet with a 16-h photoperiod and 60 µE/m^2/s light intensity. Small holes were made in the plastic bags and left for 7 to 8 days for plant acclimatization. After 2 weeks, these plants were transferred to the 20 cm diameter pots containing autoclaved field soil (Better, HIEUGIANG Co., Ho Chi Minh, Vietnam) and shifted to the greenhouse with 26 to 30°C/20 to 25°C day/night temperatures and about a 10 to 12 h photoperiod for flowering and seed set. The plants were irrigated with nutrient solution (TANNONGPHAT Co., Ha Noi, Vietnam) once a month and gradually with fresh water whenever required.

2.3 Data collection growth, development, and productivity

2.3.1 Time of growth and development of transgenic peanut

Tissue-cultured peanuts have enough stems, leaves, and roots, plants with 6 – 8 cm stem height were used to grow under

greenhouse conditions. Peanut line growth was calculated from the planting in the greenhouse to the stage when 50% of the total plants have 5 mature leave, level 1 branches, and appear the first flower per stem. Further, the total duration of growth was calculated from planting to the stage when 80 – 85% of peanuts are ripe, the veins of the hull are prominent, the inside of the hull has turned dark and the leaves turn yellow.

Plant height was determined by a ruler from the plant's base to the top of the highest point. It was collected in 10 randomly chosen plants. Further, the number of leaves and number of branches were calculated at the end of the growth period.

2.3.2 Factors that constitute yield and yield components

The number of mature pods/plants was calculated by counting pods of ten sample plants of each plot (with three replications). Further, the weight (g) of one hundred pods and one hundred seeds was obtained by weighing a random sample of 100 pods and 100 seeds, respectively.

2.4 Statistical Analysis

All numerical data accumulated from this study were subjected to statistical analysis and significance tests. All data were subjected to statistical analysis using Duncan's test with SPSS (ver. 20.0) (IBM, Armonk, NY, USA). Differences reported as significant are at $p < 0.05$.

3 Results and Discussion

3.1 Time of growth and development of transgenic peanut

Transgenic peanuts after 18 to 20 days of planting in the greenhouse began to form real leaves (Table 1) while in the case of seed-grown plants, real leaves start appearing only after 15 days of seed sown. Transgenic peanut lines grow and develop well under greenhouse conditions. The time from planting to the first branch appearing is 27 to 30 days with chitinase transgenic peanut lines (Table 1) while in the case of non-transgenic peanuts growing from seed (NC-1), the first branch appeared 20 days after planting. Chitinase transgenic peanut lines were harvested in 140 – 144 days after planting in the greenhouse while this period was reported as only 122 days for NC-1. These results have shown that the transgenic peanut lines had a longer growth period compared to the peanuts grown from seed. This is comprehensive because changing from *in vitro* to *in-vivo* conditions requires more time to adapt plants in the soil. These results are in agreement with the findings of Minh and Hieu (2012) who reported a 125 to 140 days period between planting and harvesting in peanut cultivar L14.

3.2 Plant height

The results presented in Table 2 showed that plant height ranges from 9.3 to 10.6 cm when the plant reached to 5 leave stage after planting. In general, plant height among transgenic and non-transgenic plants did not show any statistical deviation at this stage. At a full-bloom stage, the plant height of *syncodChi42-2* transgenic peanut lines varied from 17.6 to 17.8 cm while in the case of *syncodChi42-1* and *Chi42* transgenic peanut lines, it varied from 16.8 to 17.4 cm, 15.8 to 15.9 cm, respectively. Plant height continuously increased until the plant reaches the end of flowering. At this stage, plant height ranges from 20.8 to 23.0 cm for *syncodChi42-2* transgenic peanut lines and 20.5 to 21.8 cm, 20.4 to 20.8 cm, and 17.9 cm for *syncodChi42-1*, *Chi42*, NC, respectively. Among the tested transgenic peanut lines, the highest plant height of 30.0 cm was reported for the S2-4 line (Table 2). From the results of the current study, it can be concluded that the plant

Table 1 Time of growth and development of chitinase transgenic peanut lines

Gene	Transgenic peanut lines	Growth and development (days)				
		5 leaves	Level 1 branches	Beginning of Flowering	End of flowers	Harvest
syncodChi42-2	S2-2	18	27	57	77	142
	S2-4	18	27	59	77	140
	S2-6	19	27	59	75	140
syncodChi42-1	S1-1	19	30	59	75	144
	S1-2	20	29	57	77	142
	S1-3	20	30	57	77	142
Chi42	WT-1	18	30	59	77	144
	WT-2	19	30	59	77	142
	WT-3	18	30	58	77	144
NC	NC-1	15	20	43	64	122
	NC-2	20	30	58	77	145

Note: NC: non-transgenic peanut from *in vitro*.

Table 2 Plant height of transgenic peanut lines (cm) during the study period

Gene	Transgenic peanut lines	Plant height (cm) at different time after planting			
		5 leaves	Full-bloom	End of flowers	Harvest
syncodChi42-2	S2-2	$10.6^{a} \pm 0.42$	$17.8^{a} \pm 1.30$	$20.8^{b} \pm 1.92$	$29.3^{ab} \pm 0.97$
	S2-4	$9.8^{ab} \pm 0.84$	$17.8^{a} \pm 1.30$	$23.0^{a} \pm 1.87$	$30.0^{a} \pm 1.60$
	S2-6	$10.6^{a} \pm 0.89$	$17.6^{a} \pm 1.52$	$23.0^{a} \pm 1.73$	$29.8^{a} \pm 1.27$
syncodChi42-1	S1-1	$10.6^{a} \pm 0.55$	$17.4^{ab} \pm 1.34$	$20.6^{b} \pm 0.82$	$28.1b^{c} \pm 0.74$
	S1-2	$9.9^{ab} \pm 0.65$	$16.8^{ab} \pm 0.84$	$21.8^{ab} \pm 1.15$	$27.9^{bc} \pm 1.78$
	S1-3	$9.3^{b} \pm 0.57$	$17.4^{ab} \pm 1.52$	$20.5^{b} \pm 0.87$	$29.0^{ab} \pm 0.94$
Chi42	WT-1	$9.7^{ab} \pm 0.67$	$15.9^{b} \pm 1.02$	$20.6^{b} \pm 1.82$	$26.1^{d} \pm 1.34$
	WT-2	$10.0^{ab} \pm 1.22$	$15.9^{b} \pm 1.24$	$20.4^{b} \pm 1.52$	$26.0^{d} \pm 0.71$
	WT-3	$9.6^{ab} \pm 0.89$	$15.8^{b} \pm 1.10$	$20.8^{b} \pm 1.92$	$26.8^{cd} \pm 1.15$
NC		$10.1^{ab} \pm 0.55$	$14.2^{c} \pm 0.45$	$17.9^{c} \pm 1.24$	$23.3^{e} \pm 1.48$

Here a-e Means with different superscripts in the same column that followed the mean and standard deviation are significantly different ($p < 0.05$), NC: non-transgenic peanut from *in vitro*.

Table 3 Number of leaves per plant during the study period

Gene	Transgenic peanut lines	Number of leaves per plant at different times after planting				
		Flowering	Full-bloom	End of flowering	Harvest	Number of green leaves at harvest
syncodChi42-2	S2-2	$12.4^{a} \pm 0.55$	$15.2^{ab} \pm 0.45$	$17.2^{bc} \pm 0.45$	$21.2^{a} \pm 0.45$	$4.4^{a} \pm 0.55$
	S2-4	$12.8^{a} \pm 0.45$	$16.0^{a} \pm 0.45$	$18.4^{a} \pm 0.45$	$21.8^{a} \pm 0.45$	$4.4^{a} \pm 0.55$
	S2-6	$12.6^{a} \pm 0.55$	$15.6^{a} \pm 0.55$	$17.6^{b} \pm 0.55$	$21.6^{a} \pm 0.55$	$4.4^{a} \pm 0.55$
syncodChi42-1	S1-1	$12.4^{a} \pm 0.55$	$15.4^{a} \pm 0.55$	$17.4^{b} \pm 0.55$	$21.4^{a} \pm 0.55$	$4.2^{a} \pm 0.45$
	S1-2	$12.4^{a} \pm 0.55$	$15.4^{a} \pm 0.55$	$17.4^{b} \pm 0.55$	$21.4^{a} \pm 0.55$	$4.2^{a} \pm 0.45$
	S1-3	$12.4^{a} \pm 0.55$	$15.6^{a} \pm 0.55$	$17.6^{ab} \pm 0.55$	$21.6^{a} \pm 0.55$	$4.2^{a} \pm 0.45$
Chi42	WT-1	$11.0^{b} \pm 1.00$	$14.4^{b} \pm 0.89$	$16.4^{d} \pm 0.55$	$20.0^{ab} \pm 1.00$	$4.2^{a} \pm 0.45$
	WT-2	$10.8^{b} \pm 0.84$	$14.6^{b} \pm 0.89$	$16.4^{d} \pm 0.55$	$20.0^{ab} \pm 0.71$	$4.2^{a} \pm 0.45$
	WT-3	$11.2^{b} \pm 0.84$	$14.6^{b} \pm 0.55$	$16.4^{d} \pm 0.55$	$20.2^{b} \pm 0.84$	$4.2^{a} \pm 0.45$
NC		$9.8^{c} \pm 0.84$	$13.0^{c} \pm 1.00$	$15.6^{d} \pm 1.14$	$19.2^{c} \pm 0.84$	$4.0^{a} \pm 0.55$

Here a-d Means with different superscripts in the same column that followed the mean and standard deviation are different ($p < 0.05$), NC: non-transgenic peanut from *in vitro.*

height of chitinase transgenic peanut lines was higher than the non-transgenic peanut at different times after planting, and these differences were statistically significant ($p<0.05$). L14 is a peanut with a balanced shape, strong growth, and larger plant height, which will affect flowering and pod formation. Therefore, the plant height of peanuts will create a premise for flowering, better pod formation, and higher peanut yield corresponding to transgenic peanut lines. Improved plant height of transgenic cotton plants were also reported by Bashir et al. (2022), who used the barley chitinase I and chitinase II gene to create resistance against fungi.

3.3 Number of leaves per plant

A perusal of the data presented in Table 3 revealed that different transgenic peanut lines (*Chi42, syncodChi42-1*, and *syncodChi42-2)* showed a significant effect on the number of leaves per plant. Overall, the experimental result showed that the number of leaves was found higher in the S2A-12 line at all growth stages starting from beginning to the flowers production (12.8 leaves) and harvesting (21.8 leaves), and these differences are statistically significant ($p < 0.05$) as compared to the non-transgenic peanut lines. The number of leaves increased during the pod development phase and decreased during harvest. Leaves are the essential source from which the photosynthates are channeled to the sink. During the pod development phase, leaves provide nutrition to pods and a higher number of leaves contribute to higher pod yield. According to Yang et al. (2020) research, transgenic soybean plants weren't witnessing any detrimental impacts on growth and development by the overexpression of the chitinase gene *CmCH1*. Similarly, Zaynab et al. (2017) informed that transgenic potatoes expressing the rice chitinase gene had a higher number of leaves per plant than non-transformed plants.

Table 4 Number of branches per plant during the study period

Gene	Transgenic peanut lines	Number of level 1 branches/ plant		Total number of branches/plant	
		Full-bloom	Harvest	Full- bloom	Harvest
syncodChi42-2	S2-2	$3.4^{ab} \pm 0.55$	$4.6^{a} \pm 0.55$	$4.8^{ab} \pm 0.45$	$6.4^{a} \pm 0.55$
	S2-4	$3.6^{a} \pm 0.55$	$4.8^{a} \pm 0.45$	$5.0^{a} \pm 0.00$	$6.4^{a} \pm 0.55$
	S2-6	$3.4^{ab} \pm 0.55$	$4.8^{a} \pm 0.45$	$4.8^{ab} \pm 0.45$	$6.4^{a} \pm 0.55$
syncodChi42-1	S1-1	$3.4^{ab} \pm 0.55$	$4.6^{a} \pm 0.55$	$4.8^{ab} \pm 0.45$	$6.0^{ab} \pm 0.00$
	S1-2	$3.6^{a} \pm 0.55$	$4.4^{a} \pm 0.55$	$5.0^{a} \pm 0.00$	$6.2^{ab} \pm 0.45$
	S1-3	$3.2^{ab} \pm 0.45$	$4.4^{a} \pm 0.55$	$4.8^{ab} \pm 0.45$	$6.4^{a} \pm 0.55$
Chi42	WT-1	$3.2^{ab} \pm 0.45$	$4.4^{a} \pm 0.55$	$4.8^{ab} \pm 0.45$	$6.0^{ab} \pm 0.45$
	WT-2	$3.2^{ab} \pm 0.45$	$4.4^{a} \pm 0.55$	$5.0^{a} \pm 0.45$	$6.2^{ab} \pm 0.00$
	WT-3	$3.6^{a} \pm 0.55$	$4.6^{a} \pm 0.55$	$4.8^{ab} \pm 0.00$	$6.2^{ab} \pm 0.45$
NC		$2.8^{b} \pm 0.45$	$3.4^{b} \pm 0.55$	$4.4^{b} \pm 0.55$	$5.6^{b} \pm 0.55$

Here a-b Means with different superscripts in the same column that followed the mean and standard deviation are different ($p < 0.05$), NC: non-transgenic peanut from *in vitro.*

Table 5 Yield parameters of transgenic peanut lines

Gene	Transgenic peanut lines	Yield parameters of transgenic peanut lines		
		Number of mature pods/plant	100 pods weight i(g)	100 seeds weight (g)
syncodChi42-2	S2-2	$8.6^{a} \pm 0.55$	$114.84^{ab} \pm 1.21$	$35.22^{ab} \pm 0.61$
	S2-4	$9.0^{a} \pm 0.71$	$115.48^{a} \pm 0.58$	$36.22^{ab} \pm 0.93$
	S2-6	$8.4^{ab} \pm 0.55$	$115.32^{a} \pm 0.58$	$35.62^{ab} \pm 1.32$
syncodChi42-1	S1-1	$8.4^{ab} \pm 0.55$	$112.58^{ab} \pm 2.38$	$33.66^{c} \pm 0.88$
	S1-2	$8.4^{ab} \pm 0.55$	$114.88^{ab} \pm 1.29$	$34.54^{bc} \pm 1.05$
	S1-3	$8.6^{a} \pm 0.55$	$114.70^{ab} \pm 0.78$	$34.54^{bc} \pm 0.77$
Chi42	WT-1	$8.6^{a} \pm 0.89$	$111.86^{b} \pm 2.64$	$33.06^{d} \pm 0.96$
	WT-2	$8.2^{ab} \pm 0.84$	$111.86^{b} \pm 2.42$	$33.10^{d} \pm 0.51$
	WT-3	$8.4^{ab} \pm 0.55$	$111.68^{b} \pm 2.07$	$32.28^{d} \pm 0.44$
NC		$7.6^{b} \pm 0.55$	$107.26^{c} \pm 4.81$	$31.76^{e} \pm 1.01$

Here a-e Means with different superscripts in the same column that followed the mean and standard deviation are different ($p < 0.05$). NC: non-transgenic peanut from *in vitro.*

3.4 Number of branches per plant

The number of level 1 branches and the total number of branches per plant at different chitinase transgenic peanut lines varied from 3.2 to 3.6 and 4.8 to 5.0, respectively for the full-bloom stage (Table 4). There weren't any visible changes in the total number of branches per plant in transgenic peanut lines compared to the untransformed control at full bloom and harvest, except for line S2-4 (Table 4). At the maturity stage, in comparison with the control, the number of level 1 branches and the total number of branches/plant of all transgenic lines were remarkably increased, particularly for *syncodChi42-2* genes associated with S2-4 (Table 4). According to Dapaah et al. (2014) number of branching in peanuts may positively impact the final yield. Similarly, Cuong et al. (2019) studied the impact of level 1 branches in cultivar L14 and found a significant association with the final yield, and in this manner findings of this study are in agreement with the findings of these studies.

3.5 Yield parameters of transgenic peanut lines

Differences in yield parameters among transgenic and non-transgenic test lines are shown in Table 5. Regarding the number of mature pods per plant, all transgenic peanut lines had a significantly higher number of pods as compared to the non-transgenic plants (Table 5). In the case of 100 pods and 100 seeds weight, all chitinase transgene peanut lines have significantly higher averaged pod and seed mass as compared to the non-transgenic. Among the various tested lines, *syncodChi42*-2 (S2-2, S2-4, and S2-6) transgenic peanut lines have a higher number of

mature pods per plant (10.5 – 18.4%) as compared to the non-transgenic cultivar (Table 5). Further, *syncodChi42-1* and *Chi42* transgenic peanut lines showed an enhanced number of mature pods per plant (7.9 – 13.2%) compared with non-transgenic cultivars. Further, the S2-4 line showed significantly higher yield parameters as compared to the other transgenic lines (Table 5).

According to Nagpure et al. (2014), a crop's productivity is diminished by the effects of chitinase in improving the crop's defense mechanisms against several types of stresses. Jeong et al. (2013) created OsNACS transgenic rice plants with the control of RCc3 and GOS2 promoters. Crop yields under normal conditions increased from 9% to 26%. In addition, the investigation also mentioned that RCc3:OsNAC5 plants had a larger grain yield of 22 – 63% under water deficiency conditions.

Growth and yield characteristics including plant height, number of leaves/plant, number of branches/plant, number of mature pods/plant, pod and seed weights of the peanut lines were evaluated under greenhouse conditions and found statistically comparable between the transformed and non-transformed plant lines. Similar types of results had been previously recorded under both greenhouse and field conditions with other transgenic crops (Arnoldo et al. 1992; Chenault et al. 2006).

Conclusion

In this study, the total of nine transgenic peanut lines including three wild-type (*Chi42* i.e. WT-1, WT-2, WTA-3) and six synthetic gene lines (*syncodChi42-1* i.e. S1-1, S1-2, S1-3 and *syncodChi42-2* i.e. S2-2, S2-4, S2-6) were tested. The results of the study suggested that all transgenic lines did not show any major changes in the growth and development characteristics in greenhouse conditions. Peanut lines expressing chitinase 42 kDa showed significantly increased various yield parameters. This study provides a reasonable approach for the genetic improvement of peanuts to enhance resistance to the pathogen fungus *S. rolfsii*. The promising transgenic peanut lines identified in this study can be exploited as stable fungal disease-resistant peanut lines in the future for other plant breeding programs.

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Conflicts of interest

All authors declare that they have no conflicts of interest.

References

Anuradha, T.S., Divya, K., Jami, S.K., & Kirti, P.B. (2008). Transgenic tobacco and peanut plants expressing a mustard defensin show resistance to fungal pathogens. *Plant Cell Reports, 27*(11), 1777-1786. DOI: https://doi.org/10.1007/s00299-008-0596-8.

Arnoldo, M., Baszczynski, C.L., Bellemare, G., Brown, G., et al. (1992). Evaluation of transgenic canola plants under field conditions. *Genome, 35*(1), 58-63. DOI: https://doi.org/10.1139/g92-010.

Bashir, S., Yaqoob, A., Bashir, R., Bukhari, S., et al. (2022). Barley Chitinase Genes Expression Revamp Resistance Against Whitefly (*Bemisia tabaci*) in Transgenic Cotton Plants. *Research Square preprints*. DOI: https://doi.org/10.21203/rs.3.rs-1240481/v1.

Branch, W.D., & Brenneman, T.B. (1999). Stem rot disease evaluation of mass-selected peanut populations. *Crop Protection, 18*, 127-130. DOI: https://doi.org/10.1016/S0261-2194(98)00103-3.

Branch, W.D., & Brenneman, T.B. (2009). Field evaluation for the combination of white mould and tomato spotted wilt disease resistance among peanut genotypes. *Crop Protection, 28*, 595-598. DOI: https://doi.org/10.1016/j.cropro.2009.03.008.

Chenault, K.D., Melouk, H.A., & Payton, M.E. (2006). Effect of anti-fungal transgene (s) on agronomic traits of transgenic peanut lines grown under field conditions. *Peanut Science*, *33*(1), 12-19. DOI: https://doi.org/10.3146/0095-3679(2006)33[12:EOATOA]2.0.CO;2.

Chu, Y., Deng, X.Y., Faustinelli, P., & Ozias-Akins, P. (2008). Bcl-xL transformed peanut (*Arachis hypogaea* L.) exhibits paraquat tolerance. *Plant Cell Reports*, *27(1)*, 85-92. DOI: https://doi.org/10.1007/s00299-007-0444-2.

Chu, Y., Bhattacharya, A., Wu, C., Knoll, J.E., & Ozias-Akins, P. (2013). Improvement of peanut (*Arachis hypogaea* L.) transformation efciency and determination of transgene copy number by relative uantitative real-time PCR. *In Vitro Cellular & Developmental Biology-Plant*, *49*(3), 266-275. DOI: https://doi.org/10.1007/s11627-013-9518-8.

Cuong, N.L., Toan, H.K., Vu, N.X., & Huyen, T.T. (2019). Growth promotion and yield enhancement efficiency by *Bacillus* strains for groundnut under field condition in Thua Thien Hue province. *Hue University Journal of Science: Agriculture and Rural Development*, *128(3C)*, 13-22. DOI: https://doi.org/10.26459/hueuni-jard.v128i3C.5229.

Dapaah, H.K., Mohammed, I., & Awuah, R.T. (2014). Growth yield performance of groundnuts (*Arachis hypogaea* L.) in

response to plant density. *International Journal of Plant and Soil Science*, *3(9)*, 1069-1082.

FAOSTAT. (2020). Production Indices. Retrieved from https://www.fao.org/faostat/en/?#data/QI (accessed on May 13, 2022).

Ganesan, M., Bhanumathi, P., Ganesh Kumar, K., Lakshmi Prabha, A., Pill-Soon, S., & Jayabalan, N. (2009). Transgenic Indian cotton (*Gossypium hirutum*) harboring rice chitinase gene (*Chi* II) confers resistance to two pathogens. *American Journal of Biochemistry and Biotechnology*, *5(2)*, 63-74.

Hoa P.T.B., Tue N.H., Huyen, L.T.T., Linh, L.H., Nhan, N.T., et al. (2022a). Over expression of 42 kDa Chitinase Gene from *Trichoderma asperellum* SH16 in peanut (*Arachis hypogaea*). *Journal of Crop Improvement*, DOI: https://doi.org/10.1080/15427528.2022.2110346.

Hoa, P.T.B., Tue, N.H., Huyen, L.T.T., Linh, L.H., et al. (2022b). Heterologous expression of genes encoding chitinase 42 kDa from *Trichoderma asperellum* in *Arachis hypogaea* through *Agrobacterium tumefaciens*-mediated transformation. *Research Square*. DOI: https://doi.org/10.21203/rs.3.rs-1487302/v1.

Javaid, A., Ali, A., Shoaib, A., & Khan, I. H. (2021). Alleviating stress of *Sclertium rolfsii* on growth of chickpea var. *Bhakkar-2011 by Trichoderma harzianum and T. viride. The Journal of Animal and Plant Sciences*, *31*(6), 1755-1761. DOI: 10.36899/JAPS.2021.6.0378.

Jeong, J.S., Kim, Y.S., Redillas, M.C., Jang, G., et al. (2013). *OsNAC5* over expression enlarges root diameter in rice plants leading to enhanced drought tolerance and increased grain yield in the field. *Plant Biotechnology Journal*, *11(1)*, 101-114. DOI: https://doi.org/10.1111/pbi.12011.

Kishimoto, K., Nishizawa, Y., Tabei, Y., Hibi, T., Nakajima, A., & Akutsu, K. (2002). Detailed analysis of rice chitinase gene expression in transgenic cucumber plants showing diferent levels of disease resistance to graymold (*Botrytis cinerea*). *Plant Science*, *162*, 655-662. DOI: https://doi.org/10.1016/S0168-9452(01)00602-1.

Kumar, D.P., Anupama, P.D., Singh, R.K., Thenmozhi, R., Nagasathya, A., Thajuddin, N., & Paneerselvam, A. (2012). Evaluation of extracellular lytic enzymes from indigenous *Bacillus* isolates. *International Research Journal of Microbiology*, *3(2)*, 060-065.

Lin, W., Anuratha, C.S., Datta, K., Potrykus, I., Muthukrishnan, S., & Datta, S.K. (1995). Genetic engineering of rice for resistance to sheath blight. *Nature Biotechnology*, *13*, 686-691. DOI: https://doi.org/10.1038/nbt0795-686.

Livingstone, D.M., Hampton, J.L., Phipps, P.M., & Grabau, E.A. (2005). Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barely oxalate gene. *Plant Physiology*, *137(4)*, 1354-1362. DOI: https://doi.org/10.1104/pp.104.057232.

Loc, N.H., Quang, H.T., Hung, N.B., Huy, N.D., Phuong, T.T.B., & Ha, T.T.T. (2011). *Trichoderma asperellumChi42* genes encode chitinase. *Mycobiology*, *39(3)*, 182-186. DOI: https://dx.doi.org/10.5941%2FMYCO.2011.39.3.182.

Luong, N.N., Tien, N.Q.D., Huy, N.X., Tue, N.H., et al. (2021). Expression of 42 kDa chitinase of *Trichoderma asperellum* (Ta-CHI42) from a synthetic gene in *Escherichia coli*. *FEMS Microbiology Letters*, *368*(16), DOI: fnab110.https://doi.org/10.1093/femsle/fnab110.

Mehan, V.K., Mayee, C.D., & McDonald, D. (1994). Management of *Sclerotium rolfsii*-caused stem and pod rots of groundnut-a critical review. *International Journal of Pest Management*, *40*, 313-320. DOI: https://doi.org/10.1080/09670879409371906.

Minh, H.K., & Hieu, N.M. (2012). Study on the effect of sowing time on growth and yield of peanut cultivar L14 in Winter-Spring crop on sandy soil in Quang Binh province. *Science and Technology Journal of Agriculture & Rural Development*, *10*, 12-20.

Nagpure, A., Choudhary, B., & Gupta, R.K. (2014). Chitinases: in agriculture and human healthcare. *Critical Reviews in Biotechnology*, *34*(3), 215-232. DOI: https://doi.org/10.3109/07388551.2013.790874.

Rohini, V.K., & Rao, K.S. (2001). Transformation of peanut (*Arachis hypogaea* L.) with tobacco chitinase gene: variable response of transformants to leaf spot disease. *Plant Science*, *160*, 889-898. DOI: https://doi.org/10.1016/S0168-9452(00)00462-3.

Sharf, W., Javaid, A., Shoaib, A., & Khan, I. H. (2021). Induction of resistance in chili against *Sclerotium rolfsii* by plant-growth-promoting rhizobacteria and *Anagallis arvensis*. *Egyptian Journal of Biological Pest Control*, *31*(1), 1-11. DOI: https://doi.org/10.1186/s41938-021-00364-y.

Sreeramanan, S., Maziah, M., & Xavier, R. (2009). A protocol for *Agrobacterium*-mediated transformation of banana with a rice chitinase gene. *Emirates Journal of Food and Agriculture*, *21*, 18-33. DOI: https://doi.org/10.9755/ejfa.v21i2.5161.

Sundaresha, S., Manoj Kumar, A., Rohini, S., Math, S.A., Keshsamma, E., Chadrashekar, S.C., & Udayakumar, M. (2009). Enhanced protection against two major fungal pathogens of groundnut, *Cercospora arachidicola* and *Aspergillus flavus* in transgenic groundnut over-expressing a tobacco β 1–3 glucanase.

European Journal of Plant Pathology, *126(4)*, 497-508. DOI: https://doi.org/10.1007/s10658-009-9556-6.

Takahashi, W., Fujimori, M., Miura, Y., Komatsu, T., Nishizawa, Y., Hibi, T., & Takamizo, T. (2005). Increased resistance to crown rust disease in transgenic Italian ryegrass (*Lolium multiforum* Lam) expressing the rice chitinase gene. *Plant Cell Reports*, *23*, 811-818. DOI: https://doi.org/10.1007/s00299-004-0900-1.

Tue, N. H., Tuong, T.G.C., Trang, P.T.H., Chung, N.D., Hoa, P.T.B., Tien, N.Q.D., & Loc, N.H. (2022). Cloning the root-specific Asy promoter and genes encoding chitinase 42 kDa of *Trichoderma asperellum* into the plant expression vector. *Journal of Applied Biology and Biotechnology*, *10*(3), 7-1. DOI: 10.7324/JABB.2022.100302.

Woodward, J.E., Brenneman, T.B., Kemerait, R.C., Smith, N.B., Culbreath, A.K., & Stevenson, K.L. (2008). Use of resistant cultivars and reduced fungicide programs to manage peanut diseases in irrigated and nonirrigated fields. *Plant Disease*, *92*, 896-902. DOI: https://doi.org/10.1094/PDIS-92-6-0896.

Yang, X., Yang, J., Li, H., Niu, L., et al. (2020). Overexpression of the chitinase gene *CmCH1* from *Coniothyrium minitans* renders enhanced resistance to *Sclerotinia sclerotiorum* in soybean. *Transgenic Research*, *29(2)*, 187-198. DOI: https://doi.org/10.1007/s11248-020-00190-2.

Zaynab, M., Kanwal, S., Hussain, I., Qasim, M., et al. (2017). Rice chitinase gene expression in genetically engineered potato confers resistance against *Fusarium solani* and *Rhizictonia solani*. *PSM Microbiol*, *2(3)*, 63-73.

Zhu, Q., Maher, E.A., Masoud, S., Dixon, R.A., & Lamb, C.J. (1994). Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. *Nature Biotechnology*, *12*, 807-812. DOI: https://doi.org/10.1038/nbt0894-807.

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Molecular identification of scale insect (*Eulecanium giganteum*) in *Hibiscus rosa-sinensis*

Suganthi M[1], Logeshwaran R[1], Abirami G[1], Rupa Shree B[1], Anandaraj P[1], Senthilkumar P[2*]

[1]Department of Biotechnology, Vels Institute of Science, Technology & Advanced Studies (VISTAS), Pallavaram 600117, TamilNadu, India
[2]Department of Genetic Engineering, SRM Institute of Science and Technology, Kattankulathur 603203, Chennai, TamilNadu, India

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Sequencing

ABSTRACT

Hibiscus rosa-sinensis is a widely grown evergreen valuable medicinal, ornamental species planted in India. Scale insects are small herbivorous insects found on all continents and they are serious sap sucking pests of many ornamental plants. These scale insects are undetectable due to their tiny size, basic morphology, and polyphagous feeding nature. Hence, the management of these tiny insects become a serious concern across the globe. To afford a prospective solution to the problem, an accurate, simple, and developmental-stage-independent identification method is required, hence this study attempted the molecular identification of scale insect in *Hibiscus rosa-sinensis* using mitochondrial gene Cytochrome Oxidase Subunit I (mtCOI) sequencing. The experiment was carried out by isolating insect DNA using a modified CTAB method. Through two or three rounds of error-prone PCR followed by a steady procedure to amplify a mtCOI region. This region of mtCOI has been used as a standard DNA barcode for a diverse array of taxa. The confirmation has been done by sequencing of mtCOI which suggest the highest similarities with *Eulecanium giganteum*. This study addresses the questions of biodiversity and molecular characterization of scale insects. Further, the information obtained in this study provides baseline data for future crop improvement programs and integrated pest management strategies.

* Corresponding author
E-mail: mpsenthilkumar@gmail.com (Senthilkumar Palanisamy)

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1 Introduction

Soft scales (Hemiptera: Coccoidea: Coccidae) are the most invasive group of pests and the largest family of scale insects in India, causing a notable loss in fruiting and ornamental crops. The species come under the genus Eulecanium and these are important economic pests distributed worldwide (Ben-Dov and Hodgson 1997). Usually, scale insects feed on the plant sap and excrete a sticky substance and induce sooty mold growth which causes an enormous economic loss in woody ornamentals and fruit trees (Li et al. 2002; Xie et al. 2006). Efficient management of these scale insects are limited due to the small size and inefficacy in morphological identification. Species-level identification of scale insects is often difficult and challenging due to their tiny size, reduced morphology, and high similarity in their immature stages (Watson and Kubiriba 2005). Recent studies reported that pine scales excrete non-metabolized insecticides in honeydew which has the potential to negatively affect organisms that feed on tainted honeydew such as predators, parasitoids, and pollinators (Quesada et al. 2020). *H. rosa-sinensis* known colloquially as china rose belongs to the family Malvaceae with important medicinal properties for treating diabetes, inflammation, wounds, cough, and fever, and also reducing the infections caused by bacteria and fungi (Kanthesh and Geethanjali 2021). Further, it is mainly used to prevent hair loss and gastric ulcers in several tropical countries (Missoum 2018). Due to their soothing properties, the flowers and leaves of Hibiscus have been traditionally used to treat medical conditions *viz*., gall bladder damage, cancer, to relieve dry coughs, lower blood pressure, and also to treat skin afflictions (Shashi et al. 2013).

A severe infestation of scale insects was observed in the stems of the Hibiscus plants. Scale insects vary dramatically in appearance, from very small organisms (1-2 mm) that grow beneath a wax cover of the stem to shiny pearl-like objects (about 5mm), covered with mealy wax. Usually, adult females are always immobile and attached to the stem. They secrete a waxy coating for defense, making them resemble fish scales, and so-called scale insects (Xie et al. 2006). Scale insects mainly damage the plant by sucking sap from leaves, stems, and trunks, further severely colonizing the stem and destroy which leads to the falling of leaves and stunted growth. Stems of hibiscus plants were found to be widely affected by scale insects (Chua 1997). However, there is little information on the molecular identification and species composition of scale insects infesting hibiscus in India. Generalizing the life cycle and biology of soft scales seems to be difficult since variations exist even among the subspecies.

DNA barcoding is a recent taxonomic supporting tool that uses a short gene as a marker in an organism's DNA to identify a particular species (Javal et al. 2021). DNA barcoding has several advantages over morphological identification-based taxonomy, being a relatively quick and easy identification process that uses small tissue samples and is not limited by developmental stages or gender. Insect mitochondrial DNA (mtDNA) is a small circular molecule (~16 kb in size) and has a fast mutation rate, which results in notable variation in mtDNA sequences between insect species and little variation within insect species (Wilson et al. 1985). A fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) is a conserved region and is considered a promising barcode in animals (Rodrigues et al. 2017). Molecular characterization was done using isolating the genomic DNA from the scale insect and amplifying the partial mitochondrial gene called COI . It was observed from several studies that the COI region (~710bp) can be used as a DNA barcoding tool for insect species identification. This region has provided accurate species identification even between the subspecies. The cytochrome c oxidase1 COI is used for species identification because of its high mutation speed and the highly conserved sequence of the species through which species can be easily identified even between the subspecies (Hebert et al. 2003).

In this study, a single insect was taken for insect DNA isolation. The protocol of DNA extraction is time-saving as well as economic with available laboratory chemicals, consumables, and basic equipment. Similar to the plant DNA extraction procedure, insect DNA extraction relies on a nonionic detergent like CTAB (Cetyl Trimethyl Ammonium Bromide) to lyse the insect cuticle (Saghai-Maroof et al. 1984). The amplification of the full-length sequence of the mtCOI gene was done and the sequencing of that region was performed. The present study initiated to identify the species of the *Eulecanium* genus. The resultant information may help for the insect management programs by allowing the implementation of mtCOI sequencing strategy on the insect DNA.

2 Materials and Methods

2.1 Identification and Collection of Scale insect

Scale insects were found on the stem of the *H. rosa-sinensis* plant in the area T. Nagar Chennai, Tamil Nadu. Insects were observed as thick scales and looked like a settlement of a waxy layer on the stem due to severe colonization. Adult scale insects were collected and stored in Eppendorf tubes containing 70% ethanol. Ethanol was added to the tubes to avoid bacterial or fungal formation on the insect. The tubes were stored in the refrigerator until used in further studies.

2.2 DNA isolation from the scale insect

Individual scale insect was taken and the abdomen was used to prepare the genomic DNA by following the modified cetyl tri

methyl ammonium bromide buffer (CTAB) method (Saghai-Maroof et al. 1984). The abdomen was ground by adding 1.0 mL of 2% CTAB, 1.4 M sodium chloride, 100 mM Tris-HCI (pH 8.0), and 20 mM ethylenediamine tetraacetic acid (EDTA), 0.1% of 2-mercaptoethanol and suspended in the same buffer. The suspension was incubated at 65° C for 2 hrs and chloroform: isoamyl alcohol (24:1) was added in equal volume. The suspension was centrifuged at 10,000 rpm for 10 min at 8°C. The supernatant was transferred to a fresh microcentrifuge tube without disturbing the middle protein interface. An equal volume of ice-cold 95% ethyl alcohol was added to precipitate the DNA. Further, centrifugation was carried out at 10,000 rpm for 5 min to get the precipitated DNA as a pellet. The resultant DNA pellet was washed with 70% ethanol and dissolved in 50 µL of TE buffer. Isolated DNA was further purified by adding 10 µg/100 µL of RNase mainly to remove RNA contaminants. Insect genomic DNA was resolved in 1% agarose/ ethidium bromide (EtBr) gel, visualized under UV transilluminator, and quantified using a spectrophotometer (Amersham Biosciences).

2.3 PCR amplification of COI gene

The Folmer fragment of the 5' region of COI was amplified using PCR procedure by using forward primer LCO (5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse primer HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). PCR amplification was carried out in 25 µL reaction mixture containing 10X PCR buffer (contains 25mM magnesium chloride), 2 µL dNTP mixture (0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP), 1µL of forward and reverse primer (10µmol each), 1 µL template DNA (50-150 ng), 0.3 µL Taq DNA polymerase (3U/µl), and final volume made with 16 µL distilled water. The mt COI regions were amplified in a Thermal Cycler (Eppendorf) with the PCR cycle program comprising 4 min of pre-denaturation at 94°C, followed by 35cycles of amplification (30 sec of denaturation at 94°C; 1 min of annealing at 46°C for COI; 45 sec of extension at 72°C), and final extension at 72°C for 10 min. The amplified PCR products were resolved in 1.5% agarose gel/ EtBr (0.5 µg/mL) and documented using a gel documentation system (Syngene).

2.4 COI sequencing and data analysis

The amplified bands of the COI gene were excised and purified from the gel using Gel Extraction Kit (Qiagen, Inc., Germany) by following the manufacturer's protocol. The purified PCR products were sequenced with an automated DNA sequencer with COI gene-specific primers at Barcode Biosciences, (Bangalore, Karnataka). Sequences were edited and aligned using BioEdit 7.0. The BLASTn program (http://www.ncbi.nlm.nih.gov/blast/) was used to identify the similarities between the sequences obtained and their homology in the public database. Multiple sequence alignments of the sequences were performed using CLUSTAL W and a phylogenetic tree was constructed using the Neighbor-joining tree.

3 Results

3.1 Identification and collection of scale insects from *Hibiscus* plant

Soft scales are tiny pests with a cottony, waxy surface that damage the plant by sucking sap from leaves, stems, and trunks thereby destroying the plant by reducing its growth. Scale insects were observed in the stem of *H. rosa-sinensis* plant (Fig. 1) which severely colonized the stem of the *Hibiscus* plant (Figures 1 & 2) and at the severe infection stage, it reached to leaves and lead to leaves falling and stunted growth. Figure 3 shows the stunted plant growth due to the scale insect comparing the normal growth of the plant without the insect. Insect sample was collected and transferred into 2ml of Eppendorf tube containing 70% ethanol.

Figure 1 Scale Insect identification in the *Hibiscus rosa-sinensis* plant

Figure 2 Scale Insect colonization in the stem- Close view

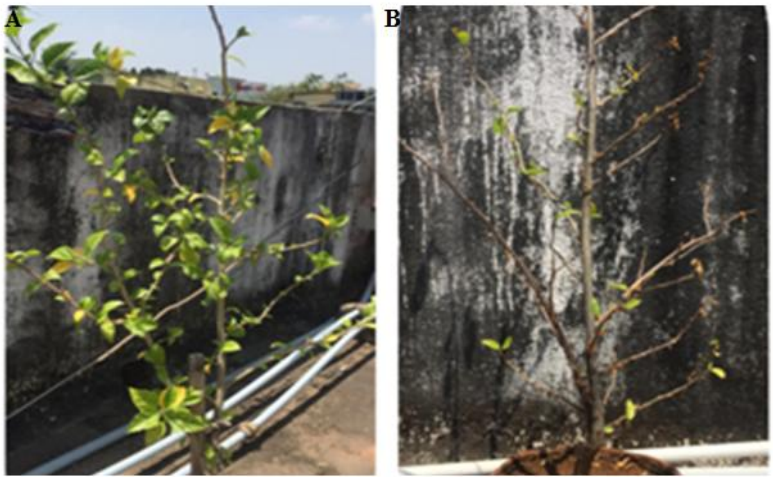

Figure 3 Comparison of the healthy plant (A) and plant affected by the scale insect (B)

3.2 Genomic DNA Isolation & PCR amplification of mtCOI gene

Genomic DNA was successfully isolated from the single-scale insect by using a modified CTAB method and the quality check was done by agarose gel electrophoresis which showed a good yield of genomic DNA under the Gel Documentation system (Figure 4). A manual CTAB method that provides high quantity and high yield DNA was used. Though the presence of RNA contamination and DNA degradation were seen, the yield quality of the DNA was good enough for the PCR amplification of the COI gene. Mitochondrial COI is a standard barcode that was used for species identification. The forward primer LCO1490 and reverse primer HCO2198 were used for the amplification of the COI gene. The PCR product was run on 1.2% Agarose gel and a 1Kb DNA ladder was used to identify the random size of the COI gene. The PCR amplification of the mtCOI gene yielded a 709 bp fragment (Figure 5).

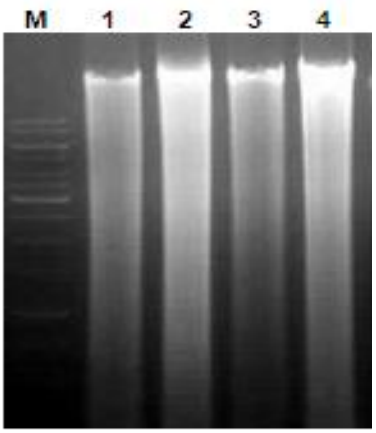

Figure 4 Genomic DNA isolation from *E. giganteum* (Lane M - DNA Marker, Lane 1-4 - Scale insect DNA with replication)

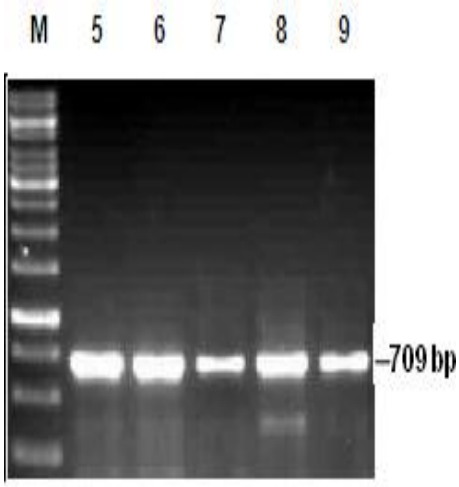

Figure 5 PCR amplification of COI gene

3.3 Sequencing analysis & PCR amplification of mtCOI gene

The sequencing of the purified PCR product was done using Sanger sequencing and a good chromatogram was obtained. mtCOI sequence analysis is essential to elucidate the presence of cryptic insect species. Using National Center for Biotechnology (BLAST) search, the mtCOI sequence was analyzed to find the regions of sequence similarity between the COI gene sequence of *Eulcaneum* and database sequences. Sequence analysis of the collected scale insect identified as *E. giganteum*. The BLAST search revealed that these insects have the highest similarity with *E. giganteum* mtCOI sequences.

Neighbor-joining phylogenetic tree (1000 bootstrap replications) constructed using mtCOI sequence of *E. giganteum* and other Genbank available mtCOI sequences. The phylogenetic tree was computed by using the Kimura-2 parameter method. Bootstrap values of more than 25 are shown. Bootstrap values are specified at each branching point. The scale bar point out the estimated genetic distance (Figure 6). Gaps are treated as missing data which are eliminated from the dataset during tree construction (Tamura et al. 2007).

4 Discussion

DNA barcoding is a major tool in the bio-surveillance of insect pests which allows rapid identification of an unknown insect

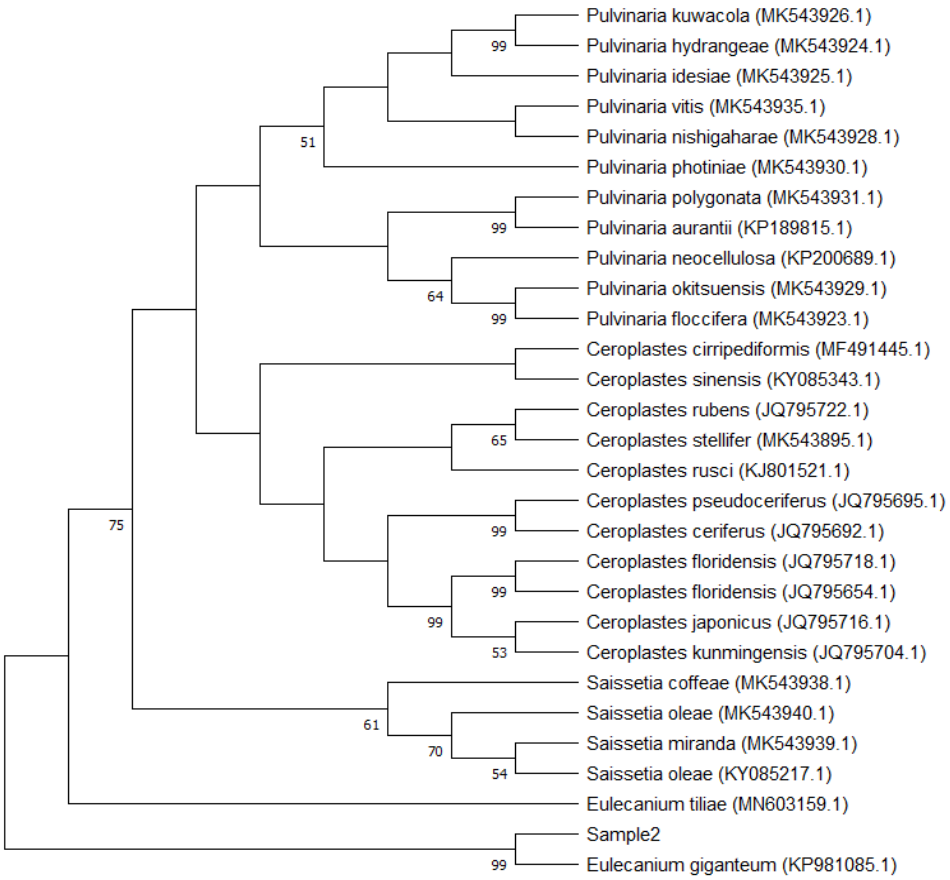

Figure 6 Phylogenetic tree analysis by using the neighbor-joining method

specimen independent of life stages (Javal et al. 2021; Hebert et al. 2003). Standardization of the PCR process for mtCOI gene amplification is the main principle in the DNA barcoding technique (Hollingsworth et al. 2011). The barcoding of an organism is done using standardized molecular techniques hence reducing any complications in morphological identification and easing the work of the researchers. The techniques that have been used for barcoding are DNA isolation, PCR amplification, and mtCOI gene sequencing (Seifert et al. 2007). DNA barcoding of insects is complicated because of the difficulty in isolating the DNA from the insect. Many successful manual methods were developed for getting a high quantity and high yield of insect DNA (Saghai-Maroof et al. 1984; Aljanab and Martinez 1997) by using tiny insect specimens. Recently, mtCOI sequencing was successfully used to identify the natural enemy of stored pests named *Xylocoris flavipes*, which promotes the effective utilization of *X. flavipes* in pest control for safe storage of grain (Zonglin et al. 2021).

In the previous study by the same author, a notorious pest of tea, the tea mosquito bug was collected from different tea plantations in India, and mitochondrial COI marker was successfully used to determine the species as *Helopeltis theivora*, which further leads to easy control measures (Suganthi et al. 2016). The tea scale (*Fiorinia theae* Green) is considered the most destructive pest of *Camellia* spp. in the southeastern U.S. (Borden and Dale 2020). Also in tea, cottony scale *Pulvinaria floccifera* was identified along with the parasitoid *Lysiphlebia* sp. by using this CO1 sequencing method (Sharma et al. 2019). In the present study, single species of *E. giganteum* was identified in the stem of the *Hibiscus* plant. Similarly, Engstrand et al. (2010) confirmed that avocado stem weevil, an important pest in avocado plantations is indeed one species named *Copturus aguacatae* using mtCOI sequencing and the result may support for successful implementation of biological control through pheromone synthesis. Higher genetic variation was observed between the species and relatively less variation within species using DNA barcodes (Hebert et al. 2003). Hence DNA barcoding has an advantage for identifying the tiny insect with less morphological polymorphism and different life stages. The mtCOI gene was used from the mitochondrial genome because it was found to be the most conservative sequence and successfully amplified for a different class of insect species (Folmer et al. 1994). In the present study, the scale insect mtCOI sequence was compared with NCBI available sequence. BLAST results showed that the sequence was matching 82% with *E. giganteum* (KP981085.1), matching 80.07% with *Aspidiotus excisus* (MK863028.1), 78.92% with *Aonidiella ensifera* (KY085356.1).

DNA barcoding was successfully utilized to identify three different thrips species *viz*., *Thrips tabaci, T. vulgatissimus*, and *T. palmi* (Karimi et al. 2010). Apart from mtCOI sequencing, the molecular diagnostic marker was developed by using the ribosomal DNA internal transcribed spacer 2 (ITS2), and this reliable molecular technique is used for the identification of various thrips species including *S. dorsalis* (Farris et al. 2010). In this study, the phylogenetic separation of *E. giganteum* (Figure 6) established the satisfactory resolution of the COI marker. In the phylogenetic tree, the mtCOI sequence of *E. giganteum* was matching 99% with already reported *E. giganteum* (KP981085.1), matching 70% with *Saissetia miranda* (MK543939.1), 65% with *Ceroplastes rubens* and *C. stelifer* (JQ795722.1 & MK543895.1) (Choi and Lee 2019; Deng et al. 2012) Hence DNA barcoding using mtCOI markers has a notable advantage where the morphological polymorphism is absent and identification of insects in various life stages.

Conclusion

In the present study, the mtCOI sequence marker was effectively used for the molecular identification of scale insects from the *Hibiscus* plant. Also, sequence analysis of the mtCOI gene confirmed that these scale insects belong to the genus *Eulcaneum* and species *giganteum*. Identification and characterization of common horticultural pests can be successfully applied in taxonomic classification. Using the DNA barcoding technique, future management of these scale insects can be more precisely implemented with accurate species identification. The study results might be useful to form the control measures since the *E. giganteum* is the notorious species of *Eulcaneum* infecting the Hibiscus.

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References

Aljanabi, S. M., & Martinez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research*, *25*, 4692-4693. https://doi.org/10.1093/nar/25.22.4692.

Ben-Dov, Y., & Hodgson, C. (1997). Soft scale insects: their biology, natural enemies and control. *World Crop Pests*, *7b*, 3-442.

Borden, M. A., & Dale, A. G. (2020). Native and Edible Ornamental Plant Congeners Enhance Ecosystem Services Through Key Pest Avoidance and Multifunctionality in Residential Landscapes. *Environmental Entomology*, *49*, 1206-1213.

Choi J., & Lee, S. (2019). Molecular phylogeny of the family Coccidae (Hemiptera, Coccomorpha), with a discussion of their waxy ovisacs. *Systemic Ecology*, *45*, 396-414.

Chua T. H. (1997). Soft Scale Insects their Biology, Natural Enemies and Control World Crop Pests. *World Crop Pests***,** *7*, 395-399.

Deng, J., Yu, F., Zhang, T. X., Hu, H. Y., et al. (2012). DNA barcoding of six Ceroplastes species (Hemiptera: Coccoidea: Coccidae) from China. *Molecular Ecology Resources*, *12*, 791-6. doi: 10.1111/j.1755-0998.2012.03152.x.

Engstrand, R. C., Tovar, J. C., Jaramillo, A. C., Kolokotronis, S. O. (2010). Genetic variation in avocado stem weevils *Copturus aguacatae* (Coleoptera: Curculionidae) in Mexico. *Mitochondrial DNA*, *21*, 38-43.

Farris, R. E., Ruiz-Arce, R., Ciomperlik. M., Vasquez, J. D., et al. (2010). Development of a Ribosomal DNA ITS2 Marker for the Identification of the Thrips, *Scirtothrips dorsalis. Journal of Insect Science*, *10*, 1-15.

Folmer, O., Black, M., & Hoeh, W. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294-299.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, *270*, 313-321.

Hollingsworth, P. M., Graham, S. W., & Little, D. P. (2011). Choosing and Using a Plant DNA Barcode. *PLoS ONE, 6*, e19254. https://doi.org/10.1371/journal.pone.0019254.

Javal, M., Terblanche, J. S., Conlong, D. E., Delahaye, N., et al. (2021). DNA barcoding for bio-surveillance of emerging pests and species identification in *Afrotropical Prioninae* (Coleoptera, Cerambycidae). *Biodiversity Data Journal*, *9*, e64499.

Kanthesh, B. M., & Bhuvaneswari, G. (2021). Miracles hidden amongst the common medicinal plants India. Green Trust – India.

Karimi, J., Hassani-Kakhki, M., & Awal, M. M. (2010). Identifying thrips (Insecta: *Thysanoptera*) using DNA Barcodes. *Journal of Cell and Molecular Research*, *2*, 35-41.

Li, Z. W., Jia, W. J., Qiao, S. Z., Li, G. M., et al. (2002). Study on biological characteristics and control techniques of *Eulecanium giganteum. Ningxia Journal of Agriculture and Forestry Science and Technology*, *4*, 25-26.

Missoum, A. (2018). An update review on *Hibiscus rosa sinensis* phytochemistry and medicinal uses. *Journal of Ayurvedic and Herbal Medicine*, *4*(3), 135-146. https://doi.org/10.31254/jahm.2018.4308.

Quesada, C. R., Scharf, M. E., & Sadof, C. S. (2020). Excretion of non-metabolized insecticides in honeydew of striped pine scale. *Chemosphere*, *249*, 126167.

Rodrigues, M. S., Morelli, K. A. & Jansen, A. M. (2017). Cytochrome c oxidase subunit 1 gene as a DNA barcode for discriminating *Trypanosoma cruzi* DTUs and closely related species. *Parasites Vectors, 10*, 488. https://doi.org/10.1186/s13071-017-2457-1

Saghai-Maroof, M. A., Solima, K. M., Jorgenson, R. A., & Allard, A. W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings National Academic Sciences, USA*, *81*, 8014-8018.

Seifert, K. A., Samson, R. A., Dewaard, J. R., & Houbraken, J. (2007). Prospects for fungus identification using COI DNA barcodes: with Penicillium as a test case study. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 3901-3906.

Sharma, R., Sharma, A., & Nadda, G. (2019). Molecular identification of a parasitoid from *Pulvinaria floccifera* infesting kangra tea of Himachal Pradesh, India. *Research journal of life science, bioinformatics, pharmaceuticals and chemical science*, *5*(1), 407-411.

Shashi, A., Sanjay Kumar, J., Amita, V., Mayank, K., et al. (2013). Pathophysiology of kidney, gallbladder and urinary stones treatment with herbal and allopathic medicine: A review. *Asian Pacific Journal of Tropical Disease, 3*, 496-504. doi: 10.1016/S2222-1808(13)60107-3

Suganthi, M., Arvinth, S., Chandrashekara, K. N., & Raj Kumar, R. (2016). Molecular characterization of tea mosquito bug from tea growing regions of India. *Mitochondrial DNA, 27*, 3504-3506.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*, 1596-1599.

Watson, G. W., & Kubiriba, J. (2005). Identification of mealybugs (Hemiptera: Pseudococcidae) on banana and plantain in Africa. *African Entomology*, *13*, 35-47.

Wilson, A. C., Cann, R. L., Carr, S. M., George, M., et al. (1985). Milochondrial DNA and two perspectives on evolutionary genetics. *Biological Journal of the Linnean Society*, *26*, 375-400.

Xie, Y. P., Xue, J. L., & Zheng, L. Y. (2006). Wax secretions of soft scale insects: their ultrastructure and chemical composition. China Forestry Publishing House Beijing China.

Zonglin, W., Shaohua, L., Jiying, L., Shiyuan, M., et al. (2021). Morphological and molecular identification of *Xylocoris flavipes* (Hemiptera: Anthocoridae) in southern China. *Grain & Oil Science and Technology*, *4*, 26-32.

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Factors Influencing Willingness to Adopt Recommended Bambara groundnut (*Vigna subterranea* L. Verdc) Agronomic Practices Among Smallholder Farmers in Semi-Arid Lands of Embu County, Kenya

Elizaphan Mboi Ombasa[1*], Phyllis Wambui Muturi[2], Bernard M. Gichimu[2], Hezron N. Isaboke[1], Josiah N. Gitari[2]

[1]Department of Agricultural Economics and Extension, University of Embu, P. O. BOX 6-60100, Embu, Kenya
[2]Department of Water and Agricultural Resource Management, University of Embu, P.O. BOX 6-60100, Embu, Kenya

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Orphaned crops

Farmer field school

ABSTRACT

The study assessed the willingness of smallholder farmers to adopt the recommended agronomic practices of Bambara groundnut in semi-arid lands of Embu County. The study was carried out in three sub-counties i.e. Mbeere North, Mbeere South, and Embu West, of Embu County. Data were extracted with the help of a well-structured questionnaire which was distributed to 384 smallholder farmers who were participants at the farmers' field schools at the three sites. The data were analyzed using means, percentages, and logistic regression. Results of the study revealed that 60.94% of the farmers were willing to adopt the recommended agronomic practices. The willingness of the farmers to adopt the recommended agronomic practices was influenced by farming experience, farm size, extension contact, participation in farmers' groups, cropping technologies adoption, and intercropping system used by the farmers. The application of the farmer participatory approach is an innovative way of introducing and promoting less popular but sustainably proven agricultural technologies among smallholder farmers. This is a climate-smart strategy to address the challenges of food in the area. Awareness creation among the farmers using the right extension channels can increase farmers' willingness to adopt climate-smart technologies such as the production of the highly nutritive and drought tolerant Bambara groundnut in dry areas.

* Corresponding author

E-mail: ombasaelizaphan@gmail.com **(**Elizaphan Mboi Ombasa)

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1 Introduction

Bambara groundnut (*Vigna subterranea* L. Verdc), is an African home-grown pulse (Puozaa et al. 2017) that is tolerant to drought, and high temperature and can thrive well in marginalized soils (Chai et al. 2016). The crop is endemic to Northeastern Nigeria and Northern Cameroon (Temegne 2018). After groundnut and cowpea, the crop is ranked third most important in Sub-Saharan Africa (SSA) (Adzawla et al. 2016). Bambara groundnut is a common diet among the people in the Western, Nyanza, and coastal regions of Kenya (Valerie and Luvembe 2016). The farmers grow Bambara groundnut mainly on small scale for subsistence use.

Although Bambara nut is a popular traditional diet in Western, Coast, and Nyanza regions in Kenya, but its adoption in other areas is dismal (Oyeyinka et al. 2017). Bambara nuts have a high possibility of improving food and nutritional security in the dry lands but it requires more publicity, both as a crop and as food (Ogwu et al. 2018). According to Nyasimi et al. (2017), effective uptake of new sustainable technologies in agriculture largely depends on the information dissemination channels employed. This study, therefore, employed the farmer participatory approach to introduce and promote Bambara groundnut production in dry lands of Embu County. This approach enabled the participants to make well-versed choices on the adoption of the crop.

There is low adoption of best agronomic practices of Bambara groundnut in the dryer parts of Eastern Kenya since the crop is less known in the region (Obura 2021). In this study, it was hypothesized that farmers' willingness to adopt the best agronomic practices on Bambara groundnut would be greatly influenced by farmers' participation in the agronomic evaluation as well as their socio-economic characteristics. Large-scale farmers are risk takers since they can put their land portions to trial (Varble et al. 2016). Researchers and extension agents play a crucial role in the uptake of new agriculture technologies (Chandio and Yuansheng 2018). Minimal contact between the target farmers and change agents has also contributed to the slow adoption trend. Tey et al. (2017) recorded always higher uptake of technologies where farmers closely associate with the change agent.

Farmer field school (FFS) is an extension teaching approach that goes beyond disseminating technical information to farmers. It is a participatory method where farmers are involved in the learning process, imparting practical skills and empowering themselves (Okeoghene 2020). Farmers' participation in extension programs enables them to consider the complexity and usefulness of the projects and therefore helps them in the decision-making (Suvedi et al. 2017). Lailogo et al. (2018) and Bhutto et al. (2018) have suggested that through the farmer participatory approach, the adoption rate has improved and farmers can achieve high and economical production since it responds directly to the needs of the farmers.

This study was anchored on the "adoption of innovation theory" that was developed by Carl Rogers (Oyeyinka et al. 2017). The theory defines adoption as choosing to practice an invention fully as the finest option. Diffusion occurs when individuals adopt new technology, product, or idea. Barrett et al. (2020) define diffusion as a process that includes innovation, communication channels, and adoption. There are two forms of communication channels; the first one is localized where communication is from local leaders and receivers of the same social system example of this type of channel are interpersonal channels while the second one is a cosmopolite channel where the sender of information is from the outside social system e.g. mass media. Another key component of adoption is the social system which is a set of correlated components involved in combined problem solving to achieve a collective objective (Al-Razgan et al. 2021).

According to Qazi et al. (2018), the innovation decision process is an insight-finding and knowledge-refining action, in which a person is encouraged to increase confidence in the advantage and shortcomings of an invention. This process has five steps i.e. knowledge, persuasion, decision, implementation, and confirmation (Sanguinetti et al. 2018). The interpersonal channels play a major role in the knowledge stage while localize channels are more significant at the persuading stage of the innovation-decision process (Ng 2020). The degree of adopting technology is greatly affected by the relative advantage of an innovation, observability, trial-ability, complexity, and compatibility (Qazi et al. 2018).

The recommended agronomic practices are optimum spacing and intercropping sorghum (cereals) with Bambara groundnuts. Farmers have been reported to use different spacing when growing Bambara groundnuts in different regions (Egbe 2016). Most farmers practice intercropping with legumes like beans, peas, and others but have neglected Bambara groundnuts (Oyeyinka et al. 2017). This research, therefore, is sought to assess the willingness of the farmers to adopt the best agronomic practices of Bambara groundnuts.

2 Materials and Methods

The experiment was carried out at Mbeere North, Mbeere South, and Embu West representing the dry parts of Embu County in the Eastern Kenya region. Mbeere North is situated in the lower midland. The elevation of the area is between 500 - 1200 m above sea level. The area is categorized as a hot, semi-arid condition with a mean temperature of 23°C and receives regular rainfall of around 800 mm annually which is usually bimodal (Kamonge et al. 2020). Mbeere South extends to lower midland within 3, 4, and 5 agro-

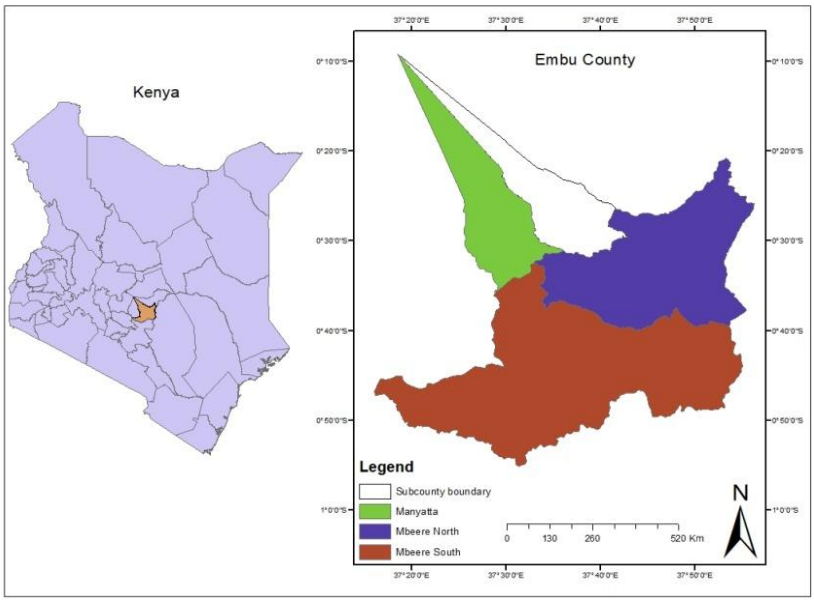

Figure 1 Map of the study site

ecological zones within the coordinates of 0° 20'N and 0° 50' South and longitude 37°16' and 37°56' East. It has an elevation of 500 to 1200 m above sea level. This area is considered as a hot, semi-arid condition with temperatures between 21.7℃ to 22.5℃ and receives medium rainfall between 700 to 900 mm per annum. Embu West site is located in Manyatta and this is found in Upper Midland 2 and 3 agroecological zones. The area has an altitude of 1440m above sea level with coordinates 0º35'25.58"S and 37º25'31.84"E. The area is categorized as a warm and humid climate with an annual temperature of about 20℃ with yearly rainfall ranging from 909 mm to 1230 mm that falls in a bimodal pattern (Kangai et al. 2021). The area of the study sites is illustrated in figure 1.

This study drew the sampling units using a multistage spatially stratified random sampling design. Sampling was done from all seven wards. The size of the sample was computed using Cochran formulae as used by Castellini et al. (2018).

$$n_o = \frac{Z^2 pq}{e^2} \quad \text{(i)}$$

Where n^0 = s size of the sample required, Z = t value at 95% confidence level from a normal table (1.96), p = probability that respondent has characteristic being measured, q = (1-p) probability that respondent has no characteristic being measured and e = 5% level of significance

The size of the sample was decided as shown

$$n_o = \frac{(1.96)^2 (0.5)(0.5)}{(0.05)^2} = 384$$

This study was carried out in two cropping periods between September 2019 and August 2020. For this, two separate experiments were done over the two periods namely September–December 2019 rainy season and March-May 2020 rainy season. The first experiment was to come up with optimum spacing for growing and yield of selected Bambara groundnut genotypes in dry lands of Embu County, Kenya. The 384 small-scale farmers were distributed in each ward of the selected three sub-counties. In each sub-county, 128 farmers were sampled and training was organized in a Farmer Field School (FFS). They were then trained on the optimum spacing, intercropping sorghum with Bambara grounds, and incorporating Bambara residues in the soil within the rows.

The study embraced the theory of the innovation-decision model up to the decision-making stage. An adoption survey was conducted at the end of the experiment to assess the willingness of the farmers to adopt the crop. A well-structured questionnaire was pretested and administered to the 384 farmers. The questionnaire was designed to assess the farmers' willingness to adopt the best agronomic practices learned at the FFS and to capture the social and economic characteristics of the small-scale growers. Data collected were related to the household head`s age, years of farming, size of the household, number of adults, size of land, farm income, gender, education level, previous technology adoption, group membership, access to extension service, cropping system adopted by the farmer and willingness to adopt the best agronomic practice of Bambara groundnuts production. To capture the farmers' views, open-ended questions were also included in the questionnaire. The responses were then coded and keyed into an excel sheet for analysis.

Data were analyzed using SPSS (version 27) in mean and percentages. A Probit model was used to examine the socio-economic characteristics that determine farmers' willingness to adopt best agronomic practices and production of Bambara groundnut. This is explained by the equation:

$$y_i * = \beta 0 + \sum_{k=1}^{k} \beta_{ki} x_{ki} + \varepsilon_i \quad \text{(ii)}$$

Where; i stands for the respondent, $X_{ki:}$ k=1 via k independent variable explaining the state for the respondent i, β_k: is the parameter that designates the result of X_k on y*, β_0: intercept that shows the probable rate of y* when all X_k=0, ε_i : stochastic error term for respondent i, The latent variable y_i^* is continuous, unobserved and ranges from $-\infty$ to $+\infty$, The variable y_i^* creates the recoded dualistic factor;

$$y_i = \begin{cases} 1 \; if \; yi * > 0, \\ 0 \; otherwise. \end{cases} \quad \text{(iii)}$$

Dealing with a willingness to adopt the best agronomic practice of Bambara groundnut production equation 1…….is interpreted as

$$y_i = \begin{cases} 1 \; if \; the \; farmer \; is \; willing \; to \; adopt \\ 0 \; if \; the \; farmer \; is \; not \; willing \; to \; adopt \end{cases}$$

The data was then tested both for multicollinearity and heteroscedasticity. The variables used in modeling factors affecting farmers' willingness to adopt the best agronomic practices of Bambara groundnut production yielded a mean-variance inflation factor (VIF) of 2.020. Each of the variables had a VIF value of less than 10 but greater than 1. According to Marie et al. (2020), VIF values less than 10 show the non-existence of a multicollinearity problem. The contingent valuation method was used in the quantification of the farmers' willingness to adopt the best agronomic practice of Bambara groundnut production.

3 Results and Discussion

3.1 Adoption of the Recommended Bambara Agronomic Practices

The majority (66.67%) of the farmers had previously adopted agricultural technologies and 70.83% were organized into various farmer groups. All the selected farmers were accessing extension services but in various frequencies, where 22.40% received monthly services, 32.29% received the services annually, while 45.31% received irregular services. Consequently, the farmers practiced irregular cropping systems with most of them (49%) practicing crop rotation whereas 21, 13, 9, and 6 percent practiced mixed cropping, intercropping, mono-cropping, and multiple cropping systems respectively. The majority (58.07%) of the farmers preferred the system where Bambara groundnut was intercropped with cereals and its residues incorporated into the soil. However, 31.25% of the farmers were not impressed by any of the intercropping systems. Eventually, 60.94% of the participants were found to be willing to adopt the best agronomic practices of Bambara groundnuts which comprised 47% male and 53% female.

Table 1 Adoption of recommended Bambara agronomic practices

Variable	Description	Percentage(%)
Previous technology adoption	Yes	66.67
Farmers' groups	Yes	70.83
Extension services	Monthly	22.40
	Once a year	32.29
	Irregularly	45.31
Cropping system	Crop rotation	49.22
	Mono-cropping	9.38
	Intercropping	13.8
	Mixed cropping	21.09
	Multiple cropping	6.51
Intercropping system preferred	Groundnut residues removed	10.68
	Groundnut residues incorporated	58.07
	None	31.25
Willingness to adopt Bambara groundnut	Willing	60.94

Table 2 Factors influencing farmers' willingness to adopt the production of Bambara groundnut

Variable	Marginal effects	Standard Error	Z Score
Age of the farmer	-0.0887	0.0825	-1.08
Household head gender	0.3848	0.2190	1.76
Level of education of the farmer	0.0943	0.1142	0.08
Farming experience	0.4414	0.2049	2.15*
Size of the household	0.4118	0.4173	-0.99
Source of labor	0.5326	0.3343	1.59
Household farm size (acres)	0.5667	0.2151	2.63*
Access to extension services	0.2287	0.8242	2.77
Membership in farmers' groups	0.4648	0.1597	2.91
Type of cropping system	-0.1948	0.6789	-2.87
Household income	0.0001	0.0001	1.87
Off-farm income	-0.2260	0.1419	-1.59
Access to credits	0.5844	0.1074	-0.54
Previous adoption of technology	0.2660	0.5025	5.30
Irrigation method	0.5949	0.3420	1.74
Time of planting	-0.7502	0.2665	-2.81
Intercropping system preference	0.0953	0.2196	0.43

*P≤0.05; Prob > chi (1) = 0.0000; R-squared = 60.43

3.2 Factors Influencing Farmers' Willingness to Adopt Bambara Groundnut

Results presented in table 2 indicate the factors influencing the willingness of farmers to adopt Bambara groundnut production. The probit model has binary dependent variables (1= willing to adopt best agronomic practices of Bambara groundnuts, 0= not willing to adopt best agronomic practices of Bambara groundnuts).

The farming experience was significant (0.4414) in the farmers' willingness to adopt Bambara groundnut production. This means that an increase in the farmers' experience in farming by one year increases the chances of the farmer adopting Bambara groundnuts by 44.14%. This is because as farmer accumulates experience over time, they switch from conventional agriculture to modern agricultural practices (Paustian and Theuvsen 2017). Barnes et al. (2019) recorded that increase in the farmers' experience increases the adoption rate.

The size of the farm had a positive marginal effect (0.5667) and is significantly related to the willingness of the farmer to adopt the Bambara groundnut production. This means that when the size of the farm increases by a unit it resulted in farmers' willingness to adopt Bambara groundnut production increasing by 56.67%. Consistent results were recorded by Ntshangase et al. (2018) who found that growers who have greater parcels have higher chances of adopting new agricultural technologies compared to those with small parcels of land.

Extension services access was significant (0.2287) on the willingness of the farmers to adopt the best agronomic practices of Bambara groundnut. This hinted that regular extension service access by the farmers enhanced their willingness to adopt Bambara groundnut production by 22.87%. This underpins the importance of change agents when promoting the uptake of new ideas in agriculture. Findings by Suvedi et al. (2017) recorded that increase in farmer-extension contacts enhanced farmers' education and adoption of agricultural technologies. Group membership had a positive marginal effect (0.4648) on the willingness of the farmers to adopt the best agronomic practices of Bambara groundnut production. The willingness of the farmer to adopt the best agronomic practices of Bambara groundnuts increased (46.48%) with group membership. Most of the farmers who adopt new agricultural technologies are members of various agricultural organized groups (Mango et al. 2017).

The previous adoption of various cropping technologies was significant (0.2660) in the willingness of the farmer to adopt Bambara groundnut production. When the farmers had previously adopted other cropping technologies, they are more willing to

adopt Bambara groundnut production by 26.6%. Singh et al. (2016) recorded that those farmers who had previously adopted new cropping technologies are risk takers and early adopters of new agricultural technologies.

Finally, 60.94% of the participants have the willingness to adopt the best agronomic practices of Bambara groundnuts which comprised 47% male and 53% female. Based on gender, this is contrary to a study by Rola-Rubzen et al. (2020) who reported that more men adopt technologies in agriculture than women. Verkaart et al. (2019) recorded that for a technology to be adopted widely, it must be environmentally sustainable and has more economic benefits than conventional methods.

Conclusion and Recommendations

Results of the study can be concluded that farmers were willing to adopt the best agronomic practices of Bambara groundnut production. Results showed that farming experience, household farm size, extension contact, participation in farmers' group, type of cropping system, and intercropping system used by the farmer were found to be significant in the willingness of the farmers to adopt best agronomic practices and production of the legume. Awareness creation among the farmers using the right extension channels can increase farmers' willingness to adopt climate-smart technologies such as the production of the highly nutritive and drought tolerant Bambara groundnut in dry areas. Therefore, policy interventions should target the training of farmers and promotion of best agronomic practices of Bambara groundnut production, as a drought intervention mechanism to promote food security, especially in dry areas. Additionally, the ministry of agriculture should focus on the increasing frequency of contact between agricultural extension personnel and farmers since this will enable farmers to be informed on current agricultural technologies and innovations.

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Declaration

The authors did not report any conflict of interest

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References

Adzawla, W., Donkoh S.A., George N., Reilly P., et al. (2016). Adoption of Bambara Groundnut Production and its effects on farmers' welfare in Northern Ghana. *African Journal of Agricultural Research, 11* (7), 583-594.

Al-Razgan, M., Alrowily, A., Al-Matham, R. N., Alghamdi, K. M., Shaabi, M., & Alssum, L. (2021). Using diffusion of innovation theory and sentiment analysis to analyze attitudes toward driving adoption by Saudi women. *Technology in Society*, *65* (23), 175-186.

Barnes, A. P., Soto, I., Eory, V., Beck, B., et al. (2019). Exploring the adoption of precision agricultural technologies: A cross regional study of EU farmers. *Land use policy*, *80*, 163-174.

Barrett, C. A., Pas, E. T., & Johnson, S. L. (2020). A cost analysis of the innovation–decision process of an evidence-based practice in schools. *School Mental Health*, *12*(3), 638-649.

Bhutto, N., Rahman, A., Nahiyoon, A., Khan, A., & Zaman, B. (2018). Role of farmers' training on cotton production through farmer field school (FFS) approach in Sanghar, Sindh Pakistan. *International Journal of Farming and Allied. Science*, *7*(1), 18-22.

Castellini, G., Bruschettini, M., Gianola, S., Gluud, C., & Moja, L. (2018). Assessing imprecision in Cochrane systematic reviews: a comparison of GRADE and Trial Sequential Analysis. *Systematic Reviews*, *7*(1), 1-10.

Chai, H.H., Massawe F., & Mayes, S. (2016). Assessment of a segregating population for the improvement of drought tolerance in Bambara Groundnut. *Plant Breeding in Horticulture*, *2* (4), 15–28.

Chandio, A. A. & Yuansheng, J. (2018). Determinants of adoption of improved rice varieties in northern Sindh, Pakistan. *Rice Science*, *25*(2), 103-110

Egbe, M. O. (2016). Nodulation, nitrogen yield and fixation by Bambara Groundnut (*Vigna Subterranea* (L.) Verdc.) Landraces Intercropped with Cowpea and Maize in Southern Guinea Savanna of Nigeria. *Agricultural Science*, *1*(4), 15–28.

Kamonge, S., Kamau, L., & Muhoho, N. (2020). Prevalence of Intestinal Parasitic Infections and Transmission Risk Factors in Primary School Children in Mbeere North Sub-County, Embu County, Kenya. *International Journal of Pathogen Research*, 4(3), 1-7

Kangai, R., Chitechi, E. W., Koske, J., Waswa, B., & Ngare, I. (2021). Determinants of climate change adaptation and perceptions among small-scale farmers of Embu County, Eastern Kenya. *African Journal of Environmental Science and Technology*, *15*(4), 167-178

Lailogo, O. T., Suva, M. M., Torres, C. S., Jamias, S. B., & Baconguis, R. B. (2018). Participatory communication among

farmers in rice integrated crop management-farmer field School (Ricm-Ffs), East Nusa Tenggara Province, Indonesia. *International Journal of Agriculture Innovations and Research*, *6*(4), 71-74.

Mango, N., Makate, C., Tamene, L., Mponela, P., & Ndengu, G. (2017). Awareness and adoption of land, soil and water conservation practices in the Chinyanja Triangle, Southern Africa. *International Soil and Water Conservation Research*, *5*(2), 122-129

Marie, M., Yirga, F., Haile, M., & Tquabo, F. (2020). Farmers' choices and factors affecting adoption of climate change adaptation strategies: evidence from northwestern Ethiopia. *Heliyon*, *6*(4), e03867.

Ng, Y. M. M. (2020). Re-examining the innovation post-adoption process: The case of Twitter discontinuance. *Computers in Human Behavior*, *103*, 48-56.

Ntshangase, N. L., Muroyiwa, B., & Sibanda, M. (2018). Farmers' perceptions and factors influencing the adoption of no-till conservation agriculture by small-scale farmers in Zashuke, KwaZulu-Natal Province. *Sustainability*, *10* (2), 555-569.

Nyasimi, M., Kimeli, P., Sayula, G., Radeny, M., Kinyangi, J., & Mungai, C. (2017). Adoption and dissemination pathways for climate-smart agriculture technologies and practices for climate-resilient livelihoods in Lushoto, Northeast Tanzania. *Climate*, *5*(3), 63-70.

Obura, M. (2021). Effect of phosphorus fertilizer rates and priming treatments on seed quality of Bambara Groundnut (*Vigna subterranea* (L.) Verdc.). Unpublished PhD thesis submitted to the University of Eldoret, Kenya.

Ogwu, M. C., Ahana, C. M., & Osawaru, M. E. (2018). Sustainable food production in Nigeria: a case study for Bambara Groundnut (*Vigna subterranea* (L.) Verdc. Fabaceae). *Journal of Energy and Natural Resource Management*, *4*(2), 187-196

Okeoghene, S. (2020). Management of cocoa black pod disease by farmers in Edo State, Nigeria: The role of farmer field school. *Development*, *10*(2), 528-540.

Oyeyinka, A. T., Pillay, K., Siwela, M. (2017). Consumer awareness and acceptability of Bambara groundnut as a protein source for use in complementary foods in rural KwaZulu-Natal. *South African Journal of Clinical Nutrition*, *30*(4), 87–92.

Paustian, M., & Theuvsen, L. (2017). Adoption of precision agriculture technologies by German crop farmers. *Precision Agriculture*, *18*(5), 701-716

Puozaa, D., Jaiswal, S., Dakora, F. (2017). African origin of Bradyrhizobium Populations Nodulating Bambara Groundnut (*Vigna Subterranea* L. Verdc) In Ghanaian and South African Soils. *Plos One* 12 (9). https://doi.org/10.1371/journal.pone.0184943.

Qazi, W., Raza, S. A., & Shah, N. (2018). Acceptance of e-book reading among higher education students in a developing country: the modified diffusion innovation theory. *International Journal of Business Information Systems*, *27*(2), 222-245.

Rola-Rubzen, M. F., Paris, T., Hawkins, J., & Sapkota, B. (2020). Improving gender participation in agricultural technology adoption in Asia: from rhetoric to practical action. *Applied Economic Perspectives and Policy*, *42*(1), 113-125.

Sanguinetti, A., Karlin, B., & Ford, R. (2018). Understanding the path to smart home adoption: Segmenting and describing consumers across the innovation-decision process. *Energy Research & Social Science*, *46*, 274-283.

Singh, P. K., Barman, K., & Varshney, J. G. (2016). Adoption Behaviour of Vegetable Growers Towards Improved Technologies. *Indian Research Journal of Extension Education 11*(21) 62-65.

Suvedi, M., Ghimire, R., & Kaplowitz, M. (2017). Farmers' participation in extension programs and technology adoption in rural Nepal: a logistic regression analysis. *The Journal of Agricultural Education and Extension*, *23*(4), 351-371.

Temegne, N. C., Gouertoumbo, W. F., Wakem, G. A., Nkou, F. T. D., Youmbi, E., & Ntsomboh-Ntsefong, G. (2018). Origin and Ecology of Bambara Groundnut (*Vigna subterranea* (L.) Verdc.): A Review. *Journal of Ecology & Natural Resources*, *2*, 1-10.

Tey, Y. S., Li, E., Bruwer, J., Abdullah, A. M., et al. (2017). Factors influencing the adoption of sustainable agricultural practices in developing countries: a review. *Environmental Engineering & Management Journal*, *16*(2), 167-176

Valerie, P., & Luvembe, S. W. (2016). Influence of farmers' socio-economic characteristics on adoption of Bambara Nut Production in Western Kenya. *Asian Journal of Agricultural Extension, Economics & Sociology*, 14(1) 1-10.

Varble, S., Secchi, S., & Druschke, C. G. (2016). An examination of growing trends in land tenure and conservation practice adoption: Results from a farmer survey in Iowa. *Environmental management*, *57*(2), 318-330.

Verkaart, S., Mausch, K., Claessens, L., & Giller, K. E. (2019). A recipe for success? Learning from the rapid adoption of improved chickpea varieties in Ethiopia. *International journal of agricultural sustainability*, *17*(1), 34-48.

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Madin-Darby Canine Kidney (MDCK) Cell line permeability of Curcumin loaded Phycocyanin nanosponges - *In-Vitro* study

Manjuladevi Kasirajan*, Ramaiyan Velmurugan, A.Vijayalakshmi

School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai- 600 117, Tamil Nadu

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ABSTRACT

Blood Brain barrier (BBB) is a natural protective wall in the brain to restrict the invasion of xenobiotics or toxic chemicals. This, in turn, becomes a major obstacle for researchers and industry people in formulating new drugs to treat brain disorders like brain tumors, Alzheimer's disease, multiple sclerosis, meningitis, and so on. The purpose of this research is to study the *in-vitro* cytotoxicity & BBB permeation of curcumin-loaded phycocyanin nanosponges (Cur-PC NS) using Madin-Darby Canine Kidney (MDCK) cell lines. Cell viability of Cur-PC NS was performed using 3-(4,5-dimethylthiazol-2-yl)-2.5- diphenyltetrazolium bromide (MTT) assay, the transepithelial electrical resistance (TEER) values, and permeability coefficient were measured to test the integrity of monolayer of MDCK cell line. Results of the current study showed that Cur-PC NS at 50µM, 85% of MDCK cells are more viable and there was a significant ($p<0.01$) reduction in TEER values up to 48 hours when compared to the curcumin. The permeability coefficient of nanosponges produced a 2.5-fold increase in enhancement ratio with a Papp value of $1.94\pm0.11\times10^{-6}$ cm/s and $4.86\pm0.04\times10^{-6}$cm/s for curcumin and Cur-PC NS respectively. Results of the study can be concluded that phycocyanin nanosponges can be used as a carrier for curcumin to permeate the BBB which may play a major role in the treatment of various brain disorders. Future studies are needed to substantiate the exact mechanism of permeability with clarification of efflux transporters presented in BBB.

* Corresponding author

E-mail: mdevi.sps@velsuniv.ac.in (Manjuladevi Kasirajan)

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1 Introduction

Blood- Brain barrier (BBB) is a protective and dynamic wall that restricts the entry of any harmful substance. This barrier system protects the brain from xenobiotics which is also a major obstacle to delivering drugs into the central nervous system (Pardridge 2005; Kadry et al. 2020; Neumaier et al. 2021; Sanchez-Dengra et al. 2021). Efflux transporters play a vital role in fluxing the drugs and limit the BBB penetration (Loscher and Potschka 2005; Neumaier et al. 2021; Sanchez-Dengraetal 2021). The Key barriers that interfere with the permeation of drugs are brain microvascular endothelial cells (BMECs) with tight junctional proteins & metabolizing enzymes like cytochrome P450 and transport barriers like P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and organic anion transporting polypeptide. Over the decade's, pharmaceutical scientists had to overcome the hurdles in the invention of drugs for central nervous system (CNS) disorders (Banerjee et al. 2016). With the knowledge of the complex nature of the blood-brain barrier, a drug can be designed by implementing nanotechnology to treat CNS-related disorders (Suresh et al. 2020).

The BBB permeability of a drug can be predicted using *in vitro* models. Various non-cerebral and cerebral origin *In-vitro* models are available. In recent studies, MDCK cell lines were more preferred when compared to $CaCo_2$ cell lines. The advantages of MDCK cells are a shorter duration for maturation time in culture, low risk of infection, low expression of P-glycoprotein (P-gp), and experimental reproducibility (Horio et al. 1989; Irvine et al. 1999; Polli et al. 2000; Jiang et al. 2022).

In the last two decades, various research studies have been performed to deliver curcumin through nanotechnology to resolve the failure of curcumin therapy in the concept of poor bioavailability and fast metabolism (Yallapu et al. 2013; MalekiDizaj et al. 2022). In current MDCK cell lines was used to study the permeability and integrity of tight junctions of curcumin-loaded phycocyanin nanosponges and compared with pure curcumin.

2 Materials and Methods

For the curcumin-loaded phycocyanin nanosponges, curcumin was obtained from Sigma Aldrich, Phycocyanin from TCI Chemicals Pvt. Ltd, and 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAA) solution, penicillin-streptomycin solution, and trypsin solution were purchased from Sigma-Aldrich. MDCK cell line was obtained from NCCS, Pune and Poly vinyl alcohol, double distilled water, ethanol and all other reagents and chemicals used are analytical grades.

Curcumin-loaded phycocyanin nanosponges (Cur-PC-NS) (Prathima and Sreeja 2013; Velmurugan et al. 2019) was prepared using the emulsion solvent evaporation technique. The final nanosponges were characterized as per Manjuladevi and Velmurugan (2020) and used for this study. The *In-vitro* cell line permeability study was performed using the MDCK cell line (Wang et al. 2014).

2.1 MDCK cell culture

For the transport studies, MDCK cells (Madin-Darby Canine Kidney cells) were cultivated in standard conditions with DMEM medium supplemented with 10% FBS, 1% NEAA, and 1% penicillin–streptomycin and was cultured in a humidified incubator at 37°C with 5% CO_2 and (80% confluent) were seeded at a density of 10^5 cell/mL on the upper side of 12 well plate filters (1.131 cm^2 growth area). The culture medium was replaced every three days following the two days of seeding to be ready for experimental use. The quality of the monolayers was assessed by measuring their transepithelial electrical resistance (TEER) at 37 °C using an EVOM epithelial Voltmeter with an Endohm electrode (World Precision Instruments, INC., Sarasota, FL.). The TEER shows the impedance to the passage of small ions through the physiological barrier and is recognized as one of the most accurate and sensitive measures of BBB integrity. Only monolayer's displaying TEER values above 400 Ω was used in the experiments (Wang et al. 2014).

2.2 Cytotoxicity study

The cell viability of nanosponges was performed using an MTT assay. MDCK cells were seeded in 96- well plates at a density of $5X10^3$ cells/ well and cultures for 24 hr. Nanosponges were also added to the wells at various concentrations. After allowing 2 hrs co-incubation at 37°C, 100 µL of MTT (0.5 mg/mL) was added to each well and incubated for a further 4 h at 37°C. After incubation, the medium was removed, and 100 µL of dimethylsulfoxide (DMSO) was added to the residual precipitates. The absorbance was determined at 590 nm using a microplate reader. Cell viability was expressed as a percentage of the absorbance relative to that of the control. Control cells were not exposed to any materials. Experiments were performed with three replicate wells for each sample and control (Wang et al. 2014).

2.3 Monolayer integrity testing (TEER measurement)

The cell lines were used after pre-incubating at 37°C in 5% CO_2 conditions for 2 days. TEER was measured to confirm the functionality of the tight junctions. The assays were carried out using the Blood Brain Barrier cell layers with TEER values in the range between 400 and 2000 Ω cm^2. After establishing that MDCK display extremely tight barrier properties, nanosponges were suspended in a 0.2 mL assay medium or the free drug to the apical side of the Blood

Brain Barrier layers and cultured in the model. The TEER values were monitored at a fixed time (Nikandish et al. 2016).

2.4 Drug uptake and Calculation of permeability (P*app*)

At the end of the study, samples were collected from the apical and basolateral sides of the BBB model and the concentration using HPLC-UV methods was measured. To calculate the apparent permeability coefficient (transport capacity), the following formula was used

$$P_{app} = (dQ/dt) \times (1/A.Co)$$

The enhancement ratio was calculated by dividing the Papp value of curcumin-loaded phycocyanin nanosponges and pure drug, dQ/dt is the transferred drug per time; A is the surface area of the filter (1.131 cm^2); Co is the initial concentration of nanosponges in apical side (Taub et al. 2002)

2.5 Statistical analysis

The results were presented as mean and Standard deviation (SD) with n=3. Statistical significance of differences was processed by Wilcoxon matched-pairs test and student t-test (Paired and unpaired). The value of *p<0.05 & ** p<0.01 was statistically significant. All the statistical calculations were performed using the software Graph Pad prism version 9.1.2.

3 Results

3.1 Cytotoxicity study

The Cytotoxicity of Cur-PC- NS was performed using MTT assay using MDCK cell lines after 24-hrs exposure to free drug and curcumin-loaded phycocyanin nanosponges. Results of the study showed that the Cur-PC-NS at a concentration of 25µg/ml & 50µg/ml have no adverse effect on cell viability (Figure 1).

3.2 Monolayer integrity testing

Results presented in figure 2 revealed that Cur-PC-NS decreased the integrity of the Blood Brain Barrier model cell line, proving that it can open the tight junction of Blood Brain Barrier for movement of curcumin loaded phycocyanin nanosponges into the brain. The amount of curcumin and curcumin loaded phycocyanin nanosponges after crossing the monolayer is shown in figure 3.

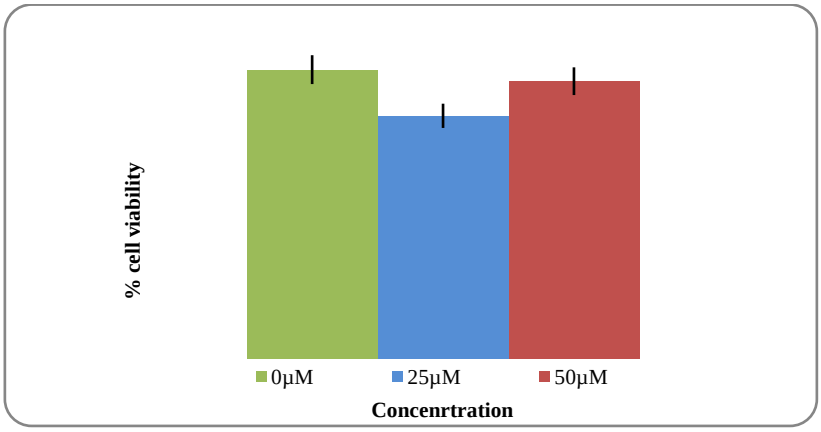

Figure 1 MDCK cell viability % of nanosponges at 24hr, the data represent mean ± SD (n=3 different monolayer)

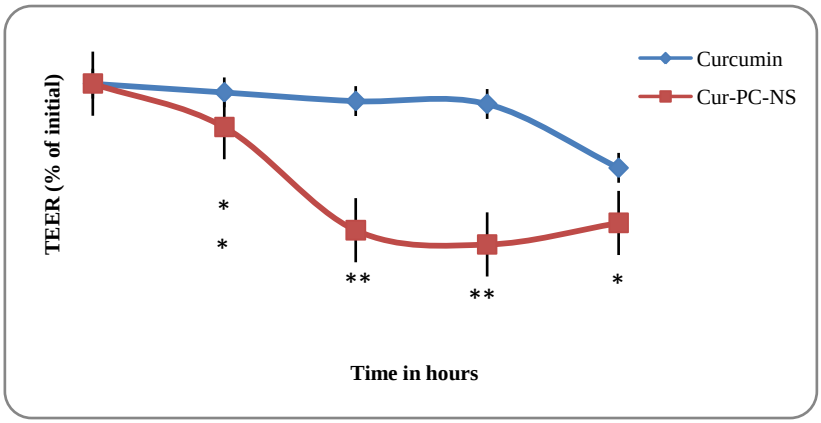

Figure 2 Effect of Cur-PC-NS and curcumin with TEER measuring. The data represent mean ± SD (n=3); **p<0.01

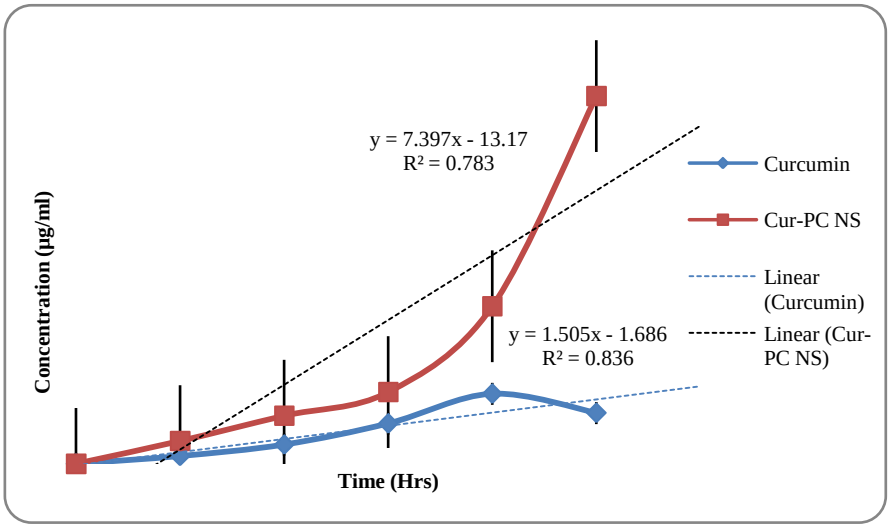

Figure 3 In-vitro cell line permeability investigation using MDCK cell line, the data's represent mean ± SD; **p<0.01

3.3 Permeability coefficient (P*app*)

The permeability coefficient was found $1.94\pm0.11\times10^{-6}$ cm/s and $4.86\pm0.04\times10^{-6}$ cm/s for curcumin and Cur-PC-NS, respectively. The enhancement ratio showed that Cur-PC-NS exhibited 2.5-fold increase in positive effect than the pure drug curcumin on MDCK cell line permeability.

4 Discussion

Curcumin loaded phycocyanin nanosponges was used to improve the BBB permeation, more entrapment, and target delivery which in turn to treat various CNS-related disorder based on earlier research (Tejashri et al. 2013; Gharakhloo et al. 2020; Suresh et al. 2020). In this study, the MDCK model was chosen, as it mimics the structure of BBB and also due to its advantage over the human colorectal adenocarcinoma cell line (Caco-2) in sense of shorter culture time (4 days vs. 21 days) drug permeability across BBB (Irvine et al. 1999; Polli et al. 2000). The rationale behind using phycocyanin as a carrier for curcumin is due to its wide range of application in the pharmaceutical field (Jiang et al. 2017), have proved to target TAM (Tumor-associated Macrophages) as photosensitizer (Wan et al. 2017).

Our results showed that the curcumin loaded phycocyanin nanosponges (Cur-PC-NS) had a positive effect and exhibited 96% of cells are viable at 50µg/ml which shows high biocompatibility of our nanosponges and no toxic effect on cells. Our findings are supported by previous research works exhibiting that curcumin has a non- cytotoxic effect on cells at lower concentrations (Zanotto-Filho et al. 2012; Gharakhloo et al. 2020; Susanna et al. 2020). The tight junction integrity of monolayer is also loosened or decreased to 45% of the initial TEER value in our results suggesting that the increased permeability of curcumin nanosponges, regain of the tight junction at 48hrs i.e., 52%, and the reversal of TEER value indicates the reconstruction of cell junctions (Yeh et al. 2011; Wang et al. 2014). Hence it suggests that curcumin loaded phycocyanin nanosponges are safe and compatible. The enhancement ratio of Cur-PC-NS permeability coefficient (Papp) is 2.5-fold when compared to curcumin alone, which is also supported by the research work of Pushpalatha et al. (2018) in curcumin nanoformulation. Thus, curcumin nanoformulation can be used for treating glioblastoma as a single therapy or along with chemotherapeutic agents due to its safety, biocompatibility, and pharmacological property (Neil and Sandeep 2016; Del Prado-Audelo et al. 2019). Further studies are needed to prove the exact mechanism of penetration, whether passive or endocytosis and transcellular transport of nanosponges and P-glycoprotein substrate mediated inhibition or efflux of the drug.

Conclusion and Future Perspective

Our results proved that the formulated nanosponges have no toxicity and decreased the TEER values thereby increasing the permeation of the drug into the blood brain barrier. Thus, it can be concluded that phycocyanin nanosponges can be used as a carrier to deliver curcumin into the brain. We have completed the animal study for our nanosponges along with biomarker estimation, which will be published in the future. However, future studies are needed to confirm the exact mechanism of permeation across the blood brain barrier in presence of various efflux transporters.

Acknowledgment

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Conflicts of Interest and Financial Disclosure

The authors declare that there is no conflict of interest and have not received any funds from any source for this research.

References

Banerjee, J., Shi, Y., & Azevedo, H. S. (2016). In vitro blood–brain barrier models for drug research: state-of-the-art and new perspectives on reconstituting these models on artificial basement membrane platforms. *Drug Discovery Today*, *21*(9), 1367-1386

Del Prado-Audelo, M. L., Caballero-Florán, I. H., Meza-Toledo, J. A., Mendoza-Muñoz, N., et al. (2019). Formulations of curcumin nanoparticles for brain diseases. *Biomolecules*, *9*(2), 56

Gharakhloo, M., Sadjadi, S., Rezaeetabar, M., Askari, F., Rahimi, A., et al. (2020). Cyclodextrin-based nanosponges for improving solubility and sustainable release of curcumin. *Biological Chemistry & Chemical Biology*, *Chemistry Select*, *5*, 1734–1738

Horio, M., Chin, K.V., Currier, S J., & Goldenberg, S., et al. (1989). Transepithelial Transport of Drugs by the Multidrug Transporter in Cultured Madin-Darby Canine Kidney Cell Epitheli. *Journal of Biological Chemistry*, *264*(25), 14880-14884

Irvine, J D., Takahashi, L., Lockhart, K., Cheong, J., et al. (1999). MDCK (Madin-Darby canine kidney) cells: A tool for membrane permeability screening. *Journal of Pharmaceutical Sciences*, *88*, 28-33

Jiang, L., Kumar, S., Nuechterlein, M., Reyes, M., et al. (2022). Application of a high-resolution in vitro human MDR1-MDCK assay and in vivo studies in preclinical species to improve prediction of CNS drug penetration. *Pharmacology Research & Perspectives*, *10*(1), e00932

Jiang, L., Wang, Y., Yin, Q., Liu, G., et al. (2017). Phycocyanin: A Potential Drug for Cancer Treatment. *Journal of Cancer*, *8*(17), 3416–3429

Kadry, H., Behnam, N., & Luca, C. (2020). A blood–brain barrier overview on structure function, impairment and biomarkers of integrity. *Fluids Barriers CNS*, *17*, 69

Löscher, W., & Potschka, H. (2005). Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. *NeuroRx: The Journal of the American Society for Experimental Neuro Therapeutics*, *2*(1), 86–98

MalekiDizaj, S., Alipour, M., DalirAbdolahinia, E., Ahmadian, E., et al. (2022). Curcumin nanoformulations: Beneficial nanomedicine against cancer. *Phytotherapy Research*, *36*(3), 1156– 1181

Manjuladevi, K., & Velmurugan, R., (2020). A perspective view on formulation and optimization of curcumin loaded phycocyanin nanosponges. *International Journal of Research in Pharmaceutical Sciences* , *11*(4), 8119-8123

Neil, V. K., & Sandeep, M., (2016). Therapeutic potential of curcumin for the treatment of brain tumors. *Oxidative Medicine and Cellular Longevity*, *1*, 1-14

Neumaier, F., Zlatopolskiy, B.D., & Neumaier, B. (2021). Drug Penetration into the Central Nervous System: Pharmacokinetic Concepts and In Vitro Model Systems. *Pharmaceutics*, *13*, 1542

Nikandish, N., Hosseinzadeh, L., HematiAzandaryani, A., & Derakhshandeh, K. (2016). The Role of Nanoparticle in Brain Permeability: An in-vitro BBB Model. *Iranian journal of Pharmaceutical Research*, *15*(2), 403–413.

Pardridge W. M. (2005). The blood-brain barrier: bottleneck in brain drug development. *NeuroRx: The Journal of the American Society for Experimental NeuroTherapeutics*, *2*(1), 3–14

Polli, J.W., Humphreys, J.E., Wring, S.A., Burnette, T C., et al. (2000). Comparison of MDCK and bovine brain endothelial cells (BBECs) as a blood-brain barrier screen in early drug discovery. In M. Balls, A.M. van Zeller, & M Halder (Eds.) Progress in the Reduction, Refinement and Replacement of Animal Experimentation (pp 271–289), *New York: Elsevier Science*

Prathima, S., & Sreeja, K. (2013). Formulation and Evaluation of Voriconazole loaded nanosponges for oral and topical delivery. *International Journal of drug delivery and research*, *5*(1), 55-69

Pushpalatha, R., Selvamuthukumar, S., & Kilimozhi, D. (2018). Cross-linked, cyclodextrin-based nanosponges for curcumin delivery - Physicochemical characterization, drug release, stability and cytotoxicity. *Journal of Drug Delivery Science and Technology*, *45*, 45–53

Sánchez-Dengra, B., González-Álvarez, I., González-Álvarez, M., & Bermejo, M. (2021). New *In-Vitro* methodology for kinetics distribution prediction in the brain. An additional step towards an animal-free approach. *Animals*, *11*(12), 3521

Suresh, T., Fong, Y. C., & Chia, H. S. (2020). Advancements in the Blood–Brain Barrier Penetrating, Nanoplatforms for Brain Related Disease Diagnostics and Therapeutic Applications. *Polymers*, *12*, 3055

Susanna, G., Alice, C., Andrea, B., Riccardo A.. et al. (2020). Nanosponges for the protection and release of the natural phenolic antioxidants quercetin, curcumin and phenethyl caffeate. *Materials Advances*, *1*, 2501-2508

Taub, M. E., Kristensen, L., & Frokjaer, S. (2002). Optimized conditions for MDCK permeability and turbidimetric solubility studies using compounds representative of BCS classes I-IV. *European Journal of Pharmaceutical Sciences: official journal of the European Federation for Pharmaceutical Sciences, 15*(4), 331–340

Yallapu, M. M., Jaggi, M., & Chauhan, S. C. (2013). Curcumin nanomedicine: a road to cancer therapeutics. *Current Pharmaceutical Design, 19*(11), 1994–2010

Tejashri, G., Bajaj, A., & Jain, D. (2013). Cyclodextrin based nanosponges for pharmaceutical use: A review. *Acta Pharmaceutica, 63*, 335–358

Velmurugan, R., Manjuladevi, K., Keerthi, G., Yamuna, R., et al. (2019). A method to enhance blood brain barrier permeability of curcumin. *The Patent office Journal No. 46/2019*, Application No 201941045090A, 53972

Wan, D. H., Zheng, B. Y., Ke, M. R., Duan, J. Y., et al. (2017). C-Phycocyanin as a tumour-associated macrophage-targeted photosensitiser and a vehicle of phthalocyanine for enhanced photodynamic therapy. *Chemical Communications (Cambridge, England), 53*(29), 4112-4115

Wang, M., Zhang, Y., Sun, B., Sun, Y., et al. (2014). Permeability of exendin-4-loaded chitosan nanoparticles across MDCK cell monolayers and rat small intestine. *Biological & pharmaceutical bulletin, 37*(5), 740–747

Yeh, T. H., Hsu, L. W., Tseng, M. T., Lee, P L., et al. (2011). Mechanism and consequence of chitosan-mediated reversible epithelial tight junction opening. *Biomaterials, 32*, 6164–6173

Zanotto-Filho, A., Braganhol, E., Edelweiss, M. I., Behr, A G., et al. (2012). The curry spice curcumin selectively inhibits cancer cells growth in vitro and in preclinical model of Glioblastoma. *The Journal of Nutritional Biochemistry, 23*(6), 591-601

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Ameliorating Direct Blue Dye Degradation Using *Trametes versicolor* Derived Laccase Enzyme Optimized through Box–Behnken Design (BBD) via Submerged Fermentation

Umamaheswari Ramasamy[1], Ramkumar Lakshmanan[1*], Mythili Ravichandran[1,3], Prabu Periasamy[2], Shanmugam Sengodan[1]

[1]Department of Microbiology, K.S. Rangasamy College of Arts and Science (Autonomous), Tiruchengode, Namakkal, India
[2]Department of Biotechnology, Periyar University PG Extension Center, Dharmapuri, India
[3]Department of Microbiology, Vivekanandha Arts and Science College for Women, Sankari, Namakkal, India

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KEYWORDS

Laccase

Trametes versicolor

Fruit peels

Dye decolorization

Box-Behnken design

ABSTRACT

The major intend of this study was to elucidate the laccase production by *Trametes versicolor* under submerged fermentation using fruit waste peel as substrate. The textile dye was decolorized by the procured crude enzymatic extract using the response surface methodology. The submerged media with organic fruit peel waste extract (jackfruit, pineapple & kaffir) supplemented with gypsum, calcium carbonate, and nutrient broth were considered superior for laccase production. The produced laccase enzyme was used in dye decolorization at the optimum conditions using the Box-Behnken design. Subsequently, the experiment was designed with four variables (dye concentration, pH, temperature & time) with three factors to achieve the maximum direct blue dye decolorization. The highest laccase activity level was obtained from jackfruit peel extract with 3.86U/ml on 15th day at 25^{o}C with pH 5.0 when compared to the other two extracts. The maximum laccase activity with guaiacol was obtained at optimum pH 4 and 40^{o}C. The predicted value was experimentally validated by attaining 81.25% of dye color removal. From the result, the optimum conditions for direct blue color removal were: dye concentration 40ppm, pH 4.0, temperature 40^{o}C at 24 hours. From the results of this study, it was concluded that the jack fruit peel was a more suitable substrate for laccase production. The dye decolorization results were recommended that Box-Behnken design for parameters optimization. The *T. versicolor* laccase was more proficient for textile dye decolorization. The opportunity was created by using the laccase enzyme for the biological treatment of textile dyeing effluent before discharging into the environment.

* Corresponding author
E-mail: lakshmananram75@gmail.com (L. Ramkumar)

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1 Introduction

A Phenomenon of economic expansion and urbanization progression led to the inundation of toxic materials discharged from the industry. Typically, the disposal of the complex pollutant deposition was hazardous to environmental health (Crawford et al. 2022) and also a challenging task (Shaban et al. 2017). Various options were available to control these pollutants. Unfortunately, the physical-chemical methods led to immense expenses towards investments such as the ozonation plant which was a batchwise setup (de Boer et al. 2022), the operation cost of running an anaerobic reactor for a long duration (Muthukumar et al. 2004), poor stability of electrodes (Bayramoglu et al. 2007). Even though these methods had a good beginning but faced unfavorable ends like deposits of sludge and toxic by-products many countries, including India (Szostek et al. 2022; Ali et al. 2013), impractical electrodes maintenance (Macedo et al. 2021), low efficiency besides all types of dyes (Jebapriya and Gnanadoss 2013), needed more improvement in efficiency by filtration process (Cescon and Jiang 2020), election and cleaning of filtration, and sometimes the effluent flow rate might affect the reactor (Muthukumar et al. 2004). Occasionally, the intricate matrix in the textile effluent hold noxious properties (Jalal et al. 2021) which would affect the microorganisms and lead to an elongated process (Husain 2010) and nevertheless if disintegrated the chemicals in the environment. But some reports confirmed that the metabolites obtained during degradation become more vicious than parent dye compounds (Manavalan et al. 2013). However, the biological option could make a viable one.

Over the past few decades, enzymatic treatment for textile dye decolorization was widely practiced. The major advantages of this method were it could react in a wide range of pH, different temperatures, substrate specificity, no sludge accumulation, and appropriate for various effluents (Sathishkumar et al. 2010; Songserm et al. 2012; Manavalan et al. 2013; Cordova-Villegas et al. 2019). Singh et al. (2022) also proved that biological treatment had a potential way than standard methods. Despite its dye degradation potential, the earlier reports described the predilection of laccase superiority over the physical-chemical methods. In addition to recycling, stability, and lifetime of laccase by immobilization (Shokri et al. 2021), no radical mechanisms are needed to cleave the bond of the dye structure (Upadhyay et al. 2016), and eradication of dye lethal properties (Shekher et al. 2011). Furthermore, the laccase could endure in both phenolic and non-phenolic substances, and due to high tolerance towards noxious waste led to simplifying the man-made dye (Madhavi and Lele 2009).

Laccase (EC 1.10.3.2: p-diphenol: dioxygenoxidoreductase), a blue multicopper protein belonged to an oxidase group and existed in four categories of living organisms like bacteria, and insects, fungi, and higher plants (Shekher et al. 2011; Agustin et al. 2021). Perhaps, due to attentiveness toward polyphenol group oxidation and substrate specificity, laccase was used in bioremediation, dye color removal, pulp and paper bleaching, and wastewater treatment (Okwara et al. 2021). Freshly, laccase had gained application in food processing as a wine stabilizer, juice stabilizer, and as biocatalyst (Backes et al. 2021). However, the enzyme played a crucial role in bioremediation as a multifarious mixture of chemical endocrine disruptors (Becker et al. 2017; Villalba-Rodriguez et al. 2022). *T. versicolor* was the most excellent laccase producer when compared with other white-rot fungi (Sun et al. 2021).

A massive enhancement of industrial activities led to the accumulation of waste in the surroundings. The accumulated organic wastage would become a major perplexity associated with environmental pollution. The agro-industrial waste mainly consisted of valuable components (Freitas et al. 2021) like carbohydrates, protein, a complex polysaccharide, and polyphenol components (Yusuf 2017). Owing to the poor waste management system, the deployment of this valuable component had converted into valued integrated products now established the world over (Levin et al. 2012). Thus the economy changed its way from the "take-make-dispose" to the "take-make-use" model (Russo et al. 2021). The fruit wastes from the pabulum industries were utilized as substrates for engendering the enzymes, which made it less investment for the industrialist (Yusuf 2017). Using *T.versicolor*, the fruit peel like jackfruit, pineapple, and kaffir was taken as substrates for laccase production by submerged fermentation. The main objective of this study is to obtain the laccase enzyme from the waste fruit peels and incorporate it into the textile dye to achieve higher potential decolorization.

2 Materials and Methods

2.1 Media formulation and submerged fermentation

T.versicolor (MTCC No: 138) was collected from Chandigarh, India, and was periodically subcultured and maintained at 4^{o}C on Potato Dextrose Agar (PDA) slant for further processing. For laccase screening, the five days old fungus was inoculated in the PDA plate with 2mM of guaiacol. These plates were incubated at 25^{o}C for 5 days with free guaiacol as control.

The lignocellulose organic substrates of fruit peels *viz.*, jackfruit, pineapple, and kaffir were soaked in 83.17 mM KOH solution for one hour and dehydrated at 60^{o}C in a tray drier (Rosales et al. 2002). The 10g of dried substrates were boiled separately in 500ml distilled water till they attain 100ml. Then, the substrates had undergone homogenization and filtration. A submerged fermentation culture was created in a 250ml Erlenmeyer flask each containing 100ml homogenized extract, 1.3g of nutrient broth, and 1g of both gypsum and calcium carbonate. The pH was adjusted to

5.0. Then, the flask was autoclaved at 121°C for 15 minutes, inoculated with five days old fungal mycelium, and incubated at 25°C for 30 days (Xin and Geng 2011).

2.2 Downstream process and enzyme assay

For this, 20 ml of sterile distilled water was added into the fermentation flask, mixed thoroughly for 20 minutes in the shaker, and filtered through a muslin cloth. The extraction was centrifuged at 5,000 rpm for half an hour to remove the slurry (Patel and Gupte 2016). The obtained supernatant was known as crude enzyme and was stored in the sterile container at -20ºC for further work. The laccase enzyme activity was determined using guaiacol as substrate under a double beam UV spectrophotometer at 450 nm (Desai et al. 2011).

2.3 Effect of Laccase activity at different pH and temperature

The effect of pH on laccase activity was analyzed using different pH ranges of 3 to 11 with sodium acetate buffer at 30°C for 15 minutes. The effect of temperature on laccase activity was measured by varying the incubation temperature between 20°C - 60°C at the optimum pH for 15 minutes and the residual activity was dignified using guaiacol as substrate. All the experiment was performed in triplicate.

2.4 Optimization of dye decolorization

In the present study, the decolorization was carried out in 10ml of the test tube with enzyme volume under static conditions. The dye decolorization effectiveness was achieved by the partially purified enzyme. The 5ml of the reaction mixture in the test tube consisted of an equal volume of dye solution and crude laccase enzyme and 3ml of 50 mM of sodium acetate buffer and incubated at 30°C. The percentage of color removal was detected under a UV spectrophotometer at 587 nm (Sathishkumar et al. 2010).

The direct blue azo dye decolorization was optimized by RSM based Box-Behnken method employed by Statease Design Expert software (version 11) to minimize the number of experiments carried out to analyze the data.. Dye concentration (30, 40, 50 ppm), pH (2, 4, 6), temperature (30°C, 40°C, 50°C), and time (12, 24, 36 hours) were expressed as X_1, X_2, X_3, X_4 respectively to evaluate the decolorization as a response. The four major independent variables could be approached by the quadratic model equation as given.

$$Y= b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4$$

Where Y response, b_0 constant, X_1, X_2, X_3, and X_4 were dye concentration, pH, temperature, and time respectively, b_1, b_2, b_3, and b_4 were linear coefficients, b_{11}, b_{22}, b_{33}, and b_{44} were quadratic coefficients, b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, and b_{34} were cross-product coefficients. To barricade the unexpected variability in the visually examined replications the experimental runs were accomplished randomly.

The dye decolorization was recorded for the entire factors using Box-Behnken Method. Following that, it was statistically analyzed by ANOVA (Two-way table) to analyze the interaction between the independent variables. In addition, it was also assessed the fitness of the model based on their interactions.

3 Results

3.1 Medium optimization on laccase activity

T.versicolor was grown in the guaiacol plate for laccase screening test. The growth of the fungi had been deferral due to the effect of the guaiacolsubstrate destruction. After a week of incubation, the culture had developed brown color around the colony. It was indicated that the *T.versicolor* was a laccase producer. Furthermore, the *T.versicolor* was cultivated on three different lignocelluloses organic substrates along with nutrient broth, gypsum, and calcium carbonate to enhance its growth.

The laccase production by *T.versicolor* on lignocellulose organic substrates under submerged fermentation was observed and the result was recorded (Figure 1). The enzyme activity was quantified every alternative day from the third day of inoculum till the enzyme level truncates. The laccase enzyme activity was gradually incremented from the 5th day and progressively increased up to the 15th day and then the activity declined from the 16th to 22nd day. It was found that the laccase enzyme activity on jackfruit peels was 3.86 U/ml, which was higher than pineapple peel at 2.96 U/ml, and kaffir peel at 2.66 U/mlon 15th day. For further incubation after15th day, the level of the enzyme was diminished.

3.2 Effect of Laccase activity at different pH and temperature

The pH was an essential and significant factor, that influenced the extracellular laccase production during fermentation. The optimum pH was determined by changing the pH range from 3-11 in sodium acetate buffer solution. In this study, the laccase activity was increased at pH 4.0. By increasing in pH range above pH4.0, a sharp decline was reported in the enzyme activity (Figure 2).

Another important factor was the temperature which thermal stability was determined with different temperatures ranging from 20°C - 60°C with 10°C intervals at optimum pH. The enzyme activity versus temperature showed a great increased activity at 40°C when compared to 20°C and 30°C. After a prolonged increase in the temperature above 40°C, the laccase activity was diminished (Figure 3).

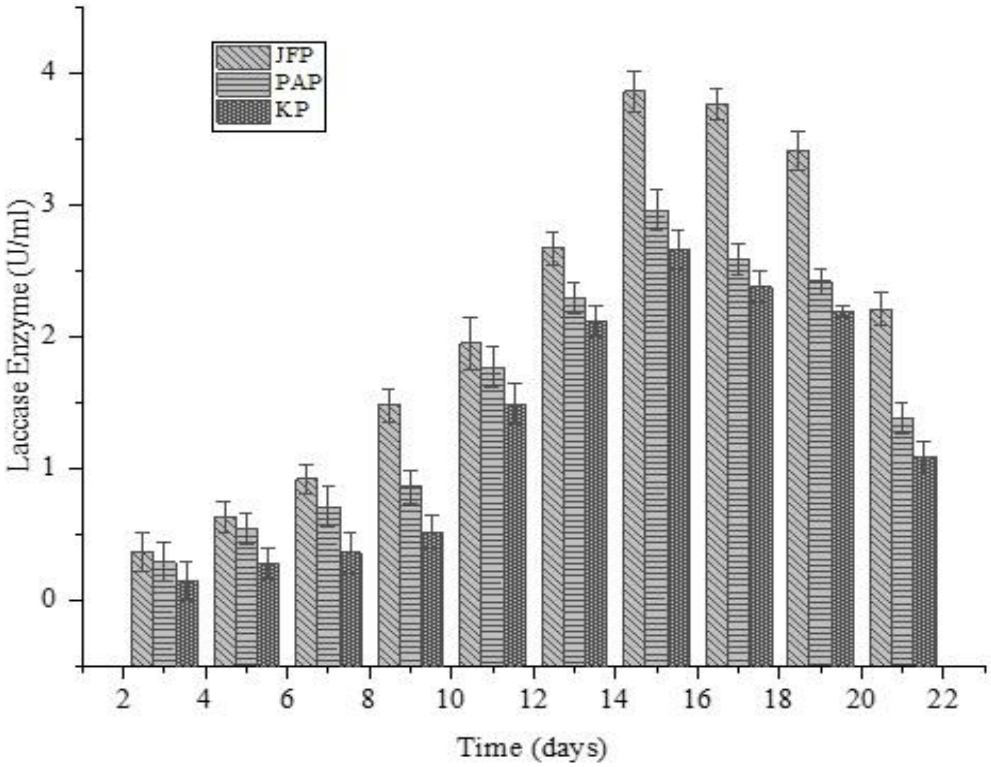


Figure 1 Production of Laccase under submerged fermentation using three different organic fruit peel wastes

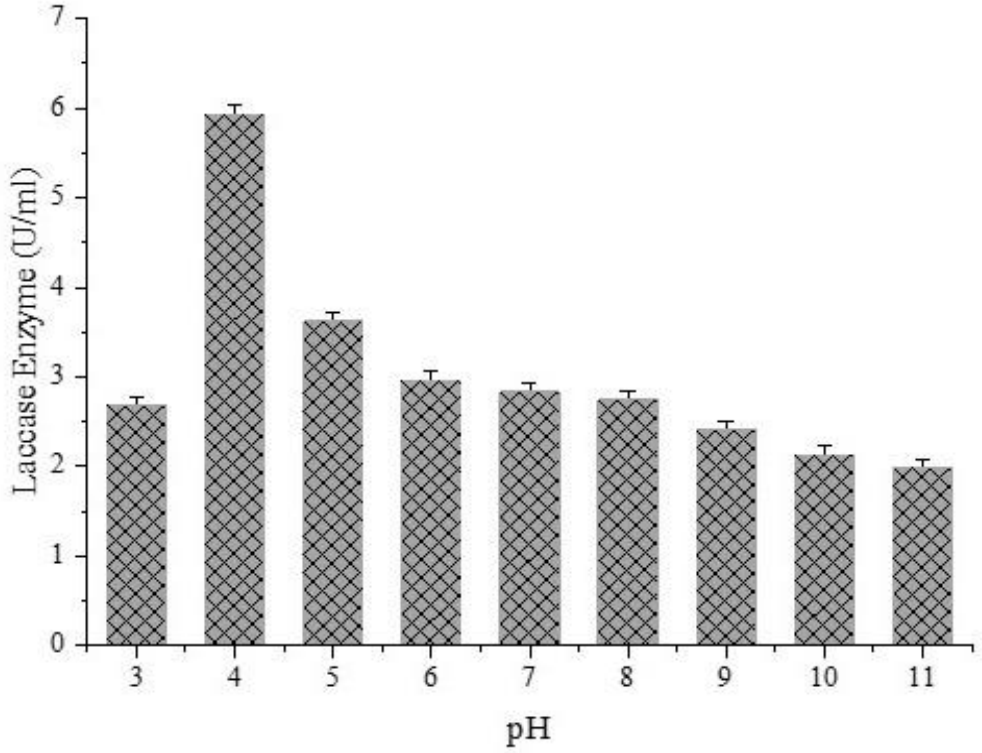


Figure 2 Effect of different pH on laccase activity

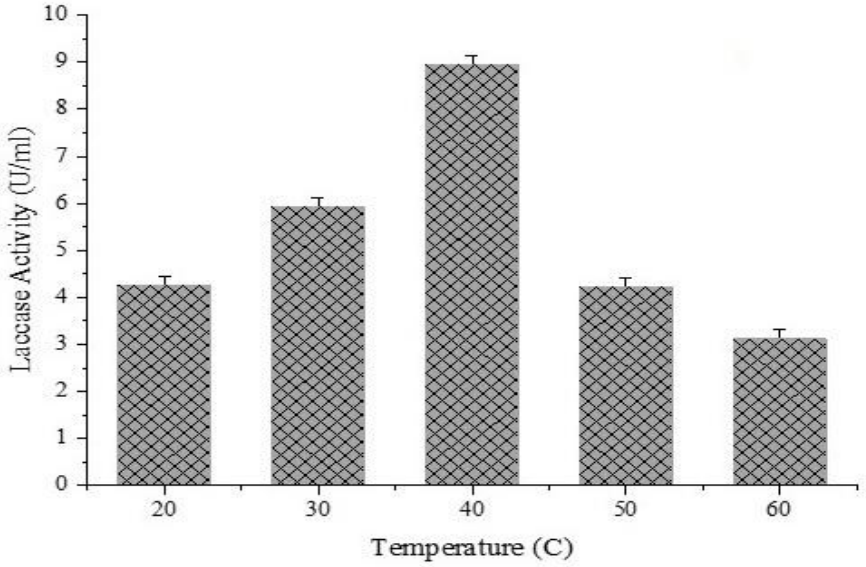


Figure 3 Effect of different temperatures on laccase activity

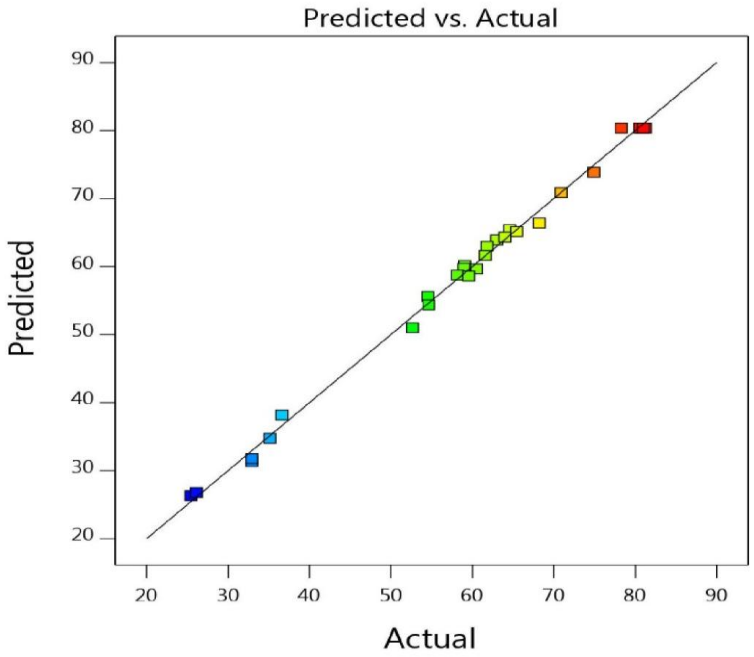


Figure 4 Box-Behnken design plot for actual versus predicted values for dye color removal

3.3 Dye decolorization Box-Behnken Method

By using the Box-Behnken design, the effect of independent variables *viz*., dye concentration (X_1), pH (X_2), temperature (X_3), and time (X_4) were systematically investigated by running 29 experiments. The level of independent variables used in the decolorizing of direct blue dye was given in table 1. In our study, the obtained result (Figure 4) was stated that, the actual and predicted response for direct blue dye decolorization. The experiment data were analyzed using ANOVA (Two-way table) to ascertain the interaction between the independent variables and the response by the quadratic model. The first and second-degree effects of all the variables were significant except for initial dye concentration, which was not omitted for the model. The second-order polynomial equation had been fitted to the data by the multiple regression procedure. This observation had been related to the response with the four factors as the below equation:

$Y(\%) = +80.36 - 0.9308X_1 + 16.13X_2 - 0.5308X_3 + 5.93X_4 - 31X_1^2 - 22.89X_2^2 - 12.28X_3^2 - 8.86X_4^2 + 0.7800X_1X_2 + 0.4275X_1X_3 + b_{14}X_1X_4 - 3.83X_2X_3 + 0.2175X_2X_4 + 1.78X_3X_4$

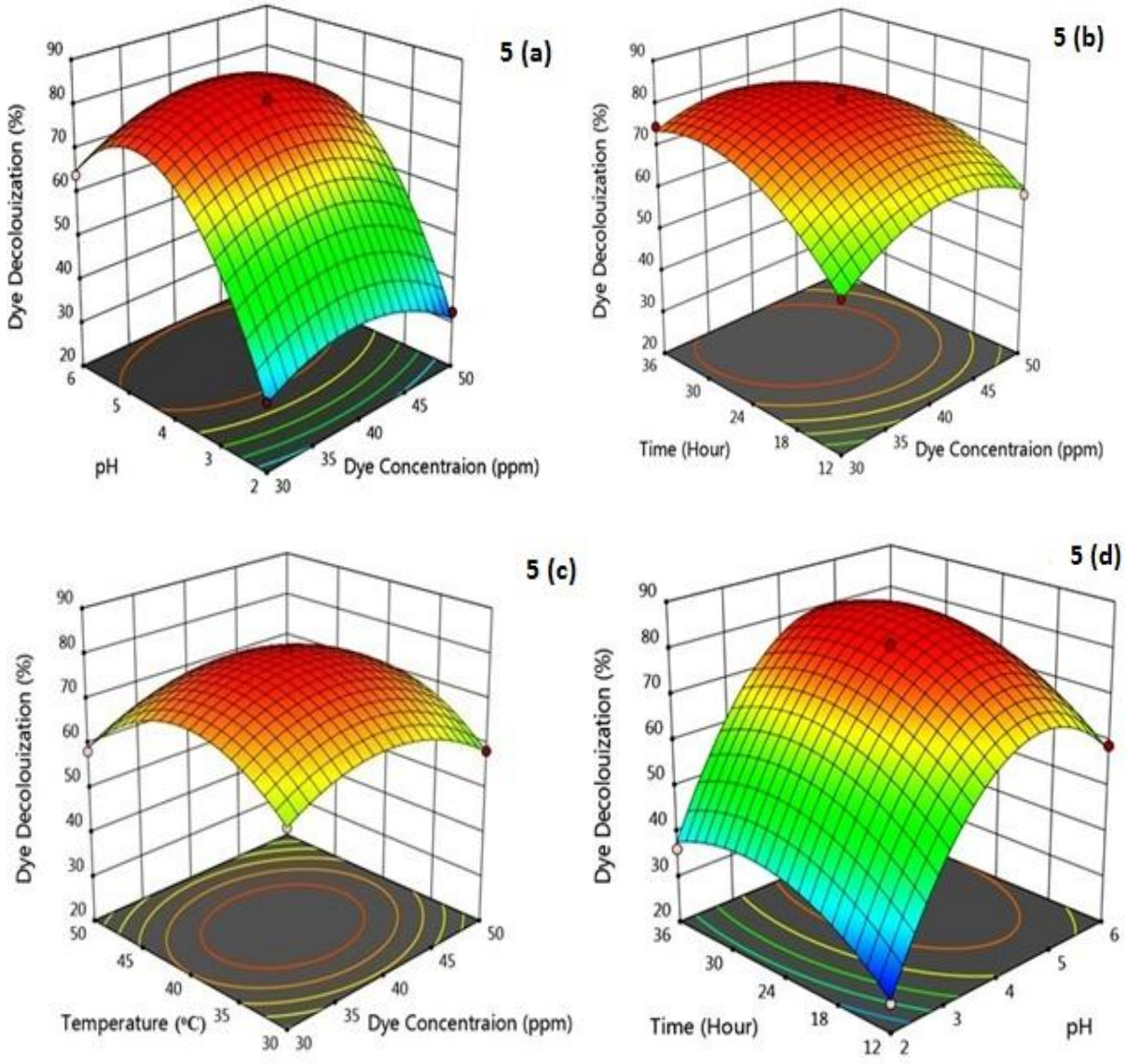
Furthermore, the ANOVA had been engaged to examine the fitness of the model. The high F-value of 264.61 and P-value < 0.0001 indicated that the model was significant which was less than 0.05 with the value of R^2= 0.9962. In addition, the experimental value of predicted R^2 = 0.9814 was coincidental with the adjusted R^2= 0.9925. The ANOVA result was satisfied with the experimental data of the quadratic model.

The ANOVA result was satisfied with the experimental data of the quadratic model for the independent variables involved in

Table 1 Box–Behnken design matrix for Direct Blue dye decolorization with a comparison of theoretical and experimental values

Run	Independent Variables				Response		
	DB Dye Concentration (ppm)	pH	Temperature (°C)	Time (Hour)	DB Dye Decolorization (%)	Predicted Value	Residue
	X_1	X_2	X_3	X_4	Y	Y^1	
1	40	6	40	36	70.89	70.89	-0.0008
2	40	2	30	24	32.91	31.79	1.1246
3	50	2	40	24	32.91	31.31	1.5963
4	40	4	40	24	81.25	80.36	0.8880
5	40	6	30	24	60.49	59.65	0.8362
6	40	6	50	24	61.79	62.99	-1.1971
7	50	4	30	24	59.06	58.94	0.1225
8	50	4	40	12	59.06	60.16	-1.1038
9	40	4	30	36	62.98	63.90	-0.9238
10	30	4	30	24	61.58	61.65	-0.0742
11	40	2	50	24	25.42	26.33	-0.9088
12	40	2	40	36	36.62	38.19	-1.5725
13	40	4	40	24	80.75	80.36	0.3880
14	40	4	40	24	78.29	80.36	-2.0720
15	40	4	50	12	52.64	50.99	1.6513
16	30	2	40	24	35.15	34.74	0.4146
17	40	4	40	24	80.54	80.36	0.1780
18	50	4	50	24	58.15	58.73	-0.5808
19	40	4	40	24	80.98	80.36	0.6180
20	40	4	50	36	68.21	66.40	1.8129
21	30	4	40	12	54.64	54.37	0.2746
22	50	6	40	24	65.45	65.14	0.3129
23	50	4	40	36	64.01	64.36	-0.3471
24	40	4	30	12	54.52	55.61	-1.0854
25	40	2	40	12	26.12	26.77	-0.6542
26	30	6	40	24	64.57	65.44	-0.8688
27	30	4	50	24	58.96	59.74	-0.7775
28	40	6	40	12	59.52	58.60	0.9175
29	30	4	40	36	74.91	73.88	1.0313

decolorization. Besides, the regression equation was characterized as three-dimensional surface plots of graphical representations. Figure 5 (a-f) represented the interaction between the two variables and the other two was constant at the central values. The central values of all the variables were dye concentration of 40 ppm, pH 4, the temperature of 40^{o}C, and the time interval of 24 Hrs. Figure 5a showed that the maximum decolorization was obtained at acidic pH whereas the percentage decreases at pH 6. The decolorization was not achieved at basic pH and also increase in dye concentration ensured a change in the color removal. The surface plot of Figure 5b was shown when the time duration increased up to 24 hours for an increase in the decolorization process due to the depletion of enzyme volume. In our study, the maximum result was obtained in 24 hours and there was no change in percentage by prolonged incubation. Figure 5c designated that the decolorization had increased when the temperature increased up to 40^{o}C. The decolorization was faintly improved at temperature versus dye concentration when compared to pH versus dye concentration. The increase in the dye concentration did not affect the decolorization as did the pH and temperature affected. Figure 5d represented the change in both reaction time and pH providing an increment in decolorization. Figure 5e illustrated that the decolorization was affected when temperature and pH increased up to a certain level. The optimum pH and temperature contributed to a better response to decolorization. Figure 5f expounded that the decolorization had increased with the time duration and it also had increased to a particular hour and then equilibrium was sorted out.



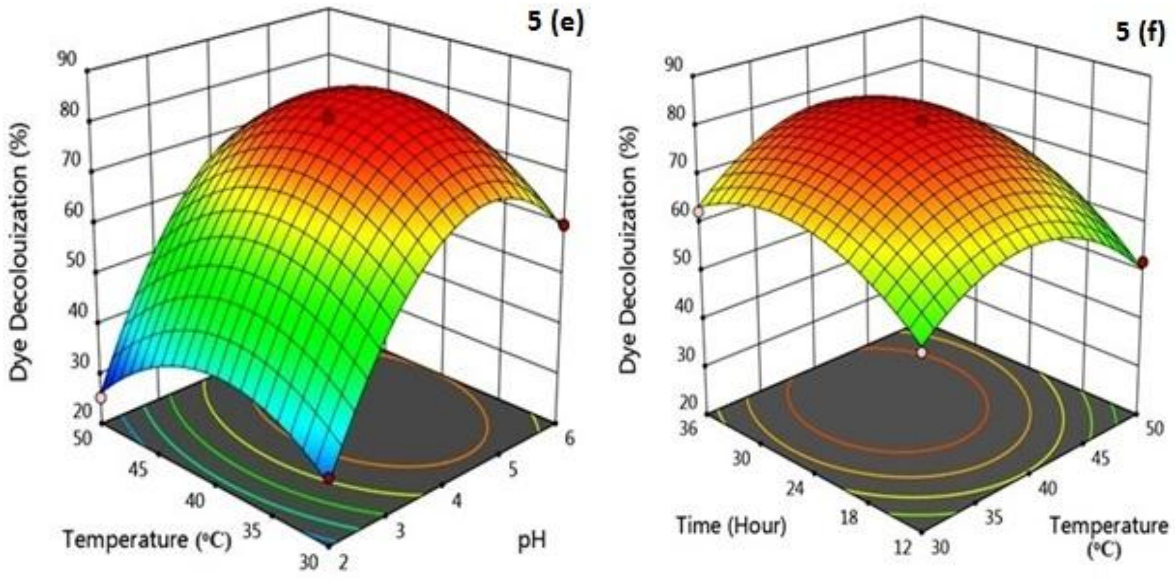


Figure 5 (a-f) Surface graph of direct blue dye decolorization displaying the interaction of the parameters like dye concentration, pH, temperature, and time interval.

The Direct Blue dye spectrum in the visible section exhibited a maximum peak at 587 nm. The UV visible scan of the treated dye showed the desertion of the band and a substantial decrease in the peak compared to the untreated band which directed the significant amendment in the dye structure due to the oxidation of guaiacol substrate as revealed in Figure 6. In this study, the optimum pH value was 4 and the detected temperature was 40°C for direct blue dye with 40 ppm dye concentration at 24 hours. The results of the study showed that the rate of decolorization of crude laccase enzyme was recorded to be 81.25% and it was obvious that there was an increased rate of decolorization than the purified one, which was recorded to be 49%.

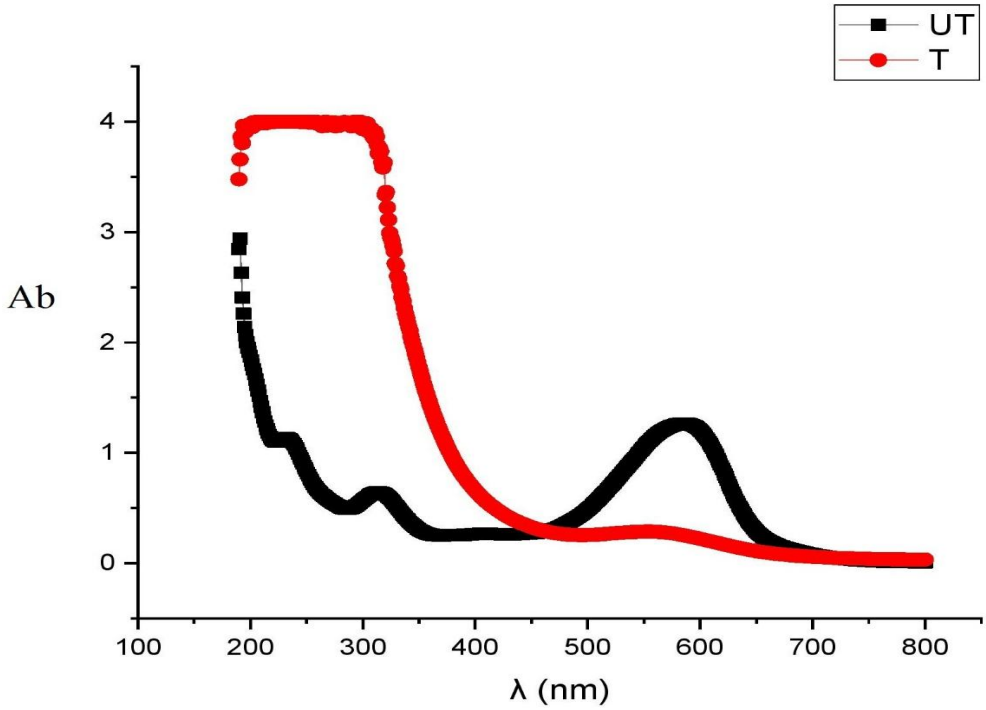


Figure 6 UV –visible absorption spectra of direct blue dye before (UT) and after (T) decolorization by crude laccase enzyme

4 Discussion

The *T.versicolor* involved in this study was proved as a laccase producer based on its efficacy. Most of the research studies proved that the *T.versicolor* was one of the potent laccase producers among the white-rot fungi (Okwara et al. 2021). Ramkumar et al. (2010, 2011) exhibited that the inorganic substrates like gypsum and calcium carbonate encouraged the high yield of enzyme when incorporated into lignocellulose organic substrate and also additionally performed as additives for the mycelium growth. The nutrient broth contained peptone, yeast extract, and beef extract which acted as nitrogen and vitamin sources that enhanced the fungal growth and thus the laccase engendered (Garzillo et al. 1992). The optimum pH range and temperature for *T.versicolor* were 4 - 6 and 25°C - 30°C respectively (Jo et al. 2010; Dos Santos Bazanella et al. 2013; Latif et al. 2022). Moreover, it was worth probing for incipient substrates like jackfruit peel, pineapple peel, and kaffir peel, especially if they were available in generously voluminous amounts, allowing the white-rot fungi to produce a maximum peak of enzymes. Romelle et al. (2016) reported that the cellulose content in jackfruit peel (27.75%) was higher than in pineapple (14.80%) and kaffir fruit peel (12.72%). Results of this study suggested that jackfruit peel could be used as a lignocellulosic organic substrate for further study. In the year 2013, Dos Santos Bazanella et al. (2013) reported that the maximum laccase activity by *Pleurotus* sp was obtained using pineapple peel when compared to wheat bran. *T.versicolor* had obtained laccase activity of 60.73 U/g using pineapple crowns as substrate (Backes et al. 2022)

The best laccase activity was determined at pH 4. It was obvious that the enzyme activity was greater under acidic conditions than in the alkaline condition. The change in the optimum pH strongly depended on the substrates involved in the fermentation media. The nutritional composition in the medium was affected due to an increase in pH which leads to low microbial growth (Braunschmid et al.2021). This was due to hydroxide anion bonding with T_2/T_3copper site subsequent inhibition in the enzyme activity which affected electron transfer during oxidation (Sousa et al. 2021). Amari et al. (2021) reported that the laccase from *T.versicolor* had an optimum pH in acidic conditions and correlated with the current result.

Like pH, the temperature was energy which played a vital role in the enzymatic reaction. The maximum laccase activity was achieved at 40°C due to an increased speed in kinetic energy and also the enzyme interaction between protein molecules and substrates to a certain temperature range (Kurniati et al. 2022). The reason for declined activity towards high temperature was due to protein denature and also loss of the three-dimensional structure. The preceding report also defined the optimum temperature for free laccase was retained at 55°C and 65°C for immobilized laccase (Amari et al. 2021). From these results, the optimum temperature obtained could be successfully used for industrial effluent treatment, which would be below 60°C (Zang et al. 2022; Ivanka et al. 2010).

Box-Behnken Method (BBM) was one of the principles of Response Surface Methodology (RSM), as a potential empirical evidence tool for decolorization. It was evaluated with mathematical and statistical methods, which related between independent variables and responses (Akar et al. 2021). The previous report of Cordova-Villegas et al. (2019) proved that the maximum removal of azo dye color was obtained at the optimum pH of 3-5. The same report was also proved by Birhanli et al. (2022) for the effective decolorization of azo dye. Generally, the fungal laccase was more active at acidic pH whereas bacterial laccase was active at basic and neutral pH (Coria-Oriundo et al. 2021) The other study reported that the maximum laccase activity was at acidic pH and low activity was observed at basic pH (Iqbal et al. 2021). However, the increase in dye concentration might affect the enzyme activity due to the toxicity of the concentration (Barathi et al. 2022; Hafshejani et al. 2014), which was directly linked to our results. The Direct blue dye was decolorized at 24 hours and no more decolorization was observed after 48 hours which was correlated with the report of Darvishi et al. (2018). The change in both reaction time and pH provides an increment in decolorization. Wikee et al. (2019) proved that the temperature for laccase stability was up to 50°C and if it increased after this the enzyme would be unstable. However, the decolorization was affected when temperature and pH were increased up to a certain level. It led to enzyme inactivation due to high temperature and the dispersion of dye molecules across the matrix due to the increase in pH (Ranimol et al. 2021). The decolorization increases with the time duration and equilibrium was sorted out and the temperatures above 40°C inhibit the enzyme activity leading to diminishing the decolorization (Iqbal et al. 2021). The antecedent reports proved that null decolorization occurs below 25°C and above 50°C (Hafshejani et al. 2014).

Based on the analysis, the pH, temperature, dye concentration, and time interval were considered as the most predominant parameters for the decolorization of the textile dye. In this study, an increase in these parameters was excepted dye concentration to brink value, led to an increase in decolorization, and proved the quadratic model. The crude laccase enzyme decolorization efficacy showed more activity than the purified one based on the typical characteristics of the laccase, which would be lacking in purified conditions (Madhavi and Lele 2009; Kandasamy et al. 2022). The results were closely related to Murugesan et al. (2007) who reported that purified laccase required mediator HBT (1-hydroxy benzotriazole) for certain dyes. Hou et al. (2004) also proved that

decolonization was increased from 66% to 90% by the addition of mediator ABTS (2,2′- Azino-di-(3-ethylbenzothialozin-6-sulfonic acid). The decolorization with purified laccase was obtained with 49%, which indicated crude laccase act as a potential candidate to degrade textile dyes in near future.

Conclusion

The obtained result of this study demonstrated the potential production of laccase enzyme through submerged fermentation by *T.versicolor* using agro-waste such as organic peels. Based on this study, the potent lignocellulose organic substrate was identified by comparing the three-waste substrate and from the results of this study, the maximum yield was obtained at 25^{o}C as 3.86U/ml by Jack fruit peel. The maximum laccase activity was recorded at pH 4 and 40^{o}C. The color removal efficiency of the Direct Blue azo dye was 81.25% at dye concentration 40 ppm, pH 4, temperature 40^{o}C at 24 hours by Box-Behnken Method. It was practically proved than a purified one. The prospects of laccase enzyme in decolorization were emphasized more than other endeavors of physical and chemical treatment. Besides the articulated needs of this laccase enzyme in bioremediation, capable of solving environmental pollutants problems like textile effluent treatment and massive application in food industries, the lucrative economic way for the enzyme production using organic waste was undoubtedly proved. The transformation of agro-industrial residues to essential substances might not only provide future aspects to researchers but it also would diminish the existing environmental hazards and enzyme production in the commercial market.

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Conflict of Interest

The authors declare that they have no conflict of interest concerning this work.

References

Agustin, M. B., de Carvalho, D. M., Lahtinen, M. H., Hilden, K., Lundell, T., & Mikkonen, K. S. (2021). Laccase as a Tool in Building Advanced Lignin-Based Materials. *ChemSusChem*, 14(21), 4615-4635. DOI: https://doi.org/10.1002/cssc.202101169

Akar, S.T., Koc, E., Sayin, F., Kara, I., & Akar, T. (2021). Design and modeling of the decolorization characteristics of a regenerable and eco-friendly geopolymer: Batch and dynamic flow mode treatment aspects. *Journal of Environmental Management*, *298*, 113548. DOI: https://doi.org/10.1016/j.jenvman.2021.113548

Ali, L., Algaithi, R., Habib, H.M., Souka, U., Rauf, M.A.,& Ashraf, S.S. (2013). Soybean peroxidase-mediated degradation of an azo dye–a detailed mechanistic study. *BMC biochemistry*,*14*, 1-14.

Amari, A., Alzahrani, F. M., Alsaiari, N. S., Katubi, K. M., Rebah, F. B., & Tahoon, M. A. (2021). Magnetic metal organic framework immobilized laccase for wastewater decolorization. *Processes*, *9*(5), 774. DOI: https://doi.org/10.3390/pr9050774

Backes, E., Kato, C.G., da Silva, T.B., Uber, T.M., Pasquarelli, D.L., Bracht, A., & Peralta, R.M. (2022). Production of fungal laccase on pineapple waste and application in detoxification of malachite green. *Journal of Environmental Science and Health, Part B*, *57*(2), 90-101. DOI: https://doi.org/10.1080/03601234.2022.2025739

Backes, E., Kato-Schwartz, C.G., Corrêa, R.C.G., Moreira, R.d.F.P.M., et al. (2021). Laccases in food processing: Current status, bottlenecks and perspectives. *Trends in Food Science & Technology*, *115*, 445-60.DOI: https://doi.org/10.1016/j.tifs.2021.06.052

Barathi, S.,Aruljothi, K.N., Karthik, C., Padikasan, I.A., & Ashokkumar, V. (2022). Biofilm mediated decolorization and degradation of reactive red 170 dye by the bacterial consortium isolated from the dyeing industry wastewater sediments. *Chemosphere*, *286*, 131914. DOI: https://doi.org/10.1016/j.chemosphere.2021.131914

Bayramoglu, M., Eyvaz, M., & Kobya, M. (2007). Treatment of the textile wastewater by electrocoagulation: economical evaluation. *Chemical Engineering Journal*, *128*, 155-161. DOI: https://doi.org/10.1016/j.cej.2006.10.008

Becker, D., Rodriguez-Mozaz, S., Insa, S., Schoevaart, R., et al. (2017). Removal of endocrine disrupting chemicals in wastewater by enzymatic treatment with fungal laccases. *Organic Process Research & Development*, *21*, 480-491. DOI: https://doi.org/10.1021/acs.oprd.6b00361

Birhanlı, E., Noma, S.A.A., Boran, F., Ulu, A., Yeşilada, Ö., & Ateş, B. (2022). Design of laccase–metal–organic framework hybrid constructs for biocatalytic removal of textile dyes. *Chemosphere*, *292*, 133382. DOI: https://doi.org/10.1016/j.chemosphere.2021.133382

Braunschmid, V., Binder, K., Fuerst, S., Subagia, R., Danner, C., Weber, H., & Guebitz, G. M. (2021). Comparison of a fungal and a bacterial laccase for lignosulfonate polymerization. *Process*

Biochemistry, *109*, 207-213. DOI: https://doi.org/10.1016/j.procbio.2021.07.001

Cescon, A., & Jiang, J. Q. (2020). Filtration process and alternative filter media material in water treatment. *Water*, *12*(12), 3377. DOI: http://dx.doi.org/10.3390/w12123377

Cordova-Villegas, L.G., Cordova-Villegas, A.Y., Taylor, K.E., & Biswas, N. (2019). Response surface methodology for optimization of enzyme-catalyzed azo dye decolorization. *Journal of Environmental Engineering*,*145*(5), 04019013.

Coria-Oriundo, L.L., Battaglini, F., & Wirth, S.A. (2021). Efficient decolorization of recalcitrant dyes at neutral/alkaline pH by a new bacterial laccase-mediator system. *Ecotoxicology and Environmental Safety*, *217*, 112237. DOI: https://doi.org/10.1016/j.ecoenv.2021.112237

Crawford, S.E., Brinkmann, M., Ouellet, J.D., Lehmkuhl, F., Reicherter, K., Schwarzbauer, J., & Hollert, H. (2022). Remobilization of pollutants during extreme flood events poses severe risks to human and environmental health. *Journal of hazardous materials*, *421*, 126691. DOI: https://doi.org/10.1016/j.jhazmat.2021.126691

Darvishi, F., Moradi, M., Jolivalt, C., & Madzak, C. (2018). Laccase production from sucrose by recombinant Yarrowia lipolytica and its application to decolorization of environmental pollutant dyes. *Ecotoxicology and environmental safety*,*165*, 278-283. DOI: https://doi.org/10.1016/j.ecoenv.2018.09.026

de Boer, S., González-Rodríguez, J., Conde, J.J., & Moreira, M. T. (2022). Benchmarking tertiary water treatments for the removal of micropollutants and pathogens based on operational and sustainability criteria. *Journal of Water Process Engineering*, *46*, 102587. DOI:https://doi.org/10.1016/j.jwpe.2022.102587

Desai, S.S., Tennali, G.B., Channur, N., Anup, A., Deshpande, G., & Murtuza, B.A. (2011). Isolation of laccase producing fungi and partial characterization of laccase. *Biotechnol Bioinf Bioeng*, 1, 543-549.

Dos Santos Bazanella, G.C., de Souza, D.F., Castoldi, R., Oliveira, R.F., Bracht, A., & Peralta, R.M. (2013). Production of laccase and manganese peroxidase by Pleurotus pulmonarius in solid-state cultures and application in dye decolorization. *Folia microbiologica*, *58*, 641-647.

Freitas, L. C., Barbosa, J. R., da Costa, A. L. C., Bezerra, F. W. F., Pinto, R. H. H., & de Carvalho Junior, R. N. (2021). From waste to sustainable industry: How can agro-industrial wastes help in the development of new products? *Resources, Conservation and Recycling*, *169*, 105466. DOI: https://doi.org/10.1016/j.resconrec.2021.105466

Garzillo, A.M.V., Di Paolo, S., Burla, G., & Buonocore, V. (1992). Differently-induced extracellular phenol oxidases from *Pleurotus ostreatus*. *Phytochemistry*, *31*, 3685-3690. DOI: https://doi.org/10.1016/S0031-9422(00)97509-5

Hafshejani, M.K., Ogugbue, C.J., & Morad, N. (2014). Application of response surface methodology for optimization of decolorization and mineralization of triazo dye Direct Blue 71 by *Pseudomonas aeruginosa*. *3 Biotech*, 4(6), 605-619.

Hou, H., Zhou, J., Wang, J., Du, C., & Yan B. (2004). Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. *Process Biochemistry*, *39*(11), 1415-1419.DOI: https://doi.org/10.1016/S0032-9592(03)00267-X

Husain, Q. (2010). Peroxidase mediated decolorization and remediation of wastewater containing industrial dyes: a review. *Reviews in Environmental Science and Bio/Technology*,*9*, 117-140.

Iqbal, K., Nadeemm, A., & Zafar, U. (2021). Biostoning of textile effluent with laccase enzyme. *Bangladesh Journal of Scientific and Industrial Research*, *56*(2), 115-24. DOI: https://doi.org/10.3329/bjsir.v56i2.54318

Ivanka, S., Albert, K., & Veselin, S. (2010). Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. *Advances in Bioscience and Biotechnology*, *1*(3), 2010. DOI: 10.4236/abb.2010.13029.

Jebapriya, G.R.,& Gnanadoss, J.J. (2013). Bioremediation of textile dye using white rot fungi: A review. *International Journal of Current Research and Review*, 5(3), 1.

Jalal, G., Abbas, N., Deeba, F., Butt, T., Jilal, S., & Sarfraz, S. (2021). Efficient removal of dyes in textile effluents using aluminum-based coagulant. Efficient Removal of Dyes in Textile Effluents Using Aluminum-Based Coagulant. *Chemistry International*, *7*(3), 197-207. DOI: https://doi.org/10.5281/zenodo.4899952

Jo, W.S., Kang, M.J., Choi, S.Y., Yoo, Y.B., Seok, S.J., & Jung, H.Y. (2010). Culture conditions for mycelial growth of *Coriolus versicolor*. *Mycobiology*,*38*(3), 195-202.DOI: https://doi.org/10.4489/MYCO.2010.38.3.195

Kandasamy, S., Ameen, F., M. Amirul, I., Sudhakar, C., & Selvankumar, T. (2022). Laccase production from *Bacillus aestuarii* KSK using Borassus flabellifer empty fruit bunch waste as substrate and assess their malachite green dye degradation.

Journal of Applied Microbiology, 1-8. DOI: https://doi.org/10.1111/jam.15670

Kurniati, A., Puspaningsih, N.N.T., Putri, K.D.A., Damayanti, M., Purwani, N.N., Rahmah, S. A., & Sanjaya, R. E. (2022). Heterologous fusion gene expression and characterization of a novel carbohydrate binding module (Cbm36) to laccase (Lcc2). *Biocatalysis and Agricultural Biotechnology*, *102377*. DOI: https://doi.org/10.1016/j.bcab.2022.102377

Latif, A., Maqbool, A., Sun, K., & Si, Y. (2022). Immobilization of *TrametesVersicolor*laccase on Cu-alginate beads for biocatalytic degradation of bisphenol A in water: Optimized immobilization, degradation and toxicity assessment. *Journal of Environmental Chemical Engineering*, *10*(1), 107089. DOI: https://doi.org/10.1016/j.jece.2021.107089

Levin, L., Diorio, L., Grassi, E., & Forchiassin, F. (2012). Grape stalks as substrate for white rot fungi, lignocellulolytic enzyme production and dye decolorization. *Revista Argentina de microbiologia*, *44*, 105-112.

Macedo, D.S., Vepsäläinen, M., Acharya, D., Wood, C.D., et al. (2021). An unusually stable solid state Ag| AgCl reference electrode for long term continuous measurements based on a crosslinked poly (vinyl acetate)/KCl composite. *Electrochimica Acta*, *368*, 137636.DOI: https://doi.org/10.1016/j.electacta.2020.137636

Madhavi, V.,& Lele, S. (2009). Laccase: properties and applications. *BioResources*, *4*, 1694-1717.

Manavalan, T., Manavalan, A., Thangavelu, K.P., & Heese, K. (2013). Characterization of optimized production, purification and application of laccase from Ganoderma lucidum. *Biochemical Engineering Journal*, *70*, 106-114.DOI: https://doi.org/10.1016/j.bej.2012.10.007

Murugesan, K., Dhamija, A., Nam, I.H., Kim, Y.M.,& Chang, Y.S. (2007). Decolourization of reactive black 5 by laccase: optimization by response surface methodology. *Dyes and Pigments*, *75*(1):176-84. DOI: https://doi.org/10.1016/j.dyepig.2006.04.020

Muthukumar, K., Sundaram, P.S., Anantharaman, N., & Basha, C.A. (2004). Treatment of textile dye wastewater by using an electrochemical bipolar disc stack reactor. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, *79*, 1135-1141. DOI: https://doi.org/10.1002/jctb.1104

Okwara, P., Afolabi, I.S., & Ahuekwe, E.F. (2021). Application of laccase in aflatoxin B1 degradation: a review. In *IOP Conference Series: Materials Science and Engineering*, *1107*(1), 012178. DOI:https://doi.org/10.1088/1757-899X/1107/1/012178

Patel, H., & Gupte, A. (2016). Optimization of different culture conditions for enhanced laccase production and its purification from Tricholoma giganteum AGHP. *Bioresources and Bioprocessing*, *3*, 1-10.

Ramkumar, L., Ramanathan, T.,& Nedumaran, T. (2011). In Vitro effect of Organic and inorganic additives from The production of radial mycelial growth and lignocellulolytic enzyme in Lentinus Edodes (berk.) SING. *Emirates Journal of Food and Agriculture*, *23*(1), 71-79.

Ramkumar, L., Thirunavukkarasu, P.,& Ramanathan, T. (2010). Development of improved technology for commercial production and preservation of shiitak mushroom (Lentinus edodes). *American-Eurasian Journal of Agricultural & Environmental Sciences*,*7*, 433-438.

Ranimol, G., Paul, C., & Sunkar, S. (2021). Optimization and efficacy studies of Laccase immobilized on Zein-Polyvinyl pyrrolidonenano fibrous membrane in decolorization of Acid Red 1. *Water Science and Technology*, *84* (10-11): 2703–2717. DOI: https://doi.org/10.2166/wst.2021.200

Romelle, F.D., Rani, A.,& Manohar, R.S. (2016). Chemical composition of some selected fruit peels. *European Journal of Food Science and Technology*,*4*, 12-21.

Rosales, E., Couto, S.R.,& Sanromán, A. (2002). New uses of food waste: application to laccase production by *Trametes hirsuta*. *Biotechnology Letters*, *24*, 701-704.

Russo, C., Maugeri, A., Lombardo, G.E., Musumeci, L., Barreca, D., Rapisarda, A., & Navarra, M. (2021). The second life of Citrus fruit waste: A valuable source of bioactive compounds. *Molecules*, *26*(19), 5991. DOI: https://doi.org/10.3390/molecules26195991

Sathishkumar, P., Murugesan, K.,& Palvannan, T. (2010). Production of laccase from *Pleurotus florida* using agro-wastes and efficient decolorization of Reactive blue 198. *Journal of basic microbiology*, *50*, 360-367.DOI: https://doi.org/10.1002/jobm.200900407

Shaban, M., Abukhadra, M.R., Ibrahim, S.S., & Shahien, M.G. (2017). Photocatalytic degradation and photo-Fenton oxidation of Congo red dye pollutants in water using natural chromite—response surface optimization. *Applied Water Science*, *7*, 4743-4756.

Shekher, R., Sehgal, S., Kamthania, M., & Kumar, A. (2011). Laccase: microbial sources, production, purification, and potential biotechnological applications. *Enzyme research*, *2011*, 217861. DOI:https://doi.org/10.4061/2011/217861

Shokri, Z., Seidi, F., Karami, S., Li, C., Saeb, M.R., & Xiao, H. (2021). Laccase immobilization onto natural polysaccharides for biosensing and biodegradation. *Carbohydrate Polymers*, *262*, 117963. DOI: https://doi.org/10.1016/j.carbpol.2021.117963

Singh, A., Pal, D.B., Mohammad, A., Alhazmi, A., Haque, S., Yoon, T., & Gupta, V.K. (2022). Biological remediation technologies for dyes and heavy metals in wastewater treatment: New insight. *Bioresource Technology*, *343*, 126154. DOI: https://doi.org/10.1016/j.biortech.2021.126154

Songserm, P., Sihanonth, P., Sangvanich, P., & Karnchanatat, A. (2012). Decolorization of textile dyes by *Polyporus seudobetulinus* and extracellular laccase. *African Journal of Microbiology Research*, *6*, 779-792. DOI: https://doi.org/10.5897/AJMR11.988

Sousa, A. C., Martins, L. O., & Robalo, M. P. (2021). Laccases: Versatile biocatalysts for the synthesis of heterocyclic cores. *Molecules*, *26*(12), 3719. DOI: https://doi.org/10.3390/molecules26123719

Sun, K., Hong, D., Liu, J., Latif, A., et al. (2021).*Trametes versicolor* laccase-assisted oxidative coupling of estrogens: Conversion kinetics, linking mechanisms, and practical applications in water purification. *Science of the Total Environment,782*, 146917. DOI: https://doi.org/10.1016/j.scitotenv.2021.146917

Szostek, M., Kosowski, P., Szpunar-Krok, E., Jańczak-Pieniążek, M., Matłok, N., Skrobacz, K., & Balawejder, M. (2022). The Usefulness of Ozone-Stabilized Municipal Sewage Sludge for Fertilization of Maize (*Zea mays* L.). *Agriculture*, *12*(3), 387. DOI:https://doi.org/10.3390/agriculture12030387

Upadhyay, P., Shrivastava, R.,& Agrawal, P.K., (2016). Bioprospecting and biotechnological applications of fungal laccase. *3 Biotech*, *6*(1), 1-12.DOI: https://doi.org/10.1007/s13205-015-0316-3

Villalba-Rodríguez, A. M., Parra-Arroyo, L., González-González, R. B., Parra-Saldívar, R., Bilal, M., & Iqbal, H. M. (2022). Laccase-assisted biosensing constructs–Robust modalities to detect and remove environmental contaminants. *Case Studies in Chemical and Environmental Engineering*, *100180*. DOI: https://doi.org/10.1016/j.cscee.2022.100180

Wikee, S., Hatton, J., Turbé-Doan, A., Mathieu, Y., et al. (2019). Characterization and dye decolorization potential of two laccases from the marine-derived fungus *Pestalotiopsis sp*. *International journal of molecular sciences*, *20*(8):1864. DOI: https://doi.org/10.3390/ijms20081864

Xin, F., & Geng, A. (2011). Utilization of horticultural waste for laccase production by *Trametes versicolor* under solid-state fermentation. *Applied biochemistry and biotechnology*,*163*, 235-246.

Yusuf, M. (2017). Agro-industrial waste materials and their recycled value-added applications. *Handbook of Ecomaterials*, 1-11. DOI: https://doi.org/10.1007/978-3-319-48281-1_48-1

Zhang, F., Lian, M.,Alhadhrami, A., Huang, M., Li, B., Mersal, G. A., & Xu, M. (2022). Laccase immobilized on functionalized cellulose nanofiber/alginate composite hydrogel for efficient bisphenolA degradation from polluted water. *Advanced Composites and Hybrid Materials*, 1-13. DOI: https://doi.org/10.1007/s42114-022-00476-5

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Awareness and Knowledge of Vertigo among the Adult Population of Selangor, Malaysia

Shaun Lee Chun Wah[1], Vinodhkumar Ramalingam[1*], Banumathi Varadarajan[2], Jagatheesan Alagesan[3], Prathap Suganthirababu[3], Jim Brown Clements[1*]

[1]Faculty of Health and Life Science, INTI International University, Nilai, Malaysia
[2]Faculty of Health Sciences, MAHSA University, Jenjarom Selangor, Malaysia
[3]Saveetha College of physiotherapy, SIMATS, Chennai, India

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ABSTRACT

Dizziness and vertigo are common among the adult and elderly population. However, the knowledge & awareness of vertigo and the understanding of the differences between vertigo and dizziness in the adult population is seldom studied. The present study aimed to assess the level of awareness and knowledge of vertigo among the adult population living in Selangor, Malaysia. In addition, the study also focused on the participants' knowledge of differentiating dizziness and vertigo. This cross-sectional study received responses from 189 participants who were in the age range between 20 and 40 years among which 152 participants' responses met the inclusion criteria. A self-developed validated online questionnaire was used as a study tool to understand the awareness and knowledge of vertigo among the participants. Data analysis was conducted using SPSS (version 28) to obtain frequency and percentages. The results of the present study showed that 57.9% of participants had an average level of awareness of vertigo. Further, 55.3% disagreed that vertigo is the same as dizziness however only 6.6% of the participants were exactly able to identify the differences between vertigo and dizziness. The present study concludes an average level of awareness and knowledge of vertigo among most young adults of Selangor, Malaysia. However, the ability to differentiate vertigo from dizziness was very low among the participants, demonstrating a gap in their knowledge of vertigo. Hence, education about vertigo among the public must be ameliorated. Further studies are required on different age groups and within the other states of Malaysia.

* Corresponding author

E-mail: vinodh.ramalingam@newinti.edu.my (Vinodhkumar Ramalingam);
jimbrown.clements@newinti.edu.my (Jim Brown Clements)

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1 Introduction

In middle to high-income countries such as Malaysia, the aging population has drawn the attention of the healthcare system. Alongside numerous medical conditions that come with old age, it is not uncommon that a high risk of falling is associated with the elderly population, carrying a prevalence of 7.5 to 16.7% in Asia followed by 28% - 35% in Malaysia (Tungvachirakul et al. 2014; Yeong et al. 2016; Ghazi et al. 2017). Falls are the second leading cause of worldwide morbidity and mortality in the elderly population (WHO 2018), and it has become a cause of concern for society and healthcare systems. To reduce the incidence of falls, the public must be well educated about the risk factors that contribute to falls. Willadsen et al. (2016) define fall risk factors' assessment are associated with the probability of disease not necessarily recognized by the patient. Risk factors for falls can be further divided into intrinsic factors such as weakness of limbs and pre-existing medical conditions, and extrinsic factors such as poor lighting and uneven walking surfaces (Appeadu and Bordoni 2021). As the number of risk factors present in an individual increases, the risk of falling also increases (Berg and Cassells 1992; Kiel et al. 2018). Proper knowledge of the causes of falls will allow the public to identify any existing risk factors that put themselves or those under their care at a higher risk of falling, allowing them to take action to reduce those risks or seek treatment.

With an annual prevalence of 40% (Homann et al. 2013), vertigo is one of the common causes of falls globally. Based on a survey by Johns Hopkins Medicine, one-third of the American population suffer from inner-ear dysfunctions causing dizziness and loss of balance. This increases their risk of falling compared to people with a good sense of balance (Xu et al. 2021). While vertigo and inner-ear problems are commonly talked about, most people only have a vague understanding of this topic, leading to many misconceptions. One such misconception is that 'dizziness' and 'vertigo' carry the same meaning and are interchangeably used to describe a sense of altered spatial orientation and perception of movement, leading to loss of balance.

To grasp the understanding of vertigo, we must first understand dizziness. Dizziness is a vague term, used to describe a sensation of whirling or feeling a tendency to fall. It has no actual medical definition, but it is often used in place of light-headedness, presyncope, vertigo, and disequilibrium to represent these sensations. Vertigo is defined as the illusion of movement, where the patient feels their own body or the surrounding environment moving, usually in rotatory motion (Fife 2021). Hanley et al. (2001) define very clearly in their study that presyncope is a feeling of light-headedness, typically due to temporary cerebral ischemia, leading to a feeling of near fainting. Further, Sloane et al. (2001) describe disequilibrium as a sense of imbalance and typically involves the legs and trunk, without sensations of the head.

Vertigo has a lifetime prevalence of 20-30% (Neuhauser et al. 2008) and a 1-year prevalence of 4.9 % (Neuhauser 2007). Vertigo is a symptom of vestibular disorders that can be of central or peripheral pathology. Peripheral vestibular disorders causing vertigo commonly refer to benign paroxysmal positional vertigo (BPPV), Meniere's disease, and vestibular neuritis; central vestibular disorders include vestibular migraine, vertebrobasilar ischemic stroke, and insufficiency of the vertebrobasilar system (Thompson and Amedee 2009). Vertigo is typically characterized by reports of feeling a rotatory movement while not in motion that may last for seconds to hours, maybe even days or weeks (Strupp and Brandt 2008). These may be accompanied by several symptoms such as tinnitus, headache, migraine, diplopia, and dysphagia. This study focused on vertigo caused by peripheral pathologies as vertigo from central pathologies needs additional knowledge on neurological conditions which is beyond the scope of the study.

Although this knowledge may seem trivial, greater awareness and understanding of vertigo will empower the public to communicate its symptoms to their healthcare providers in a better way. Wherever necessary, an accurate diagnosis can be made to ensure that they receive proper treatment. Although vertigo is more common among the elderly, educating the young adult population on its clinical manifestations, causes, risk factors, and treatment options are equally important as a preventive measure. There are only a handful of studies done on the awareness and knowledge of vertigo (Nada et al. 2019; Alenezi et al. 2020), and none of which were done in Malaysia. A recent study on the Saudi population revealed that there was a lack of awareness and knowledge relating to various demographic factors (Alotaibi et al. 2020). This lack of awareness and knowledge of vertigo among the public will lead to the liberal use of the term 'dizziness' in cases related to and unrelated to vertigo. A study by Kroenke et al.(1992) found it useful to establish the cause of dizziness, leading up to the most appropriate management for the specific cause. However, without awareness and knowledge of vertigo among the people, healthcare providers will have great difficulty in diagnosing patients with complaints of 'dizziness' due to the inability of the patients to accurately describe and distinguish their unique experience of dizziness. The purpose of this study, therefore, was to assess the extent of awareness and knowledge of vertigo among the adult population in Selangor, Malaysia and also to find the knowledge of participants in differentiating dizziness and vertigo. The finding of the present study would provide a baseline information on the level of knowledge of vertigo among the participants and allow us to identify if there is a need to improve on the education of vertigo. The results also can be used to direct future education on vertigo,

and its diagnosis and treatment to properly equip the public with a thorough understanding of this easily overlooked topic. The present study may be the first of many to evaluate the awareness and knowledge of vertigo among Malaysians, giving researchers a baseline for future studies.

2 Materials and Methods

This cross-sectional study was conducted in Selangor, Malaysia. Selangor is a state located on the west coast of Peninsular Malaysia and comprises of 9 districts, namely Gombak, Klang, Kuala Langat, Kuala Selangor, Petaling Jaya, Sepang, Hulu Selangor, Hulu Langat, and SabakBernam. The working adult population aged between 20 - 40 years was reached in this study using an online questionnaire that utilizes true-or-false statements and a 4-point Likert rating scale in the questions to study the level of awareness and knowledge of vertigo. Furthermore, convenience sampling was used in this study due to its ease and cost-effectiveness (Elfil and Negida 2017), which involves the recruitment of participants based on their availability, accessibility, or geographical proximity. The study was approved by the INTI International University's ethical committee (INTI-IU/FHLS-RC/BPHTI/7NY12020/022). Participants of this study were recruited via social media platforms. An invitation post with instructions and the link to the questionnaire of the study was shared within Facebook groups such as INTI Physio Club. Healthy adults between the age of 20 - 40 years and those who understand the English language were chosen for the study. People who were non-Malaysians or residing out of the study area were excluded. A written invitation was shared to friends and family on WhatsApp and Instagram, requesting that they further share the study invitation with others upon completion of the questionnaire. A photo post with instructions and a link to the questionnaire was uploaded to social platforms, allowing the engagement of followers. The sample size was estimated as 385, with the confidence level set at 95%, and the z-score used was 1.96. The margin of error was set at 0.05 and the population proportion at 50% (Pourhoseingholi et al. 2013). All participants of this study were given an informed consent form and a brief explanation of the purpose and procedure of the study before recruitment. Besides, participants were assured that all information collected would be kept private and confidential.

2.1 Study Questionnaire

This study utilized a self-developed online questionnaire to obtain data from the participants. The questionnaire was developed to assess the knowledge and awareness aspects of the participants about vertigo. The questionnaire consisted of 3 sections viz., (1) demographic data of the participants, such as age, gender, race, occupation, and education level, (2) 6 questions to gauge the level of awareness of vertigo, and (3) 21 questions to gauge the understanding of vertigo, separated into 5 questions on the definition of vertigo and 16 questions on the detailed knowledge of vertigo. The questions used in the knowledge sections were compiled from a recent study on the knowledge, attitudes, and practices (KAP) relating to vertigo in Saudi Arabia (Alenezi et al. 2020). The content of the questionnaire was validated by the experts in the respective field of study, before being sent out to the target population.

The awareness section of the questionnaire was scored using a 4-point Likert scale with 'Strongly disagree' as 1, "Disagree" as 2, "Agree" as 3, and 'Strongly agree' as 4. The maximum score possible was 24 and the minimum score was 6. A score of 6 to 12 was scored as low personal awareness, 13 to 18 was scored as average personal awareness, and 19 to 24 was scored as high personal awareness.

The knowledge section of the questionnaire consists of 21 True or False questions with an extra option of "I don't know". Participants would score 1 point for each correct answer and 0 points for selecting the wrong answer or "I don't know". The participant was scored as "General awareness knowledge" when scoring 0 to 7 points, "Average knowledge" when scoring 8 to 14, and "Detailed specific knowledge" when scoring 15 to 21. The questionnaire begins with 5 questions on the definition of vertigo, aimed at assessing the participant's knowledge of the difference between vertigo and other forms of dizziness. The following 16 questions cover the causes, symptoms, and prevalence of vertigo. The target population of this study was members of the public that may or may not have medical knowledge. Thus, the questions of this questionnaire were phrased using common English, avoiding the use of medical jargon to ensure participants could fully understand the statements provided. A study by Subramaniam et al.(2017) implied that medical jargon leads to misunderstanding in the healthcare setting. The questions did not go in-depth into the medical details of vertigo, as members of the public cannot be expected to have that knowledge. Instead, the questions reflected more practical knowledge that would affect the participants' attitudes and perceptions of vertigo.

2.2 Statistical analysis

The statistical data were analyzed using the IBM® Statistical Package for the Social Sciences (SPSS) version 28. The demographic data such as gender, race, occupation, and education level, were analyzed using frequency and percentages (Table 1). Continuous variables such as age and scores obtained from the questionnaire were analyzed using the mean and standard deviation. Further, the level of awareness and level of knowledge were analyzed using frequency and percentages (Table 2 and Table 3).

Table 1 Demographic data of the participants

Variables		Frequency (%)
Age	20 – 25	98 (64.5)
	26 – 30	25 (16.4)
	31 – 35	22 (14.5)
	36 – 40	7 (4.6)
Gender	Male	50 (32.9)
	Female	102 (67.1)
Race	Chinese	133 (87.5)
	Indian	9 (5.9)
	Malay	8 (5.3)
	Others	2 (1.3)
Occupation	Arts and Entertainment	6 (3.9)
	Business	34 (22.4)
	Engineering	9 (5.9)
	Education	17 (11.2)
	Food and Beverages	3 (2.0)
	Healthcare	25 (16.4)
	Law	2 (1.3)
	Student	38 (2.5)
	Information technology	4 (2.6)
	Others	14 (9.2)
Education Level	SPM* (or equivalent)	3 (2.0)
	Pre-U	4 (2.6)
	Diploma	6 (3.9)
	Undergraduate	113 (74.3)
	Postgraduate	26 (17.1)

*SPM – Sijil Pelajaran Malaysia

Table 2 Participant's Responses for Awareness Domain

Awareness Domain	Strongly disagree n (%)	Disagree n (%)	Agree n (%)	Strongly agree n (%)
	Disagree		Agree	
I am aware of what vertigo is.	18 (11.8)	40 (26.3)	59 (38.8)	35 (23.0)
I have heard of or read about vertigo.	12 (7.9)	15 (9.9)	62 (40.8)	63 (41.4)
I have experienced vertigo.	89 (58.6)	27 (17.8)	21 (13.8)	15 (9.9)
I have heard experiences of vertigo from family members or close friends.	40 (26.3)	20 (13.2)	37 (24.3)	55 (36.2)
I have heard that vertigo will resolve on its own and does not need to be treated as a serious medical problem.	50 (32.9)	64 (42.1)	33 (21.7)	5 (3.3)
I think vertigo affects only the older population.	69 (45.4)	57 (37.5)	22 (14.5)	4 (2.6)

3 Results

3.1 Demographic features

Table 1 shows the demographic features of the participants in this study based on their age, gender, race, occupation, and education levels. A total of 189 participants responded to this study by convenience sampling. Among the 189 participants, only 152 met the inclusion criteria, aged between 20 - 40 years. The participants of the study had a mean age of 26.04 ± 4.66 years. There were 102 females (67.1%) and 50 males (32.9%). Among the participated participants, 87.5% of the participants were Chinese (n = 133), 5.9% were Indian (n = 9) and 5.3% were Malay (n = 8). The occupations of the participants varied greatly, with 25% students, 22.4% business personnel, and 16.4% in the healthcare sector. The majority of the participants had an undergraduate education level of 74.3%, followed by a postgraduate level of education of 17.1%.

3.2 Awareness of Vertigo

The present study was found to have an average personal awareness of vertigo (n = 88, 57.9%) from the participants'

responses, with a mean score of 16.61±2.95. While the remaining responses from 45 participants showed high personal awareness (29.6%), 19 participants had low personal awareness (12.5%) of vertigo. The Likert scale 4-point score for the awareness domain of the participant's response (1 and 2) considered as "Agree" and (3 and 4) considered as "Disagree" was reported in the study. In addition, most participants have heard of or read about vertigo (n = 125, 82.2%) before the study and could confidently know what vertigo is (n = 94, 61.8%). Although most participants have not experienced vertigo themselves (n = 116, 76.4%), more than half have heard of experiences from their family members or close friends (n = 92, 60.5%). Furthermore, the majority of the participants (n = 114, 75.0%) perceive vertigo to be a serious medical problem. The young adults (n=36, 23.8%) who participated in this survey experienced vertigo even before enrolling in this study. Based on the responses to this, they are aware that vertigo does not only affect the older population (n = 126, 82.9%) (Table 2).

3.3 Knowledge of Vertigo

By considering the awareness level of participants about vertigo in this study, participants mostly have an average knowledge of vertigo (n = 100, 65.8%) with an obtained mean score of 10.38 (± 4.206). Followed by 31 participants who had general awareness knowledge of vertigo (20.4%), and 21 participants who have detailed specific knowledge of vertigo (13.8%). Table 3 shows the responses of the participants to the knowledge domain of the questionnaire and the findings are divided into two sections which reflect on knowledge of vertigo and the knowledge of the differences between vertigo and dizziness.

Table 3 Participant's Responses for Knowledge Domain

	Knowledge Domain	True, n (%)	False, n (%)	I don't know, n (%)
	Knowledge on Vertigo			
1	Vertigo can be associated with problems in the inner ear. (semi-circular canals)	122 (80.3)	5 (3.3)	25 (16.4)
2	Vertigo can be associated with problems in the brain.	53 (34.9)	32 (21.1)	67 (44.1)
3	Vertigo may occur due to the use of certain medications.	42 (27.6)	20 (13.2)	90 (59.2)
4	If you have vertigo, you may feel worse when you move your head or change positions (stand up, rollover).	113 (74.3)	3 (2.0)	36 (23.7)
5	Vertigo may last for seconds, hours, or days.	110 (72.4)	3 (2.0)	39 (25.7)
6	Vertigo may be accompanied by loss of hearing and a ringing sensation in the ears.	85 (55.9)	11 (7.2)	56 (36.8)
7	Vertigo may be accompanied by seeing double, having trouble speaking or swallowing, or feeling weak.	78 (51.3)	14 (9.2)	60 (39.5)
8	Vertigo may be associated with migraines	84 (55.3)	12 (7.9)	56 (36.8)
9	Vertigo may be accompanied by a headache or sensitivity to light and noise.	106 (69.7)	10 (6.6)	36 (23.7)
10	Vertigo may be accompanied by mood swings.	34 (22.4)	36 (23.7)	82 (53.9)
11	If you have vertigo, you may feel worse when you cough or sneeze.	45 (29.6)	26 (17.1)	81 (53.3)
12	Vertigo is a disease transferred from parents to children.	3 (2.0)	92 (60.5)	57 (37.5)
13	Vertigo affects females more than males.	25 (16.4)	14 (9.2)	113 (74.3)
14	Vertigo affects the elderly more than young people.	62 (40.8)	21 (13.8)	69 (45.4)
15	Vertigo can be treated with medicine.	85 (55.9)	12 (7.9)	55 (36.2)
16	Vertigo can be treated with exercises.	49 (32.2)	19 (12.5)	84 (55.3)
	Knowledge on the Differences between Vertigo & Dizziness			
17	Vertigo is the same as dizziness	33 (21.7)	84 (55.3)	35 (23.0)
18	Vertigo is a feeling of moving or spinning when not in motion or that the world is spinning around you.	134 (88.2)	2 (1.3)	16 (10.5)
19	Vertigo is a feeling of fainting due to fear of heights.	15 (9.9)	109 (71.7)	28 (18.4)
20	Vertigo is a feeling of nausea and vomiting while in motion.	75 (49.3)	42 (27.6)	35 (23.0)
21	Vertigo is a feeling of drifting to one side while walking.	86 (56.6)	24 (15.8)	42 (27.6)

According to the section for knowledge on vertigo, most participants know that vertigo is associated with the inner ear (n = 122, 80.3%), but only a small group of them know that it can be associated with a problem in the brain as well (n = 53, 34.9%), and can occur due to the use of certain medication (n = 42, 27.6%). Further, 113 participants (74.3%) know that moving their heads or changing positions may aggravate vertigo symptoms. A larger group of participants (n=134, 88.2 %) agreed that vertigo is a feeling of moving or spinning when not in motion or that the world is spinning around you as well. 86 participants (56.6%) have agreed that vertigo is a feeling of drifting to one side while walking. Most of them (n = 110, 72.4%) agree that vertigo may last for seconds, hours, or days. Of the symptoms that accompany vertigo, more than half of the participants know that vertigo may be accompanied by loss of hearing and a ringing sensation in the ears (n = 85, 55.9%), seeing double, having trouble speaking or swallowing, or feeling weak (n = 78, 51.3%), and having migraines (n = 84, 55.3%). A larger number agreed that vertigo can lead to headache or sensitivity to light and noise (n = 106, 69.7%). However, only 34 participants (22.4%) know that vertigo can be accompanied by mood swings, while only 45 of them (29.6%) know that coughing or sneezing could also make them feel worse if they have vertigo. More than half the participants agreed that vertigo is not transferred from parents to children (n = 92, 60.5%). Only 25 participants (16.4%) know that vertigo affects females more than males, and 62 participants (40.8%) answered 'true' to vertigo affecting the elderly more than young people. Finally, 56% of the participants (n = 85) know that vertigo can be treated with medicine, and only 32.2% (n = 49) know that some exercises or maneuvers can treat vertigo.

Based on the responses of the participant's knowledge of the differences between vertigo and dizziness, 84 participants (55.3%) disagree that vertigo is the same as dizziness. However, most of the participants (n = 134, 88.2%) agreed that "vertigo is a feeling of moving or spinning when not in motion" or that "the world is spinning around you". Most of the participants (n = 109, 71.7%) disagree that vertigo is a feeling of fainting due to a fear of heights. Only a small group of the participants chose the right answer for "vertigo is a feeling of nausea and vomiting while in motion" (n = 42, 27.6%) and "vertigo is a feeling of drifting to one side while walking" (n = 24, 15.8%), which was "false". Of the 84 participants who chose vertigo as not the same as dizziness, only 10 of them (6.6%) answered all 5 questions correctly.

4 Discussion

The purpose of this study was to determine the level of awareness and knowledge of vertigo among the adult population of Selangor. The second objective was to determine the percentage of adults who understand the difference between dizziness and vertigo. A research group of young adults was selected because they would have completed their studies and are likely to be caretakers of the older population, who are more prone to vertigo. D'Amen et al. (2021) address the trend of an aging population where the responsibility of caregiving is given to the younger population. A study on the prevalence of vestibular disorders in a tertiary hospital showed that patients between 40 - 64 years old had the highest prevalence of vestibular disorders at 20.5% (Wahat et al. 2013). This indicates the need for the younger population to be better educated on common healthcare topics.

The results suggest that the majority of young adults in Selangor, Malaysia have an average awareness and knowledge of vertigo. Within the age group of 20 to 40 years, most of the participants have never experienced vertigo before, yet they have heard about it or have heard about experiences from those close to them. Notably, 23.8% of respondents in the present study experienced vertigo before, which falls between the prevalence range of 7.4% and 38.2% among vertigo patients reported from past research in Malaysia. A report by Samsudin states indicates that there is a 7.4% prevalence of vestibular problems in the Neurology clinic of Universiti Kebangsaan Malaysia Medical Centre (UKMMC) where 95 out of 1283 patients were diagnosed with some form of vestibular problems (Samsudin 2011). Another study done at UKMMC noted that 38.2% of the 777 patients in the study were diagnosed with vestibular disorders (Wahat et al. 2013). As such, many acknowledge vertigo as a serious medical problem. Benecke et al. (2013) addressed the burden of vertigo on the patient, stating that vertigo harms the patient's work performance. Patients with vertigo have reported a reduction in workload, loss of working days, or changing jobs because of their condition. The prevalence of vertigo also increases the utilization of healthcare services, adding to the burden of the healthcare system (van der Zaag-Loonen & van Leeuwen 2015; San Fillippo 2017).

In the present study, the participants were shown to have an average level of knowledge about vertigo. While 55% of the participants know that there is a difference between dizziness and vertigo, only a handful of them was able to report the difference. 10 participants (6.6%) answered all 5 of the definition knowledge questions correctly, identifying the definition of vertigo to be a feeling of moving or spinning when not in motion. Further, Stanton and Freeman (2022) describe vertigo as "feeling like the room is spinning around you". It may be argued that the other statements within that section were not false, for example, "vertigo is a feeling of nausea and vomiting while in motion". Although nausea and vomiting are often associated with vertigo, it is inaccurate to say that "vertigo is" that symptom, therefore it is deemed a false statement. Though 88.2% of participants were aware that vertigo is a rotatory sensation when at rest, it was clear that many understood vertigos to be general dizziness. In terms of knowledge of vertigo,

most of the participants were familiar with the common presentations of vertigo such as headaches, migraines, tinnitus, and vision or speech impairment. They knew that vertigo stems from problems in the semicircular canals. However, most do not know that vertigo may arise from the brain, presenting as a lesion or dysfunction of the brainstem and leading to central vertigo (Pricilia and Kurniawan 2021). 85 participants know that vertigo can be treated with medication while only slightly over half those numbers know of exercises that can treat vertigo. This typically refers to vestibular rehabilitation that may be performed by trained medical professionals and physiotherapists. Vestibular rehabilitation has been shown to improve primary symptoms of vertigo, improve balance, reduce the risk of falling, and may show a reduction in anxiety and depression in patients (Kundakci et al. 2018). Finally, the results show that the participants did not have good knowledge of the demographic prevalence of vertigo. Knowing that there is a clear lack of knowledge of vertigo, authorities need to realize the rising trends of this problem and take action to educate the public. Previous research has shown that knowledge and health education interventions are effective for the primary prevention of individuals and have been shown to improve the physical activity levels of said individuals (Ramôa Castro et al. 2017; Wang et al. 2018). With improvements in education about vertigo, greater awareness of the problem will lead to early detection of vestibular problems, enabling the patient to take control of their medical situation (Dowdal-Osborn 2002). This, in turn, will lessen the potential burden on the healthcare system.

There were a few limitations in this study; Firstly, it had a small sample size of only 189 respondents, and secondly, the sample obtained was predominantly Chinese, making the results non-generalizable to young adults of other races. This could be due to the use of convenience sampling. Apart from that, the questionnaire was administered online, making it impossible to ensure that the participants did not search for answers online or through reference books while answering the questions. If the participants have done this, it could lead to a negative skewing of the data, providing inaccurate information about the level of knowledge about vertigo. Finally, the results could not determine cause and effect as this was a cross-sectional study.

Conclusion

The findings of this study conclude that there is an average level of personal awareness and knowledge of vertigo among young adults of Selangor. Only 6.6% knew the difference between vertigo and dizziness. Most people would have encountered an instance of vertigo in their lifetime, but there is a gap in the knowledge of this condition among the public. There is a need to educate the public about vertigo, making a clear difference between dizziness and all its subtypes. More studies are needed to assess the prevalence and level of knowledge of vertigo within other states in Malaysia and with a wider age group. Future studies should aim to include more members of other races as well as different age groups to allow generalizability. A more in-depth study can be done to test and analyze if there are other correlating factors as well. This study serves as a reference point for future studies, providing a baseline for the level of awareness and knowledge of vertigo in this study population. This information would allow us to identify that there is a lack of education on this topic. This study may also put more importance on vertigo and raise its awareness among the public. As people become more aware of this, they are more likely to seek treatment for it, reducing the chances of secondary complications caused by untreated vertigo. This may, in a small way, reduce the burden on the healthcare system.

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Conflicts of interest

The authors affirm that they do not have any conflict of interest.

References

Alenezi, M. M., Almutairy, H. S., Hamoud Alokayli, A. N., Alfalah, M. A., Alnasser, H. E., & Asiri, R. N. (2020). Knowledge, attitudes, and practices relating to vertigo among newly diagnosed patients in Saudi Arabia, 2019. *Internal Journal of Medicine in Developing Countries*, *4(2)*, 403–408.

Alotaibi, S. S., Alshbiny, M. T., Alsehali, S. A., Hayat, M., et al. (2020). Knowledge and awareness of benign paroxysmal positional vertigo among Saudi population : a cross-sectional study. *Internal Journal of Medicine in Developing Countries*,*4*, 1184–1190.

Appeadu, M., & Bordoni, B. (2021). Falls and Fall Prevention In The Elderly. *StatPearls*.

Benecke, H., Agus, S., Goodall, G., Kuessner, D., & Strupp, M. (2013). The burden and impact of vertigo: findings from the REVERT patient registry. *Frontiers in Neurology*, *4*, 136.

Berg, R. L., & Cassells, J. S. (1992). Falls in older persons: risk factors and prevention. In The second fifty years: Promoting health and preventing disability. *National Academies Press* (US), 15.

D'Amen, B., Socci, M., Di Rosa, M., Casu, G., et al. (2021). Italian Adolescent Young Caregivers of Grandparents: Difficulties Experienced and Support Needed in Intergenerational Caregiving-Qualitative Findings from a European Union Funded Project. *International journal of environmental research and public health*, *19*(1), 103. https://doi.org/10.3390/ijerph19010103

Dowdal-Osborn, M. (2002). Early vestibular rehabilitation in patients with Meniere's disease. *Otolaryngologic Clinics of North America*, *35(3)*, 683–690.

Elfil, M., & Negida, A. (2017). Sampling methods in clinical research; an educational review. *Emergency*, *5(1)*, *e52*..

Fife, T. D. (2021). Approach to the history and evaluation of vertigo and dizziness. *CONTINUUM: Lifelong Learning in Neurology*, *27(2)*, 306–329.

Ghazi, H. F., Elnajeh, M., Abdalqader, M. A., Baobaid, M. F., Rosli, N. S. R., & Syahiman, N. (2017). The prevalence of falls and its associated factors among elderly living in old folks home in Kuala Lumpur, Malaysia. *International Journal of Community Medicine and Public Health*, *4(10)*, 3524–3529.

Hanley, K., O'Dowd, T., & Considine, N. (2001). A systematic review of vertigo in primary care. *British Journal of General Practice*, *51*(469), 666–671.

Homann, B., Plaschg, A., Grundner, M., Haubenhofer, A., et al. (2013). The impact of neurological disorders on the risk for falls in the community dwelling elderly: a case-controlled study. *British Medical Journal*, *3(11)*, e003367.

Kiel, D. P., Schmader, K., & Lin, F. (2018). Falls in older persons: Risk factors and patient evaluation. *UpToDate. Waltham: UpToDate Inc*.

Kroenke, K., Lucas, C. A., Rosenberg, M. L., Scherokman, B., et al. (1992). Causes of persistent dizziness: a prospective study of 100 patients in ambulatory care. *Annals of Internal Medicine*, *117*(11), 898–904.

Kundakci, B., Sultana, A., Taylor, A. J., & Alshehri, M. A. (2018). The effectiveness of exercise-based vestibular rehabilitation in adult patients with chronic dizziness: A systematic review. *F1000 Research*, *5* (7), 276.

Nada, J., Abdulaziz, D., Abdulrahman, B., Measha‌l, Z., Alhmadi, A., & Ayas, M. (2019). A Questionnaire Study to Evaluate the Knowledge of Healthcare Providers in Differentiating Between Vertigo and Dizzy Patients , in KSA. *Experiments in Rhinology & Otolaryngology*, *2*(5), 165–173.

Neuhauser, H. K. (2007). Epidemiology of vertigo. *Current Opinion in Neurology*, *20(1)*, 40–46.

Neuhauser, H. K., Radtke, A., Von Brevern, M., Lezius, F., Feldmann, M., & Lempert, T. (2008). Burden of dizziness and vertigo in the community. *Archives of Internal Medicine*, *168*(19), 2118–2124.

Pourhoseingholi, M. A., Vahedi, M., & Rahimzadeh, M. (2013). Sample size calculation in medical studies. *Gastroenterology and Hepatology from Bed to Bench*, *6*(1),14.

Pricilia, S., & Kurniawan, S. N. (2021). CENTRAL VERTIGO. *Journal of Pain, Headache and Vertigo*, *2(2)*, 38–43.

Ramôa Castro, A., Oliveira, N. L., Ribeiro, F., & Oliveira, J. (2017). Impact of educational interventions on primary prevention of cardiovascular disease: A systematic review with a focus on physical activity. *European Journal of General Practice*, *23(1)*, 59–68.

Samsudin, N. (2011). *Prevalence of Vestibular Problems in Ppukm Neurology Clinic*. Bachelor of Audiology Thesis submitted to Universiti Kebangsaan Malaysia. Retrived from http://mash.org.my/wp-content/uploads/Audio%202011%20Nor%20Syuhada%20Samsudin.pdf.

San Fillippo, D. (2017). The Impact of Vertigo on Employment and Activities of Daily Living. *Journal of Nurse Life Care Planning*, *17(1)*, 40–45.

Sloane, P. D., Coeytaux, R. R., Beck, R. S., & Dallara, J. (2001). Dizziness: state of the science. *Annals of Internal Medicine*, *134* (*9_Part_2*), 823–832.

Stanton, M., & Freeman, A. M. (2022). *Vertigo*. In *StatPearls*. StatPearls Publishing.

Strupp, M., & Brandt, T. (2008). Diagnosis and treatment of vertigo and dizziness. *Deutsches Ärzteblatt International*, *105(10)*, 173.

Subramaniam, R., Sanjeev, R., Kuruvilla, S., Joy, M. T., Muralikrishnan, B., & Paul, J. (2017). Jargon: a barrier in case history taking?-a cross-sectional survey among dental students and staff. *Dental Research Journal*, *14(3)*, 203.

Thompson, T. L., & Amedee, R. (2009). Vertigo: a review of common peripheral and central vestibular disorders. *Ochsner Journal*, *9(1)*, 20–26.

Tungvachirakul, V., Lisnichuk, H., & O'Leary, S. J. (2014). Epidemiology of vestibular vertigo in a neuro-otology clinic population in Thailand. *Journal of Laryngology and Otology*, *128*(SUPPL. S2), 31–38.

van der Zaag-Loonen, H. J., & van Leeuwen, R. B. (2015). Dizziness causes absence from work. *Acta Neurologica Belgica*, *115*(3), *345–349.*

Wahat, N. H. A., Sevasankaran, R., Abdullah, A., & Ali, R. A. (2013). Prevalence of vestibular disorders among otology patients

in a tertiary hospital in Malaysia. *Journal of Internal Medicine, 20,* 312–314.

Wang, M., Han, X., Fang, H., Xu, C., et al. (2018). Impact of health education on knowledge and behaviors toward infectious diseases among students in Gansu Province, China. *BioMed Research International*, *2018*, *6397340*, doi: 10.1155/2018/6397340.

WHO. (2018). Falls. April.

Willadsen, T. G., Bebe, A., Køster-Rasmussen, R., Ejg, D., et al. (2016). The role of diseases, risk factors and symptoms in the definition of multimorbidity–a systematicreview. *Scandinavian Journal of Primary Health Care*, *34(2):*112-21.

Xu, D., Newell, M. D., & Francis, A. L. (2021). Fall-related injuries mediate the relationship between self-reported hearing loss and mortality in middle-aged and older adults. The Journals of Gerontology: Series A, *76*(9):e213-e220.

Yeong, U. Y., Tan, S. Y., Yap, J. F., & Choo, W. Y. (2016). Prevalence of falls among community-dwelling elderly and its associated factors: A cross-sectional study in Perak, Malaysia. *Malaysian Family Physician: The Official Journal of the Academy of Family Physicians of Malaysia*, *11(1),* 7.

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In-silico designing of a potent ligand molecule against *PTEN* (Phosphatase and tensin homolog) implicated in Breast Cancer

Mukta Raghav[1], Varruchi Sharma[2], Shagun Gupta[1], Ankur Kaushal[1], Amit Vashishth[3], Hardeep Singh Tuli[1], Kuldeep Dhama[4], Anil Kumar Sharma[1,*]

[1]Department of Biotechnology, Maharishi Markandeshwar (Deemed to be University), Mullana-Ambala, Haryana, 133207, India
[2]Department of Biotechnology & Bioinformatics, Sri Guru Gobind Singh College Sector 26, Chandigarh.
[3]Department of Science and Humanities, SRM Institute of Science & Technology (Deemed to be University) Ghaziabad 201204 (UP).
[4]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, UP, India.

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Lead molecule

ABSTRACT

Breast cancer has been attributed to be the second most common malignancy in females worldwide after skin cancer associated with a significantly high mortality rate. Tumor suppressor genes have an indispensable role in maintaining genomic integrity as well as cell cycle regulation. Phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) is one of the most frequently mutated human tumor suppressor genes, implicated in cell growth, survival, and suppressing tumor formation. As the tumor progresses to more advanced stages, genetic alterations tend to increase one such alteration is the mutation of the *PTEN* gene which is linked to programmed cell death and maintenance of cell cycle regulation. There is a syndrome known as Cowden syndrome associated with a high risk of breast cancer which is a result of an outcome of germline mutations in the *PTEN* gene. Loss of *PTEN* activity, either at the protein or genomic level, has been related to many primary and metastatic malignancies including breast cancer. This study focuses on developing a potential bioavailable ligand inhibitory molecule for *PTEN*, using a computer-aided drug design approach (CADD). A library of developed ligands consisting of 50 potential molecules was screened to find a potential candidate to be used for second generation drug development. Among them, LIG28 was adjudged as the most effective and potential *PTEN* inhibitor given its maximum binding affinity of ΔG -5.96Kcal/mole with a lower RMSD value. Carmer's Rule of toxicity further revealed the compatibility and non-toxicity of the molecule. These observations underscore the importance of *PTEN* as a target in the development of tumorigenesis and the prognosis of breast cancer.

* Corresponding author
E-mail: anibiotech18@gmail.com (Anil Kumar Sharma)
Scopus Author ID: 57203774408

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1 Introduction

Cancer is attributed to the unregulated and uncontrolled division of cells, which may become malignant and spread into the neighboring tissues of the body. Genetic changes are encountered at the DNA level when cancer becomes malignant (Rajpoot et al. 2021). In the current scenario, breast cancer is known to affect one in every eight women during their life span worldwide (Momenimovahed and Salehiniya 2019). In recent times various promising inhibitors have been developed for the treatment of breast cancer targeting various molecules including mTOR and *PTEN* (Sharma et al. 2020a). *PTEN* was considered as an autonomous anticancer unit (Trotman and Pandolfi 2003; Sharma et al. 2021a,d) which acts as a tumor suppressor by antagonizing the PI3K and AKT pathways and plays an important role in cell survival, cell migration, cell and organ size control (Barbieri and Rubin 2015).

PTEN despite being called Phosphatase Tensin Homologue is also reported to be mutated in Multiple Advanced Cancer1 (MMAC1) located at chromosome 10q23.3. It encodes for 403 amino acids which display both lipid as well as protein phosphates activities. In *PTEN*, there are two functional domains (phosphate domain and a C2 domain) along with three structural regions {a short N-terminal phosphatidylinositol (PI)-4,5-bisphosphate (PIP2) binding domain and a C-terminal tail containing PEST sequences and a PDZ-interaction motif} (Sharma et al. 2016). *PTEN* acts as a negative regulator of the PI3K/AKT signaling pathway affecting cell survival, proliferation, and apoptosis directly and indirectly (Sharma et al. 2017; Ma et al. 2019). In the inositol ring, *PTEN* dephosphorylates at the 3' end of the triphosphate (PIP3) resulting in (PIP2) biphosphate, which obstructs AKT activation and downstream signaling processes (Sehrawat et al. 2021). Lack of inhibition of the AKT-dependent processes and inactivation of *PTEN* has been related to tumorigenesis in multiple human cancers (Sharma et al. 2019a), including breast cancer (Roy et al. 2010; Ram et al. 2020). During intracellular signaling, recruitment of AKT initially depends on the generation of phosphatidyl-inositol-triphosphate (PIP3) by PI3K stimulated through receptor-coupled tyrosine kinases (RTKs) (Hinz and Jücker 2019; Sharma et al. 2022a). Engagement of PIP3 to AKT leads to double phosphorylation one on the kinase domain (T308, T309, and T305 for AKT1, 2, and 3) by PDK1 and the other one on a regulatory domain (S473, S474, and S472 for AKT1, 2, and 3,) by mTOR complex 2 (Sehrawat et al. 2021). Upon activation, AKT phosphorylates its downstream targets, including tuberous sclerosis complex 2 (TSC2) (Sharma et al. 2022b), glycogen synthase kinase-3β (GSK3β), and the forkhead kinase transcription factors (FOXO), thus help in increasing cell proliferation, metabolism, and survival (Hoxhaj and Manning 2020).

It has been observed that *PTEN* emerges as a suppressor of breast cancer growth by down-regulation of PI3K which results in cell death and arrest of the G1 phase of the cell cycle. Protein structure shows a phosphatase domain that contains 1-185 residues and a C2 domain having residues 186-351 both essential for tumor suppressor function (Sharma et al. 2022c). The phoshatase domain contains the tyrosine phosphatase signature motif (H123CKAGKGR130), which forms a loop (P-loop) with the active site pocket. Inside this loop residue, C124 and R 130 are important for catalysis (Sharma et al. 2019b). *PTEN* is phosphorylated on a group of serine and threonine residues present on its C-terminal tail and results in a closed *PTEN* state in its inactive form (Singh et al. 2022) and maintains *PTEN* protein in a fixed conformation (Sheikh et al. 2020). Upon activation, the phosphatase domain of *PTEN* opens up by the de-phosphorylation of its C terminal tail, which results in increased activity of *PTEN* (Panwar et al. 2021). Considering the significance of *PTEN* in breast cancer development, prognosis, and treatment (Sharma et al. 2020b), CADD was exploited to develop a potential bioavailable ligand inhibitory molecule for *PTEN*. A library of developed ligands consisting of 50 potential molecules was screened to find a potential candidate to be used for second generation drug development (Sharma et al. 2021b,e).

2 Materials and Methods

NCBI, UniProtKB, PROCHECK, and PROSA were used to retrieve and validate the *PTEN* protein sequence. The information on the structures for *PTEN* was extracted using PDB. The models for the parent protein have been designed using iterative threading refinement (I-TASSER) (Roy et al. 2010; Zhang and Yu 2010; Yang and Zhang 2015;) program (Table 1). The designed models were then refined using PROCHECK (Wiederstein and Sippl 2007), which revealed model 1 as the most stable structure with significant core residues (Figure 1A). Further, the complete quality examination by Z- score (-12.46) indicated that the obtained model was significantly close to the template (Sharma et al. 2021c). As per the literature review, Histidine (HIS) at 123 positions, Lysine (LYS) at 125 positions, Glycine (GLY) at 127 positions, and Lysine (LYS) at 128 positions) formed the active site (Figure 1B) (Sharma et al. 2020c).

Seed molecule was explored using NCBI pub-Chem, which showed 4-Nitroquinoline as a natural ligand of the crystal structure of the *PTEN* tumor suppressor. The compound exhibits an idle molecular weight i.e 190.16 g/mol and it has the natural tendency to bind with phosphate inositol. The seed molecule was generated using Chemsketch Acd Labs (Figure 1C). The generated seed molecule was taken to AutoDock Vina autodock 4.2.6 which shows the best-fitted space of 4-Nitroquinoline close to binding tetrads. The seed molecule was positioned to interact favorably with the site (Figure 1D).

Table 1 Modelled Protein structure information with Template using ITASSER

Sr. No.	Model No.	Ramachandran Plot			Bad Contacts	G - Factor
		Core	Allowed	Disallowed		
1.	Rag 1	80.3%	15.6%	1.4%	5	-0.34
2.	Rag2	77.3%	17.7%	1.8%	31	-0.56
3.	Rag3	78.0%	16.1%	2.5%	23	-0.51
4.	Rag4	79.8%	16.0%	2.0%	5	-0.38
5.	Rag5	78.8%	14.6%	3.0%	27	-0.45

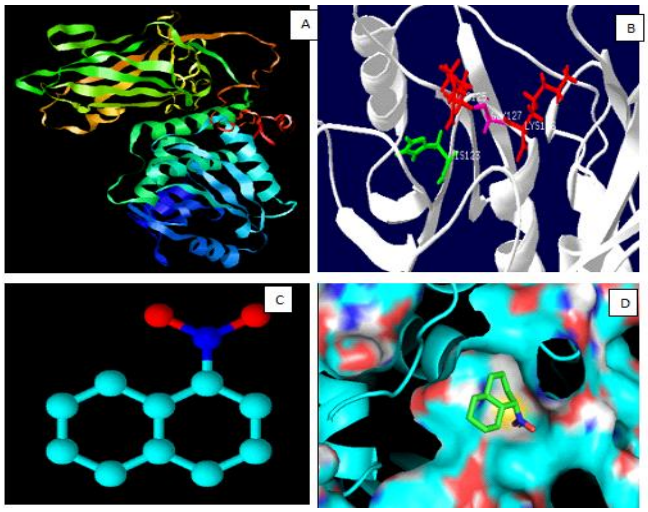

Figure 1 Predicted the best model (A) with Active site residues (B) 4-Nitroquinoline Structure (C) best binding confirmation of the lead molecule (D)

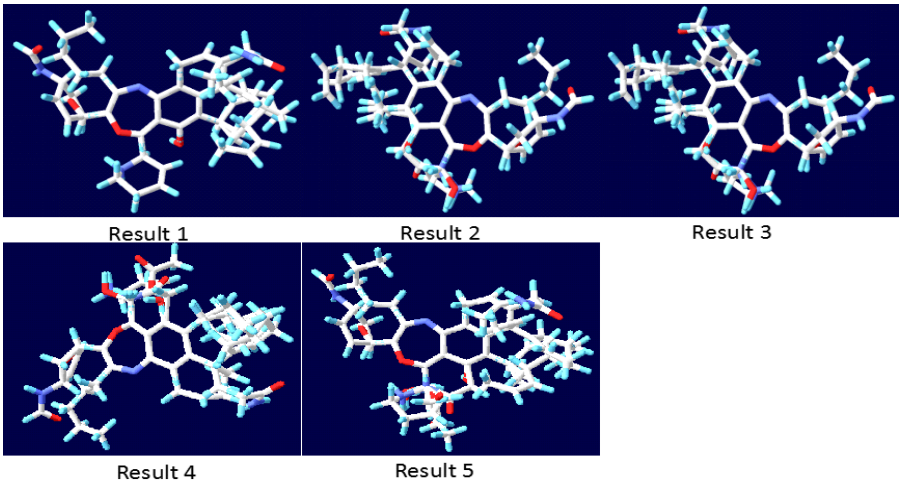

Figure 2 Best five Results obtained using the Ligbuilder

To achieve structural harmony of the site of interest, a complex of seed and target (Fused Space File) was used for fragment addition. More than 500,000 molecules were occupied by using an inbuilt library of organic fragments and a Genetic Algorithm under the Growing strategy of Ligbuilder (v1.2) which was used to get the best five results as shown in Figure 2. Through an empirical scoring function, binding affinities of the populated ligands were estimated. The screening and processing of generated molecules were done using Lipinski's rule of 5. A total of 20 unique fragment combinations were selected for further studies and the best five have been shown in Figure 2.

Table 2 Analysis of the ligands based upon the conformation, ΔG value, RMSD, and inhibition constant

S.No	Ligand Name & No.	Best Conformation No	ΔG (kcal/mol)	Ref RMSD	Inhibition Constant mM (millimolar)
1	Lig5	5	-4.98	4.85	492.10
2	Lig9	9	-3.44	3.93	422.40
3	Lig13	6	-3.98	3.55	386.14
4	LIg17	1	-4.12	3.58	410.14
5	Lig28	4	-5.96	4.90	524.28

The binding energy of grown ligands was then analyzed using MGL and Autodock tools (Panwar et al. 2021). A molecular docking experiment was done by using the Lamarckian Genetic algorithm and Local Search default parameters (Fuhrmann et al. 2010). Gibbs free energy showed significant binding between developed ligands with lower RMSD from the original conformation (Bansal et al. 2022). Activity concentration (IC50) of analyzed ligands was also found satisfactory under the micro-molar range (Table 2).

3 Results and Discussion

Structure-based drug designing approach has provided a strong platform for the researchers to perform in-silico docking and simulation studies under which they could derive *in-silico* simulation before labor-extensive wet-lab validation (Chen et al. 2012). In this study, a structure-based drug design approach was used to design a probable ligand molecule for *PTEN*. The 3D structure of the protein was modeled using ITASSER resulting in the generation of five models which were then validated using PROCHECK & PROSA. Predicted models were re-evaluated for geometry, stereochemistry checks, and energy distribution using PROCHECK. The data is in agreement with other studies reported in the literature (Wang et al. 2022). Based on the observations of validation, we have evaluated and selected the best model (Model1) with a significant C-score (80.3% core value) and goodness factor value (-0.34) (Table 1). The energy refined models of *PTEN* were generated using Iterative threading assembly refinement (ITASSER), ranked as per the cluster size. The model obtained have a higher C Score and better quality which is consistent with earlier reports in the literature as well (Zheng et al. 2021). The active site revealed HIS at 123 positions, Lysine at 125 positions, GLY at 127 positions, and LYS at 128 positions respectively. The literature search revealed that the active triad plays a crucial role in the substrate recruitment mechanism. Further seed molecules were surfed over NCBI Pub-chem. The screening and processing of generated molecules was done using Lipinski's parameters, which revealed the parametric division of the best compound having a molecular weight (MW) of 528.28, which is in agreement with earlier reports in the literature where MW fits over accepted rules of SBDD approach having many hydrogen bond donors and acceptors as HBD-3, HBA-7, with a logP value of 5.28, and the topological polar surface area (PSA) corresponds to 102.38. The molecule was reported to be the best possible ligand having distinct inhibition properties. The Genetic Algorithm under a Growing strategy of Ligbuilder (v1.2) was used with which more than 500,000 molecules populated the ligand using existing libraries in the program (Yuan et al. 2020). From the designed library of molecules, the best ten candidates have been selected for performing docking studies using Autodock which revealed Lig28 with the best -5.96 ΔG (kcal/mol) binding energy (Raghav et al. 2021). The Docking experiment was performed using the Lamarckian Genetic algorithm and Local Search default parameters with ΔG values referring to significant binding between developed ligands (Sharma et al. 2022d).

Conclusions

Despite some limitations especially with *PTEN* assessment as there are consistency and reproducibility issues with various types of assays including immunohistochemistry testing and scoring systems such as H-score, percentage of positive cells, and protein levels, still, the current in-silico study establishes the prognostic and/or predictive role of *PTEN* in breast cancer therapeutics. With many binding sites available in the *PTEN* complex, molecular modeling of the complex has been performed. Substrate recruiting tetrad was targeted with irreversible binding to arrest the substrate recruiting mechanism and hence inhibition of *PTEN* pathway. A library of developed ligands consisting of 50 potential candidates was screened for finding the best ligand having better energy and biosafety. LIG28 was found promising with enough binding affinity and the best-fitted biosafety parameters, which can act as a potential drug molecule. The study could be further extrapolated to understand the clinical utility of *PTEN*-loss in cohorts of patients. Therefore, the potential of *PTEN* as a biomarker in breast cancer is promising and deserves further investigations to establish the targeting of *PTEN* as a breast cancer therapeutic. The potent ligand molecules designed can pave the way for therapeutic implications in breast cancer especially targeting the Phosphatase and tensin homolog.

Conflict of Interest

There exists no conflict of interest amongst authors regarding the publication of this manuscript.

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References

Bansal, P., Tuli, H.S., Sharma, D., Mohapatra, R., et al. (2022). Targeting omicron (b.1.1.529) SARSCov-2 spike protein with selected phytochemicals: An in-silico approach for identification of potential drug. *Journal of Experimental Biology and Agricultural Sciences, 10*, 396-404. doi: 10.18006/2022.10(2).396.404

Barbieri, C.E., & Rubin, M.A. (2015). Genomic rearrangements in prostate cancer. *Current Opinion in Urology, 25*(1), 71-76. doi: 10.1097/MOU.0000000000000129

Chen, L., Morrow, J.K., Tran, H.T., Phatak, S.S., et al. (2012). From laptop to benchtop to bedside: Structure-based drug design on protein targets. *Current Pharmaceutical Design, 18*(9), 1217-1239. doi: 10.2174/138161212799436386

Fuhrmann, J., Rurainski, A., Lenhof, H.P., & Neumann, D. (2010). A new lamarckian genetic algorithm for flexible ligand-receptor docking. *Journal of Computational Chemistry, 31*, 1911-1918. doi: 10.1002/jcc.21478

Hinz, N., & Jücker, M. (2019). Distinct functions of akt isoforms in breast cancer: A comprehensive review. *Cell Communication and Signaling, 17*(1), 154. doi: 10.1186/s12964-019-0450-3

Hoxhaj, G., & Manning, B.D. (2020). The pi3k-akt network at the interface of oncogenic signalling and cancer metabolism. *Nature Reviews Cancer, 20*(2), 74-88. doi: 10.1038/s41568-019-0216-7

Ma, J., Benitez, J.A., Li, J., Miki, S., et al. (2019). Inhibition of nuclear *PTEN* tyrosine phosphorylation enhances glioma radiation sensitivity through attenuated DNA repair. *Cancer Cell, 35*(3), 504-518 e507. doi: 10.1016/j.ccell.2019.01.020

Momenimovahed, Z., & Salehiniya, H. (2019). Epidemiological characteristics of and risk factors for breast cancer in the world. *Breast Cancer, 11*, 151-164. doi: 10.2147/BCTT.S176070

Raghav, M., Sharma, D., Chaudhary, M., Tuli, H.S., et al. (2021). Essence of *PTEN*: A broad-spectrum therapeutic target in cancer. *Biointerface Research in Applied Chemistry, 11*, 9587-9603. doi: 10.33263/BRIAC112.95879603

Rajpoot, M., Bhattacharya, R., Sharma, S., Gupta, S., et al. (2021). Melamine contamination and associated health risks: Gut microbiota does make a difference. *Biotechnolog and Applied Biochemestry, 68*(6), 1271-1280. doi: 10.1002/bab.2050

Ram, G., Sharma, V., Sheikh, I., Sankhyan, A., et al. (2020). Anti-cancer potential of natural products: Recent trends, scope and relevance. *Letters in Applied NanoBioScience, 9*(1), 902-907.

Roy, A., Kucukural, A., & Zhang, Y. (2010). I-tasser: A unified platform for automated protein structure and function prediction. *Nature Protocols, 5*(4), 725-738. doi: 10.1038/nprot.2010.5

Sehrawat, N., Yadav, M., Singh, M., Kumar, V., et al. (2021). Probiotics in microbiome ecological balance providing a therapeutic window against cancer. *Seminars in Cancer Biology, 70*, 24-36. doi: 10.1016/j.semcancer.2020.06.009

Sharma A. K., Sharma, I., Diwan Gautami & Sharma V. (2020a). Oral squamous cell carcinoma (oscc) in humans: Etiological factors, diagnostic and therapeutic relevance. *Research Journal of Biotechnology, 15*(10), 141-151.

Sharma V, Upadhyay, S., & Sharma, A.K. (2022a). PI3kinase/Akt/mTOR pathway in breast cancer; pathogenesis and prevention with mtor inhibitors. Proceedings of *IVSRTLSB-2021, 7*(1), 184-191.

Sharma V., Panwar A., Ram G., Sankhyan A., et al. (2022b). Exploring the potential of chromones as inhibitors of novel coronavirus infection based on molecular docking and molecular dynamics simulation studies. *Biointerface Research in Applied Chemistry, 13*(2), 1-8.

Sharma, A.K., Sharma, V.R., Gupta, G.K., Ashraf, G.M., et al. (2019b). Advanced glycation end products (ages), glutathione and breast cancer: Factors, mechanism and therapeutic interventions. *Current Drug Metabolism, 20*(1), 65-71.

Sharma, V., & Sharma, A.K. (2020c). An in-silico approach for designing a potential antagonistic molecule targeting β2-adrenoreceptor having therapeutic significance. *Letters in Applied Nanobioscience, 10*(1), 2063 -2069.

Sharma, V., Kumar Gupta, G., K Sharma, A., Batra, N., et al. (2017). Pi3k/akt/mtor intracellular pathway and breast cancer: Factors, mechanism and regulation. *Current Pharmaceutical Design, 23*(11), 1633-1638.

Sharma, V., Panwar, A., & Sharma, A.K. (2020b). Molecular dynamic simulation study on chromones and flavonoids for the in silico designing of a potential ligand inhibiting mtor pathway in breast cancer. *Current Pharmacology Reports, 6*, 373-379.https://doi.org/10.1007/s40495-020-00246-1

Sharma, V., Panwar, A., & Sharma, A.K. (2021a). P13k/akt/mtor pathway-based novel biomarkers for breast cancer. *Re: GEN OPEN, 1*, 83-91.

Sharma, V., Panwar, A., Gupta, G.K., & Sharma, A.K. (2022c). Molecular docking and md: Mimicking the real biological process. *Physical Sciences Reviews*. doi: doi:10.1515/psr-2018-0164

Sharma, V., Panwar, A., Sharma, A., Punj, V., et al. (2021b). A comparative molecular dynamic simulation study on potent ligands targeting mtor/frb domain for breast cancer therapy. *Biotechnology and Applied Biochemistry*. doi: 10.1002/bab.2206

Sharma, V., Saini, P., Sheikh, I., Upadhyay, S.K., et al. (2022d) Role of plant secondary metabolites as potential antimalarial drugs. *International Journal of Mosquito Research*; *9* (3), 13-22.

Sharma, V., Sehrawat, N., Sharma, A., Yadav, M., et al. (2021c). Multifaceted antiviral therapeutic potential of dietary flavonoids: Emerging trends and future perspectives. *Biotechnology and Applied Biochemistry*.doi: 10.1002/bab.2265.

Sharma, V., Sharma, A.K., Punj, V., & Priya, P. (2019a). Recent nanotechnological interventions targeting pi3k/akt/mtor pathway: A focus on breast cancer. *Seminars in Cancer Biology, 59*, 133-146.

Sharma, V., Sharma, D.K., Mishra, N., Sharma, A.K., et al. (2016). New and potential therapies for the treatment of breast cancer: An update for oncologists. *Current Trends in Biotechnology and Chemical Research 6(1),*23-29.

Sharma, V., Sharma, N., Sheikh, I., Kumar, V., et al. (2021d). Probiotics and prebiotics having broad spectrum anticancer therapeutic potential: Recent trends and future perspectives. *Current Pharmacology Reports, 7*(2), 67-79. doi: 10.1007/s40495-021-00252-x

Sharma, V., Singh, M., Kumar, V., Yadav, M., et al. (2021e). Microbiome dysbiosis in cancer: Exploring therapeutic strategies to counter the disease. *Seminars in Cancer Biology, 70*, 61-70. doi: https://doi.org/10.1016/j.semcancer.2020.07.006.

Sheikh, I., Sharma, V., Tuli, H.S., Aggarwal, D., et al. (2020). Cancer chemoprevention by flavonoids, dietary polyphenols and terpenoids. *Biointerface Research in Applied Chemistry, 11*, 8502-8537. doi: https://doi.org/10.33263/BRIAC111.85028537

Singh, M., Kumar, V., Sehrawat, N., Yadav, M., et al. (2022). Current paradigms in epigenetic anticancer therapeutics and future challenges. *Seminars in Cancer Biology, 83*, 422-440. doi: 10.1016/j.semcancer.2021.03.013

Trotman, L.C., & Pandolfi, P.P. (2003). *PTEN* and p53: Who will get the upper hand? *Cancer Cell, 3*(2), 97-99. doi: 10.1016/s1535-6108(03)00022-9

Wang, L., Tu, H., Zeng, L., Gao, R., et.al. (2022). Identification and *in silico* analysis of nonsense snps of human colorectal cancer protein. *Journal of Oleo Science, 71*(3), 363-370. doi: 10.5650/jos.ess21313

Wiederstein, M., & Sippl, M.J. (2007). Prosa-web: Interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Research, 35*(2), W407-W410.

Yang, J., & Zhang, Y. (2015). I-tasser server: New development for protein structure and function predictions. *Nucleic Acids Research, 43*(W1), W174-181. doi: 10.1093/nar/gkv342

Yuan, Y., Pei, J., & Lai, L. (2020). Ligbuilder v3: A multi-target de novo drug design approach. *Frontiers in Chemistry, 8*. doi: 10.3389/fchem.2020.00142

Zhang, S., & Yu, D. (2010). Pi(3)king apart *PTEN*'s role in cancer. *Clinical Cancer Research, 16*(17), 4325-4330. doi: 10.1158/1078-0432.ccr-09-2990

Zheng, W., Zhang, C., Li, Y., Pearce, R., et al. (2021). Folding non-homologous proteins by coupling deep-learning contact maps with i-tasser assembly simulations. *Cell Reports Methods, 1*(3). doi: 10.1016/j.crmeth.2021.100014

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Correlation Analysis between Internet Addiction and Self-Regulation among Thai University Students

Supat Chupradit[1*], Tanaporn Tonghom[1], Priyanut Wutti Chupradit[2], Tippawan Sookruay[3]

[1]Department of Occupational Therapy, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand
[2]Educational Psychology and Guidance, Department of Educational Foundations and Development, Faculty of Education, Chiang Mai University, Chiang Mai 50200, Thailand
[3]Chiang Mai University Library, Chiang Mai University, Chiang Mai 50200, Thailand

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ABSTRACT

The self-regulation of internet activities is a challenge between technology and human interaction, particularly in adolescents. It is very important to study the relationship between self-regulation and internet addiction since humans have become closely connected to technology in recent decades. The objective of the present research was to study the relationship between internet addiction and self-regulation by assessing the habits of university students. The samples consisted of 500 first-year students residing in Chiang Mai University dormitories, and data were collected from questionnaires regarding personal information, the Internet Addiction Test (IAT), and self-regulation assessment. Pearson's correlation coefficient was used to investigate the relationship between internet addiction and self-regulation. The results of the study revealed that the level of internet addiction had a moderately positive relation with poor self-regulation, which had a correlation coefficient of 0.560 with a statistical significance level of 0.01. Further, the level of internet addiction had a low negative relation with good self-regulation, which had a correlation coefficient equal to -0.262 with a statistical significance level of 0.01. Hence, creating the necessary assistance and solutions is required to achieve a healthy balance in the behavior of young individuals.

* Corresponding author
E-mail: supat.c@cmu.ac.th (Supat Chupradit)

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1 Introduction

These days internet becomes an important part of our daily life and youths are using the internet frequently for their personal and beneficial purposes (Charoenwanit 2014; Kuss and Griffiths 2017; Cahill and Beisbier 2020). However, those who use the internet too much could cause positive and negative physical as well as mental consequences (Young 1996, 1998, 1999; Ju 2007). Further, recently it has been reported that university students use internet media for their entertainment purposes and to relieve stress, but it has been found that excessive use of the internet could cause addiction along with other problems in education or health aspects in some cases (Chupradit et al. 2019, 2020a, b; Vadivu and Chupradit 2020). It has also been reported that in some cases the absence of internet surfing or inability to use the internet might cause anxiety in active internet-using students or users (Caplan 2002; Lam 2014; Jelenchick et al. 2016; Lemmens and Hendriks 2016; Starcevic and Aboujaoude 2017; Leménager et al. 2018). One suitable solution is to surf the web properly in short intervals with limitations and change actions into workouts, reading, or other activities of interest for the benefit of one's physical and mental health (Prasertsin 2009).

Self-regulation is the ability or capability of an individual to exercise self-supervision consciously and intentionally to change his/her response to the desired standards for future rewards or to suppress automatic responses to low-value behaviors. It is a cognitive control as it signifies the ability to think before you act in an attempt to develop coexistence with self-observation, self-judgment, and self-reaction (Błachnio and Przepiorka 2016). An individual uses his/her internal standards to motivate himself/herself to conduct behavior and evaluate his/her reactions, in which everything depends on the one who acts them (Srikan 2009). The development of life at all stages requires adaptation to changes. In this regard, college life is filled with changes in many aspects, including physical, mental, emotional, and social as an individual transitions from being a high school student to a university student. University students may face difficulties in peer group activities and time management. In particular, for students living in university dorms, there are dormitory rules with which they must comply, and the need to adapt to living with others. Such factors require self-regulation in their daily activities, and time management so that student life runs efficiently.

Dormitory life is an important phase of college life where students experience lifelong learning, improve their life and social skills, and help each other with a sense of sharing and caring for one another. They learn how to live happily with others, allowing them to grow together emotionally, as well as exchange ideas and knowledge with each other, thus fostering mutual understanding and forming friendly relations. The student dormitory is a place to improve the quality of students by providing them a way of life in the dormitory as a learning mechanism (Student Dormitory Office CMU 2018).

From the occupational therapy perspective, the principle of the Person-Environment-Occupation-Performance (PEOP) Model (Charles 2005) states that the relation and balance of the 3 components, i.e. individual, environment, and lifestyle activities, will affect the behavior and ability to do good and meaningful activities. This model could be used to explain internet usage behavior among students in terms of how it affects their self-regulation in other activities and if they are obsessed with the internet. A lack of balance in these 3 components will result in impaired activities and a decreased ability to perform other activities. It has been found to affect the interest, thinking, and decision-making ability in other activities so that they are no longer capable of maintaining a balance between their needs in the working environment and participation in various activities. This makes them unsuccessful in doing activities and will affect their ability to adapt when facing challenges due to less participation in lifestyle activities (Abaoğlu et al. 2017; Cahill and Beisbier 2020).

As mentioned above, it is obvious that excessive usage of the internet by young people is a major problem that growing day by day. This made the researcher more interested in internet usage behavior and self-regulation among students by focusing on the relationship between them. In the present study, first-year students residing in the dormitories at Chiang Mai University comprising students from various faculties and programs were selected as the study population. In dormitories, students can use the unlimited internet provided by the university to access the internet everywhere. Moreover, a dormitory often has periods of access to the internet, meaning most students stay for a specified time to have more free time in the dormitory. As a result, they have the chance to use the internet for extended periods, which is advantageous for the students in university dormitories. For this reason, the researchers aimed to study the internet usage behavior and self-regulation of first-year students living in the dormitories at Chiang Mai University and to analyze the correlation between internet addiction and self-regulation.

2 Materials and Methods

2.1 Population

A total of 3,991 first-year students residing in the dormitories at Chiang Mai University, Mueang, Chiang Mai Province, who were in their first semester of the academic year 2018, participated in this study.

2.2 Samples

The samples included 500 students. The criteria for sample selection included: age of 18 years and older, no serious illnesses

or disabilities, and willingness to give consent to participate in the research. The exclusion criteria included volunteers with severe illnesses or disabilities and volunteers who did not agree to or were not ready to answer the questionnaire.

2.3 Research Tools

A well-structured questionnaire was prepared for extracting general information from the selected respondents. Further, internet usage was also evaluated during the study period. The researcher used the Internet Addiction Test (IAT) tool by Kimberly Young (Internet Addiction Test 1998), with a confidence value of 0.89, consisting of 20 questions to evaluate the level of internet usage. The samples answered of the selected questions assigned to particular numbers that matched the opinion and behaviour of the respondents, e.g. 1 = Infrequent, 2 = Occasional, 3 = Frequent, 4 = Regular, 5 = Constant, and 0 = Unable to specify. The interpretation of the internet usage assessment is as follows: A total score of 100 points divided into 4 levels including None (not addicted), Mild (mildly addicted), Moderate (moderately addicted), and Severe (severely addicted).

The self-regulation assessment form by Wattananonsakul (2009), consists of 24 questions, which are divided into two sub-measures comprising poor self-regulation in 12 positive questions, good self-regulation in 12 questions, 10 positive questions, and 2 negative questions, with 5-level rating scales. A high score means a very good level of self-regulation, while a low score means a low level of self-regulation. In this regard, this tool made use of a translation process to enable the precision quality of the tool. Both measures of good and poor self-regulation have Cronbach's alpha values of 0.82 and 0.80, respectively.

2.4 Data Analysis and Statistics

2.4.1 Data analysis with descriptive statistics

Frequency and Percentage are used to describe general information with mean and standard deviation.

2.4.2 Data analysis with inferential statistics

The researcher used Pearson's Correlation Coefficient when the data had a normal curve distribution and the Spearman Rank Difference Method when the data did not have a normal curve distribution. The criteria for determining the relation level are as per Taweerat (2000), and the detail is as (i) if the correlation coefficient is 0.80 - 1.00, is considered to be very high, (ii) if the correlation coefficient is 0.60 - 0.79, is considered to be high, (iii) if the correlation coefficient is 0.40 - 0.59, is considered to be moderate, (iv) if the correlation coefficient is 0.20 - 0.39, is considered to be low and (v) if the correlation coefficient is 0.00 - 0.19, is considered to be very low.

3 Results

3.1 The general information and characteristics of the samples

Results presented in table 1 suggested that the sample group consisted of 500 students, comprising 165 male students (33.0%) and 335 female students (67.0%). In terms of the major subject studied by respondents, the majority of the respondents (42.6%) studied human-social science as a major subject; this was followed by science-tech (38.0%) and science-health (19.4%). The samples have an age range of 18 to 21 years old with an average age is of 19.04 years.

3.2 Level of internet addiction

Results presented in table 2 suggested that the participated respondents have four levels of internet addiction. Most of them have mild internet addictions (50.2%), followed by moderate (33.6%), no addiction (15.6%), and severe internet addiction (0.6%). Among the studied 500 respondents, a total of 422 respondents (84.40%) had internet usage behavior varying from the lowest level to the severe level of internet addiction.

Table 1 General Information and Characteristics of the participating respondents

General Information and Characteristics		Number of Samples	Mean ± S.D.	Percentage	Total
Gender	Male	165	1.670 ± 0.47	33.0	500 (100%)
	Female	335		67.0	
Major subject	Science-Tech	190	2.046 ± 0.89	38.0	500 (100%)
	Science-Health	97		19.4	
	Human-Social	213		42.6	
Age	Min (y)	Max (y)		Average (y)	S.D.
	18	21		19.04	0.58

Sample size n = 500; S.D. =Standard Deviation

Table 2 Level of Internet Addiction (n = 500)

Level of Internet Addiction		Number of Samples (Percentage)	
None (0-30)		78 (15.6)	
Mild (31-49)		251 (50.2)	
Moderate (50-79)		168 (33.6)	
Severe (80-100)		3 (0.6)	
n	Min – Max (score)	Mean	SD
500	3-84	43.82	13.71

Sample size n = 500; S.D. =Standard Deviation

3.3 Self-regulation Scores of the Samples

In the case of self-regulation, the poor self-regulation scores ranged from 12 to 52 with an average self-regulation score of 27.66. While in the case of good self-regulation scores it ranges from 20 to 60 with an average score of 42.50 (Table 3).

3.4 Scores for self-regulation of the samples by gender

In the case of gender-specific self-regulations, male respondents have poor self-regulation, and their scores ranged from 12 to 49 with an average score of 29.74. Furthermore, the male's good self-regulation scores range from 20 to 60 with an average score of 41.93. In the case of females, they have poor self-regulation scores ranging from 12 to 52 with an average score of 26.64 and good self-regulation scores ranging from 20 to 60 with an average score of 42.78 (Table 4).

3.5 Correlation between internet addiction and self-regulation

The correlation coefficient of data using Pearson's correlation coefficient (Table 5) has revealed that the level of internet addiction has a moderate level of positive relation to poor self-regulation, which has a correlation coefficient of 0.56 with a statistical significance of 0.01. The level of internet addiction has a low negative relation to good self-regulation, which has a correlation coefficient equal to -0.26 with a statistical significance of 0.01.

4 Discussion

Results of the study suggested that most of the participants had a higher average score of good self-regulation. Good self-regulation is the ability of an individual to control and determine their behavior consciously and intentionally by using thoughts and reason to achieve the goals they set. During the student phase of life, a young individual goes through maturity, accepting various responsibilities such as education, living with friends, and engaging with other people in society, which in turn makes an individual learn good self-regulation skills and strive for improvement. These are factors that should motivate an individual

Table 3 Self-regulation Scores of the Samples

Self-regulation	Lowest Scores	Highest Scores	Mean	SD
Poor Self-regulation	12	52	27.66	8.35
Good Self-regulation	20	60	42.50	5.94

Sample size n = 500; S.D. =Standard Deviation

Table 4 Scores for self-regulation of the samples by gender

Gender	Samples	Self-regulation	Lowest Scores	Highest Scores	Mean	SD
Male	165	poor self-regulation	12	49	29.74	8.33
		good self-regulation	20	60	41.93	6.18
Female	335	poor self-regulation	12	52	26.64	8.18
		good self-regulation	20	60	42.78	5.80

Sample size n = 500; S.D. =Standard Deviation

Table 5 Correlation between Internet Addiction and Self-regulation

Addiction	Internet Addiction	Poor Self-regulation	Good Self-regulation
Internet Addiction	1		
Poor Self-regulation	.560**	1	
Good Self-regulation	-.26**	-.36**	1

**p < 0.01

to be more self-disciplined. Results of the current study revealed that internet usage behavior had an effect on both poor and good self-regulation with statistical significance at the level of 0.01.

In terms of poor self-regulation, it has a moderate level of positive relationship with the level of internet addiction. The correlation coefficient is 0.56. It can be summarized that students who have high scores in poor self-regulation also have a high level of internet addiction. On the other hand, students who have low scores of poor self-regulation also have a low level of internet addiction. This result is consistent with the previous study. Li et al. **(**2021**)** found that students with less self-regulation had greater internet addiction. Poor self-regulation is a lack of self-regulation by an individual to behave consciously and reasonably to achieve the goals they set, which puts an individual at risk since they perform various behaviors according to their emotions. If we look at internet usage, we could point out the usage of the internet according to emotions. Further, internet addiction and spending too much time using the internet can eventually interfere with various other activities in daily life. However, an individual with good self-regulation would likely have fewer cases of actions that result from their emotions.

Good self-regulation has a low level of negative relation to the level of internet addiction, which has a correlation coefficient equal to -0.262, indicating that students with high scores of good self-regulation have a low internet addiction. Self-regulation is achieved through mindfulness and reasoning in thinking, considering, and contemplating the result of an action. If an individual has a high level of good self-regulation, it will affect the behavior of various aspects of expression through careful thought and consideration.

Conclusions and Future Research

In conclusion, the majority of the samples who participated in the present study had a low level of internet addiction, poor self-regulation scores at a low level, and good self-regulation scores at a low to moderate level. The results of the study revealed that students with poor self-regulation behavior scores tended to have high levels of addiction to the internet, while students with high scores for good self-regulation tended to have a low level of internet addiction with statistical significance at a level of 0.01. This study revealed that most of the samples had a low level of internet usage addiction. We could make use of the internet as a medium or a tool for teaching and learning by encouraging students. This study further revealed the time and the purpose of using the internet can promote the use of the internet for education, such as organizing online learning activities, self-study, workload, and homework for academic benefits. This study examined the relationship between internet usage behavior and self-regulation. Future research should include some more variables such as self-esteem, stress, anxiety, or psychosocial factors to develop self-regulation. Further, future studies could select respondents from a different context, such as students living in off-campus residences or samples from other age groups.

Ethical Approval and Consent to Participate

This research has been certified for ethical research in humans by the Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand Project number: AMSEC-60EX-017.

References

Abaoğlu, H., Cesim, Ö. B., Kars, S., & Çelik, Z. (2017). Life Skills in Occupational Therapy. In M. Huri (Ed.), *Occupational Therapy - Occupation Focused Holistic Practice in Rehabilitation*. IntechOpen. https://doi.org/10.5772/intechopen.68462

Błachnio, A., & Przepiorka A. (2016). Dysfunction of Self-Regulation and Self-Control in Facebook Addiction. *The Psychiatric quarterly*, *87*(3), 493-500.

Cahill, S.M., & Beisbier, S. (2020). Occupational Therapy Practice Guidelines for Children and Youth Ages 5-21 Years. *American Journal of Occupational Therapy*, *74*(4), 1-48.

Caplan, S.E. (2002). Problematic Internet use and psychosocial well-being: Development of a theory-based cognitive-behavioral measurement instrument. *Computers in Human Behavior*, *18* (5), 553-575.

Charles, C. (2005). *Occupational Therapy: performance, participation, and well-being* (3rd ed.)**.** Thorofare, NJ: SLACK Incorporated.

Charoenwanit, S. (2014). Game Addiction Behaviors: Impacts and Preventions. *Thai Science and Technology Journal*, *22*(6), 871-879.

Chupradit, S., Joompathong, N., & Chupradit, P.W. (2020a). Prevalence and Correlates between Internet Use Behavior and Social Skill among University Students in Thailand. *International Journal of Psychosocial Rehabilitation*, *24*(6), 14682-14695.

Chupradit, S., Kaewmamuang, N., Kienngam, N., & Chupradit, P.W. (2019) Prevalence and Correlates between Game Addiction and Stress of Adolescents in Chiang Mai, Thailand. *Indian Journal of Public Health Research and Development*, *10(8)*,1091–1096.

Chupradit, S., Leewattana, A., & Chupradit, P.W.(2020b). The correlation analysis of internet usage and depression among undergraduate university students in Thailand: Cross-sectional

study. *Journal of Advanced Research in Dynamical and Control Systems*, *12*(6), 825-837.

Internet Addiction Test (IAT). (1998). Retrieved fromhttp://netaddiction.com/internet-addiction-test/.

Jelenchick, L.A., Hawk, S.T.,& Moreno, M.A.(2016). Problematic internet use and social networking site use among Dutch adolescents. *International journal of adolescent medicine and health*, *28*(1), 119-21.

Ju, Y.A. (2007). School-based programs for Internet addiction prevention and intervention. *International Symposium on the Counseling and Treatment of Youth Internet Addiction; Seoul, Korea*: National Youth Commission.

Kuss, D. J., & Griffiths, M.D. (2017). Social Networking Sites and Addiction: Ten Lessons Learned. *International journal of environmental research and public health*, *14*(3), 1-17.

Lam, L.T. (2014). Internet gaming addiction, problematic use of the internet, and sleep problems: a systematic review. *Current psychiatry reports*, *16*(4), 444.

Leménager, T., Hoffmann, S., Dieter J., Reinhard, I., et al. (2018). The links between healthy, problematic, and addicted Internet use regarding comorbidities and self-concept-related characteristics. *Journal of behavioral addictions*, *7*(1), 31-43.

Lemmens, J.S., & Hendriks, S.J. (2016). Addictive Online Games: Examining the Relationship Between Game Genres and Internet Gaming Disorder. *Cyberpsychology, behavior and social networking*, *19*(4), 270-276.

Li, S., Ren, P., Chiu, M. M., Wang, C., & Lei, H. (2021). The Relationship Between Self-Control and Internet Addiction Among Students: A Meta-Analysis. *Frontiers in Psychology*, *12*, 1-16.

Prasertsin, A. (2009). *Effects of the Internet on Physical and Mental Health of Thai Children and Youth: Case Study in Bangkok*. National Research Council of Thailand.

Srikan, S. (2009). *Effects of using self-directed programs on academic responsibilities of students in grade 6, Phon Prachanukul Municipality School, Phon District, Khon Kaen Province. Bangkok*: Srinakharinwirot University.

Starcevic, V., & Aboujaoude, E. (2017). Internet addiction: reappraisal of an increasingly inadequate concept. *CNS spectrums*, *22*(1), 7-13.

Student Dormitory Office CMU. (2018). *Student Dormitory Office*, Chiang Mai University.

Taweerat, P. (2000). Research Methods in Behavioral Sciences and Social Sciences. 8th ed. *Chulalongkorn University Press, Bangkok.*

Vadivu, S.V., & Chupradit, S. (2020). Psychosocial and Occupational Impact Assessment due to Internet Addiction: A Critical Review. *Systematic Reviews in Phamacy*, *11*(7), 152-155.

Wattananonsakul, S. (2009). A development of the causal models of adolescent smoking/drinking behaviors and a study of model invariance across age. Unpublished PhD thesis submitted to the Chulalongkorn University, Bankok.

Young, K.S. (1996). Internet Addiction: The Emergence of a New Clinical Disorder. *Cyber Psychology & Behavior*, *1*, 237-244.

Young, K.S. (1999). *Internet addiction: Symptoms, evaluation, and treatment innovations in clinical practice FL*: Professional Resource Press.

Young, K.S.(1998). Internet addiction: The emergence of a new clinical disorder. *Cyber Psychology & Behavior*, *1*(3), 237-244.

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Antiradical and Oxidative Stress Release Properties of *Trifolium pratense* L. extract

Lyubov S. Dyshlyuk, Maria A. Osintseva, Oksana V. Kozlova, Natalya V. Fotina, Alexander Yu. Prosekov*

Kemerovo State University, 6 Krasnaya St., Kemerovo, Russia, 650000

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Trifolium pratense L.

Callus culture

Ononin

Chlorogenic acid

Genistein

Biochanin A

Antiradical activity

ABSTRACT

Low adaptive capacity and oxidative stress are the factors leading to cellular dysfunction, protein and lipid peroxidation, and the development of diseases. In recent decades, there has been a trend toward the active use of plant-based antioxidants. *Trifolium pratense L.* is a promising plant for the pharmaceutical and food industry and has anti-radical properties. This work is devoted to studying the antiradical and oxidative stress-released properties of *T. pratense* in *Caenorhabditis elegans* under oxidative and temperature stress. The objective of this research was to evaluate the anti-radical properties of the *T. pratense* extracts and individual BAS (chlorogenic acid, ononin, biochanin A, genistein) and analysis their influences on the oxidative stress of *Caenorhabditis elegans* in the presence of paraquat. Analysis of the antiradical properties revealed that chlorogenic acid has the maximum ability to neutralize the free radical (35.49µmol). A separate analysis of oxidative stress revealed high ononin activity at concentrations of 10, 50, and 100 µmol at 48 hours of cultivation. Biochanin A increases survival by 13.1% compared to the control. The use of the extract (500µmol) contributed to an increase in survival on day 1 of incubation. Under conditions of thermal stress, ononin (50 and 200 µmol) has a positive effect on the viability of *C. elegans*. The extract and BAS of *T. pratense* are characterized by high antiradical activity. In addition, the ability to influence the viability of *C. elegans* was revealed. Therefore, it is worthwhile to further study the biological properties of *T. pratense* for use in geroprotective therapy.

* Corresponding author

E-mail: a_piskaeva@mail.ru (Alexander Yu. Prosekov)

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1 Introduction

Aging is a process of degenerative changes in cells, tissues, and organs of the body that is accompanied by reduced adaptive activity to stressful environmental conditions and an increased risk of diseases and deaths (Hou et al. 2019). One key factor influencing the development and progression of many chronic diseases is oxidative stress. Oxidative stress is a state of the body resulting from excessive production and/or low ability to eliminate free radicals (reactive oxygen species, peroxides, etc.) (Leite et al. 2020; Monteiro-Alfredo et al. 2020). ROS plays an important role in maintaining cell homeostasis. However, their increased levels cause cellular dysfunction, protein and lipid peroxidation, and DNA damage, which eventually lead to irreversible damage and cell death with the gradual development of degenerative diseases like diabetes, arthritis, cardiovascular diseases, oncology, etc.) (van der Pol et al. 2019).

A key strategy for preventing the development of degenerative diseases and aging is the inclusion of antioxidants in the diet (Roxo et al. 2020). Synthetic antioxidants have several negative effects, including allergic reactions, liver damage, etc. on the human body. Plant raw materials rich in biologically active substances (BAS) of an adaptogenic orientation can be used as an alternative to synthetic antioxidants (Hou et al. 2019). Various studies have been conducted that show the influence of adaptogenic plants on the state of the body under stressful conditions (Chen et al. 2016; Jattujan et al. 2018; Tambara et al. 2018; Wang et al. 2018). According to Brekhman and Dardymov (1969), adaptogens are characterized by various properties, including an increase in the nonspecific resistance of the body, normalizing the state of the body regardless of the nature of the pathology, do not affect the normal functions of the body more than required, and be safe (Hou et al. 2019).

From a pharmacological point of view also, plant raw materials are a suitable source of chemical compounds for the treatment of various diseases, including those caused by oxidative stress. Further, the antioxidant activity of plants is related to their chemical composition, namely the presence of BAS (polyphenols, vitamins, organic acids, etc.). Thus, the search for promising sources of BAS is relevant to normalizing the reducing-oxidizing balance in the body.

Trifolium pratense is a representative of the genus Trifolium, a forage plant that is widely used in agriculture. The main biologically active compounds of the plant include flavonoids (Quercetin, Kaempferol, Apigenin, Hyperoside), isoflavonoids (Biochanin A, Formononetin, Daidzein, Genistein, Prunetin), phenolic acids (caffeic, rosemary, chlorogenic, salicylic, n-coumaric, ferulic), etc. (Akbaribazm et al. 2020). The rich composition of secondary metabolites gives the plant a wide range of pharmacological properties, including anti-inflammatory, antioxidant, antibacterial, antiviral, antitumor, antifungal, and neuroprotective (Luo et al. 2020).

To study the role of oxidative stress and phytochemical compounds in aging processes, a model system *Caenorhabditis elegans*, a soil free-living nematode are used in vivo (Wilson et al. 2006; Thabit et al. 2019; Roxo et al. 2020; González-Peña et al. 2021; Wang et al. 2021). The nonparasitic nematode *C. elegans* is characterized by changes in behavior and physiological health indicators (stress resistance, degeneration of the nervous system, changes in the structure of muscle tissue) which are similar to humans (Wang et al. 2020). Therefore, an assessment of the adaptogenic effect on model organism *C. elegans* can provide detail related to the positive effect of plant extracts on human life expectancy. The objective of this study was to examine the antioxidant activity and stress resistance of the *T. pratense L.* extract and its individual BAS in the *C. elegans* model system.

2 Materials and Methods

Callus culture of earlier stage *T. pratense* and standard BAS individual (Sigma-aldrich, USA) were used for the preparation of extract in this study (Dyshlyuk et al. 2021). For this, *T. pratense* callus was cultured on Gamborg nutrient medium supplemented with kinetin (2.00 mg/L), 6-BAP (0.10 mg/L), IUK (2.00 mg/L), and 2.4-D (2.00 g) from seedlings (Gamborg et al. 1968). The extract was prepared as described by Dyshlyuk et al. (2021).

The antioxidant activity of *T. pratense* callus extract and individual BAS were determined to the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical as per the method given by Re et al. (1999). To determine the antioxidant activity of the callus culture extract, it was dried with the help of Mini Spray Dryer B-290 (BUCHI, Switzerland) having parameters like the temperature of 105 °C, liquid feed rate of 6.2 mL/min, and main air flow rate of ~90 m^3/h (Langrish and Premarajah 2013).

Next, the effect of *T. pratense* callus cultures extracts and individual BAS was used to analyze the stress resistance in *C. elegans* against oxidative and temperature stress. For this *C. elegans* multiplication was carried out according to the standard method given by Rathor and Pandey (2018).

Individual BAS (chlorogenic acid, ononin, biochanin A, genistein) and their dry extract were prepared in DMSO of 10 mM concentration. Test solutions of BAS at various concentrations of 10, 50, 100, and 200 µmol and callus culture extract in three concentrations of 5, 50, and 500 µmol were prepared. The test solutions were stored at 4 °C until they were used.

Oxidative Stress release in *C. elegans* and effect of various concentrations of callus culture extract and standard extract

evaluated as per Rathor and Pandey (2018). For this, after adding 15 µl of the BAS or callus culture extract and 15 µl of 1M paraquat, the nematodes inoculated plates were left to incubate at a temperature of 20 °C, and after 24 and 48 hours of incubation, first and second counting of living and dead nematodes were carried out. The results of the effect of BAS on the oxidative stress resistance of nematodes were compared with the results of control nematodes that were incubated without adding the tested compounds.

For the estimation of temperature stress in *C. elegans* in the presence of BAS, the experiment was carried out as per the method described for oxidative stress release in *C. elegans* with some required changes. Further, the experiment was carried out without the addition of papaquat; throughout the experiment, the incubation temperature was 33 °C.

All analyses were repeated 3 times and statistical data was analyzed on Microsoft Office Excel 2007. Statistical analysis of the obtained data was carried out using a single-stage Student's paired criterion for each pair of interests. The differences were considered statistically significant at $p<0.05$.

3 Results and discussion

The results presented in Figure 1 revealed the antiradical activity of various concentrations of the *T. pratense* extract and BAS compared to standard Ascorbic acid in the neutralization of the ABTS. Results of the study suggested the highest ABTS radical scavenging activity from genistein (95.76%), followed by the chlorogenic acid (91.5%) and *T. pratense* callus culture extract (87.63%), and these were at par with the standard control ascorbic acid (94.09%). Among the various tested extracts lowest ability to absorb ABTS radicals (57.40%) was reported from Biochanin A.

Table 1 shows the EC_{50} values of *T. pratense* extracts, individual BAS, and ascorbic acid (control) in ABTS radical neutralization. The highest effective concentration was determined for ascorbic acid and

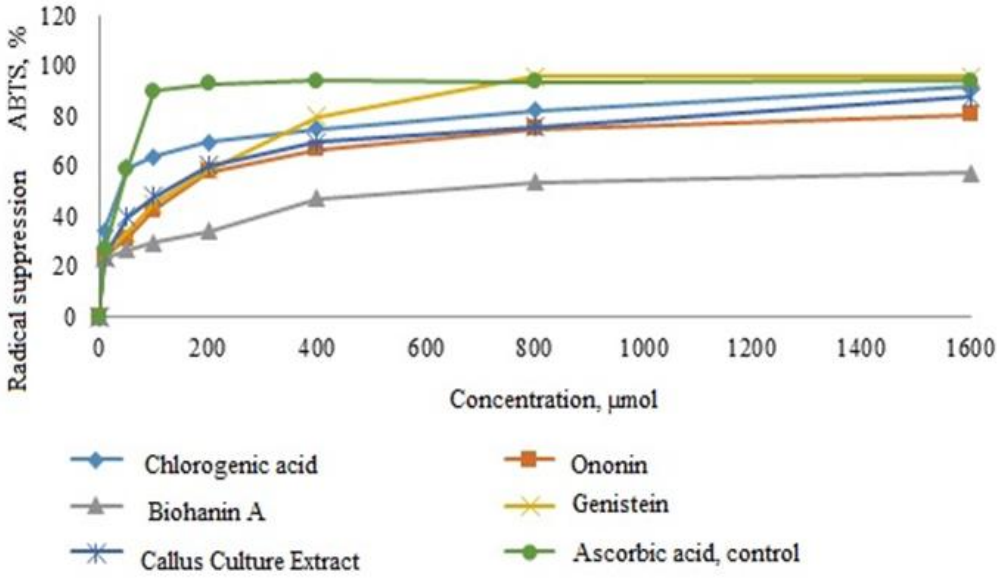

Figure 1 Antiradical activity of various concentrations of extract of callus cultures and individual BAS *T. pratense L.*

Table 1 Results of determining the effective concentration of ABTS radical suppression

No.	Name of sample	Effective concentration EC_{50}, µmol
1	Ascorbic acid (control)	38.24±1.89
2	Callus culture extract	117.60±3.62
3	Chlorogenic acid	35.49±1.59
4	Genistein	137.02±4.01
5	Biochanin A	582.20±6.64
6	Ononin	151.83±3.98

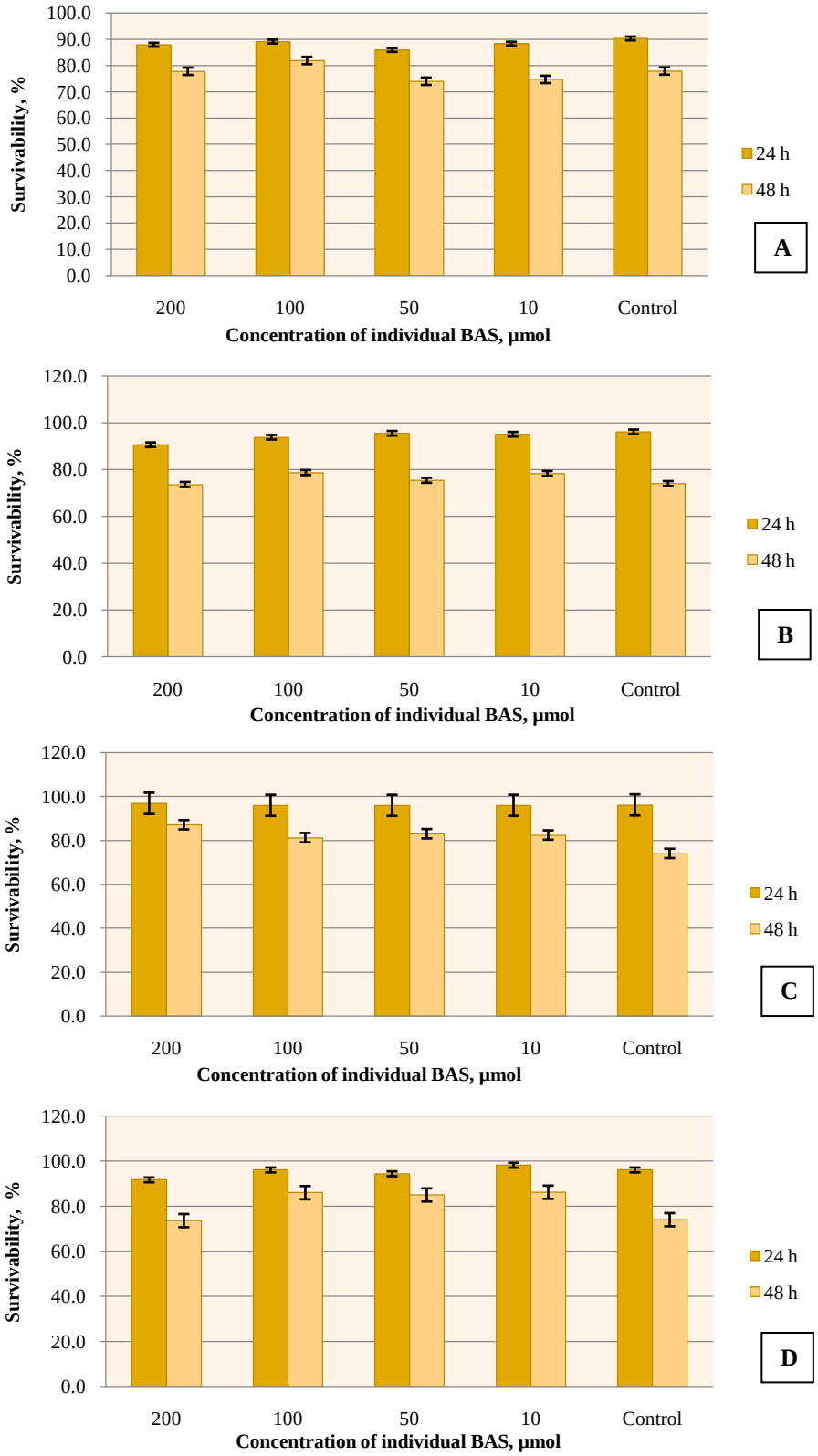

Figure 2 Effect of individual BAS on the resistance of *C. elegans* to oxidative stress (A) Effect of Chlorogenic, (B) Effect of Genistein, (C) Effect of Biochanin A, (D) Effect of Ononin

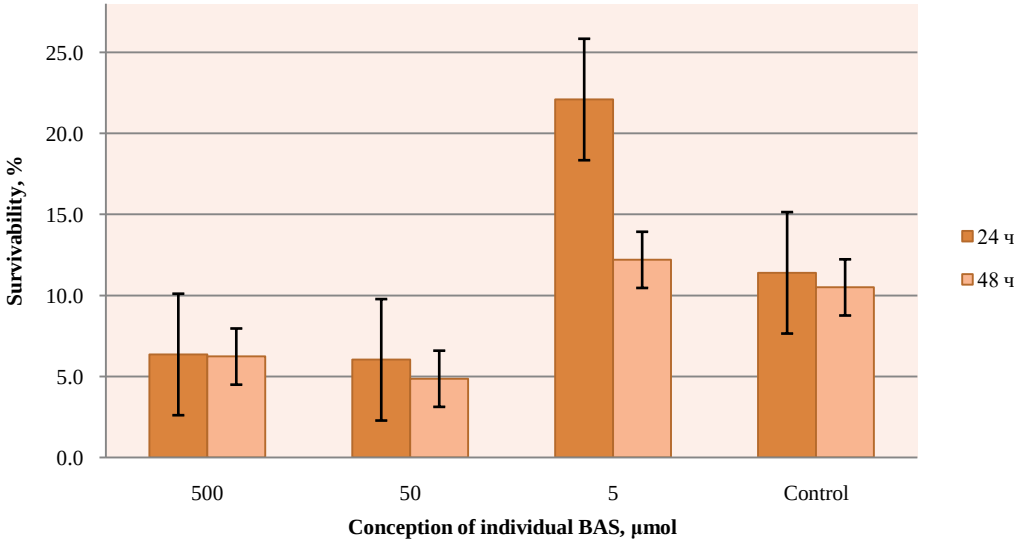

Figure 3 Effect of *T. pratense L.* callus culture extract on the resistance of *C. elegans* to oxidative stress

it is followed by chlorogenic acid (35.49 µmol), and these two are not significantly different. These results obtained are consistent with the literature data (Sun et al. 2014; Wang et al. 2021). The effective concentration of the callus culture extract was 117.60 µmol. From the studied literature, ABTS-radical absorption of methanol extract and various fractions of the plant has a value of 111.84 to more than 500 µg/ml (Esmaeili et al. 2015). Based on these indicators, it can be concluded that the extract has high antiradical activity.

To study the role of *T. pratense* callus culture extract and individual BAS (chlorogenic acid, ononin, biochanin A, genistein) in aging processes, a model system of the soil free-living *C. elegans* was used *in vivo*, since it is characterized by changes in behavior and physiological health indicators similar to humans (stress resistance, nervous system degeneration, changes in muscle tissue structure). Figure 2 presents a graphical representation of the effect of BAS tested at concentrations of 10, 50, 100, and 200 µmol on the oxidative stress resistance of *C. elegans*. The studied concentrations of individual BAS (10, 50, 100, 200 µmol) did not have any significant effect on the survival rate of *C. elegans* individuals under oxidative stress after 24 hours under the influence of 1M paraquat. After 48 hours of incubation ononin at concentrations of 10-100 microns had the greatest effect on the survival of nematodes. In addition to this BAS increased the survival rate of nematodes by 11.7% as compared to the control. The tendency to increase survival rate was also noted when using Biochanin A. The maximum survival rate was 87.1% compared to the control of 74.0%. The obtained data are in agreement with the findings of previous studies on the antioxidant properties of these BAS on other model systems (Wu et al. 2021; Dong et al. 2022). Chlorogenic acid and genistein did not affect the survival of nematodes under oxidative stress. It is important to note that there is no negative influence of chlorogenic acid and genistein on the survival of the model organism.

Figure 3 presents a graphical representation of the *T. pratense* callus culture extract for oxidative stress resistance with *C. elegans*. The results of the research revealed a positive effect of the extract in minimum concentration (500 µmol) on the survival of nematodes under oxidative stress. An increase in survival on the first day of cultivation by 93% compared to the control is associated with a wide range of compounds characterized by high antioxidant activity.

The results of the effect of individual BAS and extract on temperature stress are shown in Figures 4 and 5. At 48-hour incubation, the overall dynamics are characterized by an increase in the viability of *C. elegans* at ononin concentrations of 50 µmol and 200 µmol (by 9.4% and 8.6%, respectively). For the remaining BAS at 48 hours of incubation, there is a tendency to increase the survival rate of nematodes with an increase in the concentration of BAS. At 200 µmol of BAS, the average survival rate was increased by 11.8% as compared to the control samples. Use of the extract in a concentration of 500 µmol led to 100% death of *C. elegans* under both 24 and 48 hrs of incubation. However, when using concentrations of 5 µmol and 50 µmol, no changes in survival were reported as compared to the control. The data obtained are characterized by relatively low activity compared to data from the studied literature (Carranza et al. 2020; Cai et al. 2022). This is probably due to the deviation of the experimental conditions from the standard methodology.

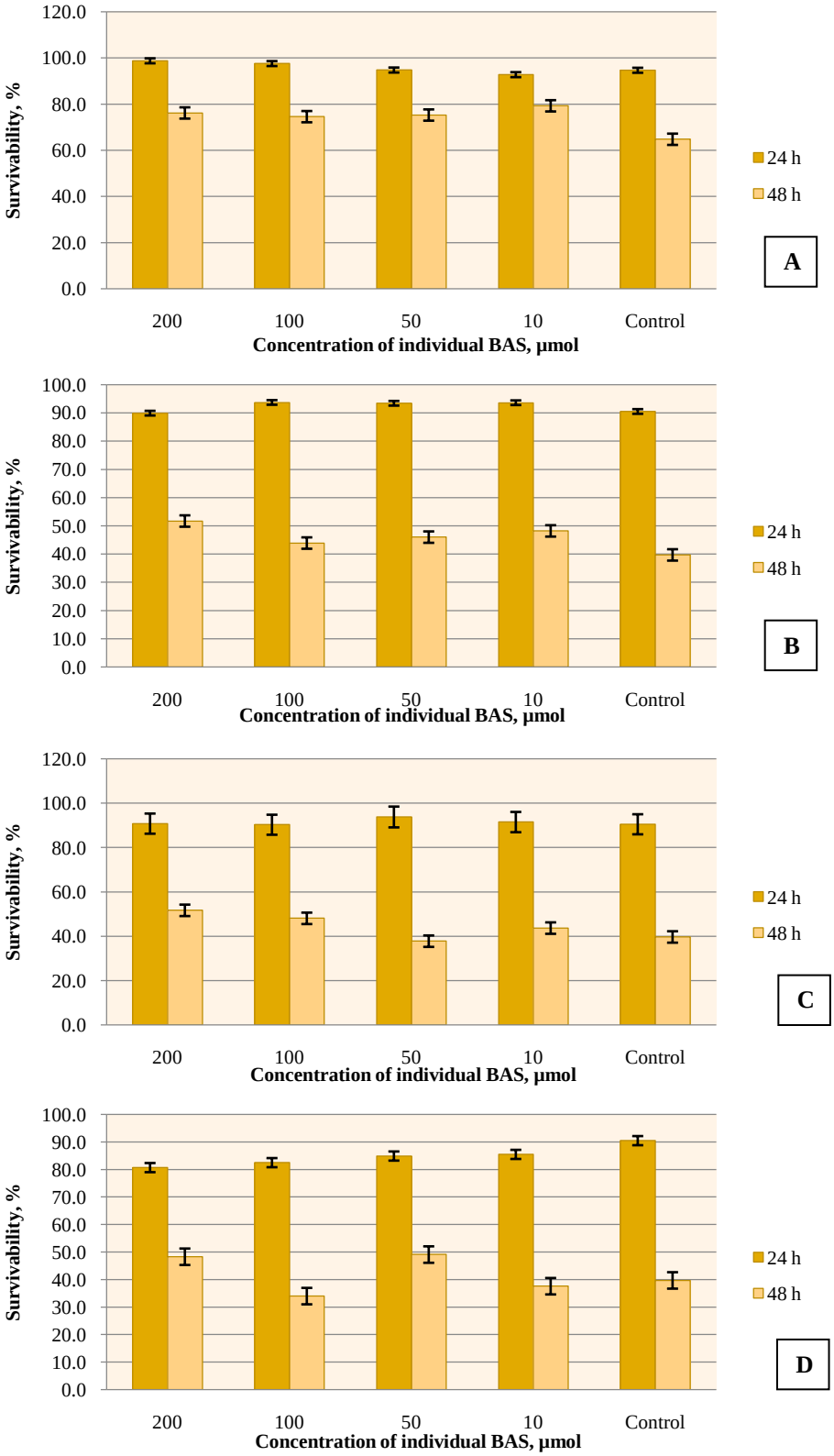

Figure 4 Effect of individual BAS of *T. pratense L.* callus culture on the resistance of *C. elegans* to temperature stress
(A) Effect of Chlorogenic, (B) Effect of Genistein, (C) Effect of Biochanin A, (D) Effect of Ononin

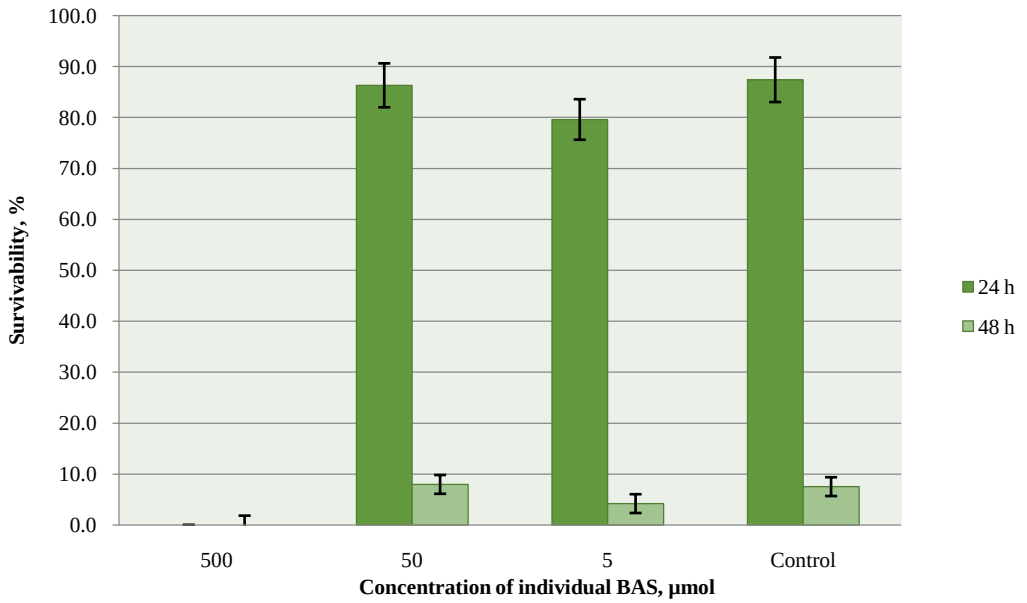

Figure 5 Effect of *T. pratense L.* callus culture extract. on the resistance of *C. elegans* to temperature stress

Conclusion

The antioxidant properties and the effect *T. pratense* callus culture extract and individual BAS (chlorogenic acid, ononin, biochanin A, genistein) on stress resistance were studied to determine the possibility of using this plant in geroprotective nutrition to increase the healthy life expectancy of the population. The data obtained showed a high antiradical activity of chlorogenic acid, followed by genistein, ononin, and callus culture extract. When studying the nematode survival under oxidative stress, callus culture extracts (5 µmol) and ononin (50 µmol) showed high indicators. When studying temperature stress, high rates are also characteristic of ononin and callus culture extract. Therefore, the results of the study suggested that *T. pratense* extract and BAS have a significant effect on the increase of the body's resistance to stressful conditions. In different cases, high rates of both antiradical activity and the ability to increase or decrease in vivo survival of the *C. elegans* were noted and leading to the further broadening of the study of the composition and biological properties of the *T. pratense* callus culture. This is relevant and promising for further use of geroprotective therapy.

Criterion of authorship

All authors are equally responsible for the research results and the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

References

Akbaribazm M., Khazaei M. R., & Khazaei. M. (2020). *Trifolium pratense* L. (red clover) extract and doxorubicin synergistically inhibits proliferation of 4T1 breast cancer in tumor-bearing BALB/c mice through modulation of apoptosis and increase antioxidant and anti-inflammatory related pathways. *Food Science & Nutrition*, *8*(8), 4276–4290. doi: 10.1002/fsn3.1724.

Brekhman I. I., & Dardymov I. V. (1969). New substances of plant origin which increase nonspecific resistance. *Annual Review of Pharmacology*, *9*, 419–430 doi: 10.1146/annurev.pa.09. 040169.002223.

Cai S. Q., Tang Z. M., Xiong C., Wu F. F., et al. (2022). The anti-inflammatory effects of apigenin and genistein on the rat intestinal epithelial (IEC-6) cells with TNF-α stimulation in response to heat treatment. *Current Research in Food Science*, *5*, 918–926. https://doi.org/10.1016/j.crfs.2022.05.011.

Carranza A. del V., Saragusti A., Chiabrando G. A., Carrari F., & Asis R. (2020). Effects of chlorogenic acid on thermal stress tolerance in *C. elegans* via HIF-1, HSF-1 and autophagy.

Phytomedicine, *66*, 153132. https://doi.org/10.1016/j.phymed.2019.153132.

Chen C., Song J., Chen M., Li Z., Tong X., et al. (2016). Rhodiola rosea extends lifespan and improves stress tolerance in silkworm, Bombyx mori. *Biogerontology*, *17*(2), 373–381 doi: 10.1007/s10522-015-9622-8.

Dong L., Yu L., Liu A., Alahmadi T. A., Almoallim H.S., & Durairaj K. (2022). Ononin mitigates streptozotocin-induced diabetic nephropathy in rats via alleviating oxidative stress and inflammatory markers. *Journal of King Saud University – Science*, *34*(6), 102029. https://doi.org/10.1016/j.jksus.2022.102029.

Dyshlyuk L. S., Fedorova A. M., Loseva A. I., & Eremeeva N. I. (2021). Callus cultures of *Thymus vulgaris* and *Trifolium pratense* as a source of geroprotectors. *Food processing: techniques and technology*, *51*(2), 423–432. doi: 10.21603/2074-9414-2021-2-423-432.

Esmaeili K. A., Taha M. R., Mohajer S., & Banisalam B. (2015). Antioxidant activity and total phenolic and flavonoid content of various solvent extracts from in vivo and in vitro grown *Trifolium pratense* L. (Red Clover). *BioMed Research International*, *643285* doi: 10.1155/2015/643285.

Gamborg O. L., Miller R. A., & Ojima. O. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, *50*, 151–158.

González-Peña M. A., Lozada-Ramírez J. D., & Ortega-Regules A.E. (2021) Carotenoids from mamey (*Pouteria sapota*) and carrot (*Daucus carota*) increase the oxidative stress resistance of Caenorhabditis elegans. *Biochemistry and Biophysics Reports*, *26*, 100989. doi: 10.1016/j.bbrep.2021.100989.

Hou L., Jiang M., Guo Q., & Shi W. (2019). Zymolytic Grain Extract (ZGE) Significantly Extends the Lifespan and Enhances the Environmental Stress Resistance of *Caenorhabditis elegans*. *International Journal of Molecular Sciences*, *20*(14), 3489. doi: 10.3390/ijms20143489.

Jattujan P., Chalorak P., Siangcham T., Sangpairoj K., & Nobsathian S. (2018). *Holothuria scabra* extracts possess anti-oxidant activity and promote stress resistance and lifespan extension in *Caenorhabditis elegans*. *Experimental Gerontology*, *110*, 158–171. doi: 10.1016/j.exger.2018.06.006.

Langrish T. A. G., & Premarajah R. (2013). Antioxidant capacity of spray-dried plant extracts: Experiments and simulations. *Advanced Powder Technology*, *24*(4), 771–779. https://doi.org/10.1016/j.apt.2013.03.020)

Leite N. R., de Araújo L., Dos Santos da Rocha P., Agarrayua D. A., et al. (2020). Baru Pulp (*Dipteryx alata* Vogel): Fruit from the Brazilian Savanna Protects against Oxidative Stress and Increases the Life Expectancy of Caenorhabditis elegans via SOD-3 and DAF-16. *Biomolecules*, 10(8), 1106. doi: 10.3390/biom10081106.

Luo L., Gao W., Zhang Y., Wang G., Wu H., & Gao W. (2020). Integrated Phytochemical Analysis Based on UPLC-MS and Network Pharmacology Approaches to Explore the Quality Control Markers for the Quality Assessment of *Trifolium pratense* L. *Molecules*, *25*(17), 3787. doi: 10.3390/molecules25173787.

Monteiro-Alfredo T., Matafome P., Iacia B. P., Antunes K. Á., et al. (2020). *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. Leaves Increase SIRT1 Levels and Improve Stress Resistance. *Oxidative medicine and cellular longevity*, *5238650*. doi: 10.1155/2020/5238650.

Rathor L., & Pandey R. (2018). Age-induced diminution of free radicals by Boeravinone B in Caenorhabditis elegans. *Experimental Gerontology*, *111*(1), 94–106. DOI: 10.1016/j.exger.2018.07.005.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., & Rice-Evans C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237.

Roxo M., Peixoto H., Wetterauer P., Lima E., & Wink M. (2020). *Piquiá Shells* (Caryocar villosum): A Fruit by-Product with Antioxidant and Antiaging Properties in *Caenorhabditis elegans*. *Oxidative medicine and cellular longevity*, *7590707*. doi: 10.1155/2020/7590707.

Sun Z. X., Liu S., Zhao Z. Q., & Su R. Q. (2014). Protective effect of chlorogenic acid against carbon tetrachloride-induced acute liver damage in rats. *Chinese Herbal Medicines*, *6*(1), 36–41. doi: 10.1016/S1674-6384(14)60004-6.

Tambara A. L., de Los Santos Moraes L., Dal Forno A. H., Boldori J. R., et al. (2018). Purple pitanga fruit (*Eugenia uniflora* L.) protects against oxidative stress and increase the lifespan in *Caenorhabditis elegans* via the DAF-16/FOXO pathway. *Food and Chemical Toxicology*, *120*, 639–650. doi: 10.1016/j.fct.2018.07.057.

Thabit S., Handoussa H., Roxo M., Cestari de Azevedo B., El Sayed N., & Wink M. (2019). *Styphnolobium japonicum* (L.) Schott Fruits Increase Stress Resistance and Exert Antioxidant Properties in *Caenorhabditis elegans* and Mouse Models. *Molecules*, *24*(14), 2633. doi: 10.3390/molecules24142633.

van der Pol A., van Gilst W. H., Voors A. A., & van der Meer P. (2019). Treating oxidative stress in heart failure: past, present and

future. *European Journal of Heart Failure*, *21*(4), 425–435. doi: 10.1002/ejhf.1320.

Wang H., Liu J., Li T., & Liu R. H. (2018). Blueberry extract promotes longevity and stress tolerance via DAF-16 in *Caenorhabditis elegans*. *Food & Function*, *9*(10), 5273–5282. doi: 10.1039/c8fo01680a.

Wang J., Deng N., Wang H., Li T., Chen L., Zheng B., & Liu R. H. (2020). Effects of Orange Extracts on Longevity, Healthspan, and Stress Resistance in *Caenorhabditis elegans*. *Molecules*, *25*(2), 351. doi: 10.3390/molecules25020351.

Wang S., Li Y., Meng X., Chen S., Huang D., Xia Y., Zhu S. (2021). Antioxidant activities of chlorogenic acid derivatives with different acyl donor chain lengths and their stabilities during in vitro simulated gastrointestinal digestion. *Food Chemistry*, *357*, 129904. doi: 10.1016/j.foodchem.2021.129904.

Wilson M. A., Shukitt-Hale B., Kalt W., Ingram D. K., Joseph J. A., & Wolkow C. A. (2006). Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*. *Aging Cell*, *5*(1), 59-68. doi: 10.1111/j.1474-9726.2006.00192.x.

Wu Q., Shen T., Liu F., & Zhang W. (2021). Biochanin A protects SH-SY5Y cells against isoflurane-induced neurotoxicity by suppressing oxidative stress and apoptosis. *NeuroToxicology*, *86*, 10–18. https://doi.org/10.1016/j.neuro.2021.06.007.

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Chemotaxonomic Significance and Environmental Implications of the Phytochemical Constituents of four *Mussaenda* L. (Rubiaceae) taxa in Nigeria

Nwafor F.I.[1,2*], Ogbonna C.E.[3], Igwe U.I.[2], Nwosu M.O.[1], Inya-Agha S.I.[2]

[1]Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Nigeria
[2]Department of Pharmacognosy and Environmental Medicines, University of Nigeria, Nsukka, Nigeria
[3]Department of Environmental Resource Management, Abia State University, Uturu, Nigeria

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KEYWORDS

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Phytochemicals

Mussaenda L.

Rubiaceae

ABSTRACT

This work investigated the phytoconstituents of some *Mussaenda* taxa (Rubiaceae) collected from Nsukka (Derived Savanna) and Uyo (Tropical Rainforest) ecological zones of Nigeria to establish their contribution as possible taxonomic and environmental monitoring markers. Fresh leaf samples used in this study were collected from plants of the same age, air-dried, and made into powder for further use. Histochemical and phytochemical tests were carried out by following the standard procedures. Results of the comparative phytochemical screening revealed the presence of flavonoids, alkaloids, glycosides, phenols, hydrogen cyanide, reducing sugars, soluble carbohydrates, saponins, steroids, terpenoids, and tannins in varying proportions. Results of the phytochemical constitute analysis revealed the presence of the cystoliths from the *M. elegans* (MEL) and *M. erythrophylla* (MER) which were absent in *Mussaenda* " Doña Aurora" (MDA) and *Mussaenda* "Doña Luz"(MDL). Further, the presence of the Raphides was unique to MEL while Gum and mucilage were reported only in MDA. Quantitatively, MEL had the highest value of terpenoids (650.88 mg/100g) while MDA had the highest values of phenols (899.27 mg/100g), alkaloids (311.01 mg/100g), reducing sugars (967.35 mg/100g), steroids (2.89 mg/100g), soluble carbohydrates (27.68 mg/100g) and tannins (393.16 mg/100g), and MDL was richest in glucosides (339.64 mg/100g), flavonoids (69.34 mg/100g) and hydrogen cyanides (1.34 mg/100g). The cluster analysis based on obtained phytochemical data revealed three (3) distinct clusters with MEL in cluster 1; MDA and MDL in cluster 2 while cluster 3 had MER. The evolutionary closeness

* Corresponding author
E-mail: felix.nwafor@unn.edu.ng (Nwafor F.I)

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of the two infraspecific and exotic species (MDA and MDL) were confirmed and their taxonomic relationship with the indigenous infrageneric taxa (MEL and MER) was established. The results also highlight the opportunity of employing plant metabolomics in ecological studies and environmental monitoring.

1 Introduction

'Phytochemotaxonomy' is an expanding area of study that focuses on utilizing chemical characteristics to improve plant classification. Phytochemical characteristics often correlate well enough with other types of characters and provide help in clearer insights into taxonomic relationships (Pandey and Misra 2008; Nwafor and Orabueze 2019). Recognition of chemical evidence in plants started, in fact, since early man and plants were classified based on their color, odor, and efficacy, obviously as a result of their chemical compositions (Nwafor and Orabueze 2019). A wide spectrum of useful biomolecules synthesized from different metabolic pathways exists in plants (Harvey, 2000). Chemical data of taxonomic value include micromolecules (both primary and secondary metabolites) and macromolecules, both non-semantide (not involved in information transfer such as starches, cellulose etc.), and semantides (molecules responsible for carrying information such as protein, RNA and). Primary metabolites are thosecompounds that take part in essential metabolic pathways, these include amino acids, aconitic acid, and citric acid. On the other hand, secondary metabolites are by-products of metabolism which are involved in non-essential roles such as protection against herbivores, insects, and harsh environmental conditions. These compounds include phenolics, alkaloids, terpenes, glucosinolates, etc. (Pandey and Misra 2008, Salim et al. 2008).

Specific chemicals or groups of compounds have been proven good chemical markers for the taxonomic delineation of plants (Nwafor and Orabueze 2019). A good example is lathyrine, which is isolated only from species of *Lathyrus* (Fabaceae), and as such, its distribution has contributed to a successful taxonomical grouping of the genus into seven distinct intrageneric taxa (Pandey and Misra 2008). Phytochemicals that are ubiquitous such as flavonoids and alkaloids are more extensively studied in chemotaxonomy. Flavonoids have been proven very useful in the classification and delimitation of the families like Asteraceae, Cactaceae, Molluginaceae, and Rubiaceae (Pandey and Misra 2008; Bhargava et al. 2013) while alkaloids are proven useful in taxonomic delimitations of the members of Apocynaceae, Papaveraceae, Solanaceae, and Fabaceae. The three genera of the family Fabaceae namely *Genista, Ammodendron,* and *Adenocarpus* contains ammondendrine-hystrine alkaloids which help in the identification of these genera. Further, morphine is present only in *Papaver somniferum* (Pandey and Misra, 2008). The presence or absence of latex vessels, resins, gums, and crystals in the wood are also features of taxonomic significance (Gott et al. 2006; Singh 2016). Many cellular contents, for example, albuminoids (*Laportea*), starch grains (*Solanum tuberosum*), protein bodies (Cactaceae), large silica bodies in epidermal cells (Arecaceae, Musaceae), calcium oxalate crystals (*Allium, Eichhornia*), cystoliths (Cannabinaceae, Moraceae, Urticaceae) and tanniniferous cells (Raptaceae, Xyridaceae) also have some bearing in plant systematics (Pandey and Misra 2008; Ekeke and Agbagwa 2014).

The genus *Mussaenda* L. is comprised of a group of flowering plants classified under the family Rubiaceae. They constitute approximately 200 individual species distributed across Asia, Australia, and Africa. It has also been introduced into Europe, South America, and North America (Nwafor et al. 2019). Most of the members of this genus are popular in the landscape industry as ornamental plants, largely due to their very attractive and colorful blooms. It is claimed that only a few ornamentals can compete favorably with *Mussaendas* when in full bloom. For instance, *M. philippica* is amongst the most cultivated ornamental plants around the globe. Hybridization and other breeding methods have further given rise to so many cultivars for improved aesthetic values, most of which bloom all seasons. Some other species have also found usefulness in other areas of human endeavor. These are also used in ethnomedicine for the treatments of cough, jaundice, liver diseases, dropsy, swellings, oedema, gout, and as febrifuge and appetizers (Burkill 1985; Stuart 2016). Phytochemicals reported from various parts of *Mussaenda* species had diuretic, antiphlogistic, antipyretic, anti-fertility (Venkatesh et al. 2013), antimicrobial (Kim et al. 1999; Jayasinghe et al. 2002), anti-tumor, analgesic, diuretic, anticonvulsant, antioxidant activities (Yaolan et al. 2004; Vidyalakshmi et al. 2007), antiviral (Sunit et al. 2003) and cytotoxic activities (Jing-Qiu et al. 2002).

Notwithstanding the multipurpose utilization of these species and their potential, especially in the area of drug discovery, there still exists a paucity of information on their taxonomic classification, and the fact that new cultivars have emerged in the last decades makes the situation even more difficult (Nwafor et al. 2019). In this study, therefore, we assessed the qualitative and quantitative phytochemical constituents of the four *Mussaenda* taxa in Nigeria (*M. elegans, M. erythophylla,* and two cultivars of *M. philippica*) for their possible contribution as chemical markers for taxonomic delimitation, ecological studies, and environmental monitoring.

2 Materials and Methods

2.1 Collection of Plant Samples

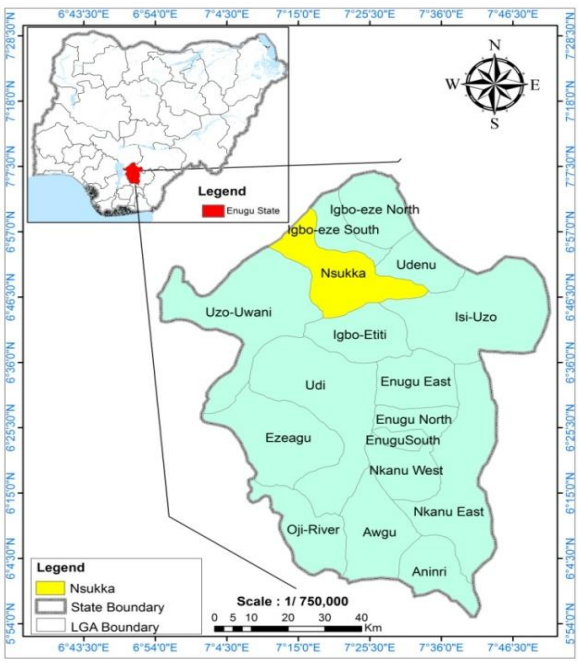
Fresh leaves of *M. elegans* (MEL), *M. erythophylla* (MER), *M.* "Dona Aurora" (MDA), and *M.* "Dona Luz" (MDL) used in this study were collected from Nsukka (Derived Savanna) and Uyo (Tropical Rainforest) ecological zones in Nigeria (Figure 1). Sampling and collection were carried out in July 2017. Nsukka is located in Enugu State, south-eastern Nigeria between Latitude 7°9'30" to 7°35'0" E and Longitude 6°41'15" to 7°5'20" N. It has a tropical climate with a mean annual temperature of 25°C and a mean annual rainfall of 1580 mm. Its vegetation type is derived savanna. Uyo is situated in Akwa Ibom State in the Niger-Delta region of Nigeria. It has a tropical rainforest climate with a mean annual temperature of 26.4 °C and a mean annual rainfall of 2500 mm. The vegetation type is rainforest and mangrove swamps.

2.2 Histochemical Studies

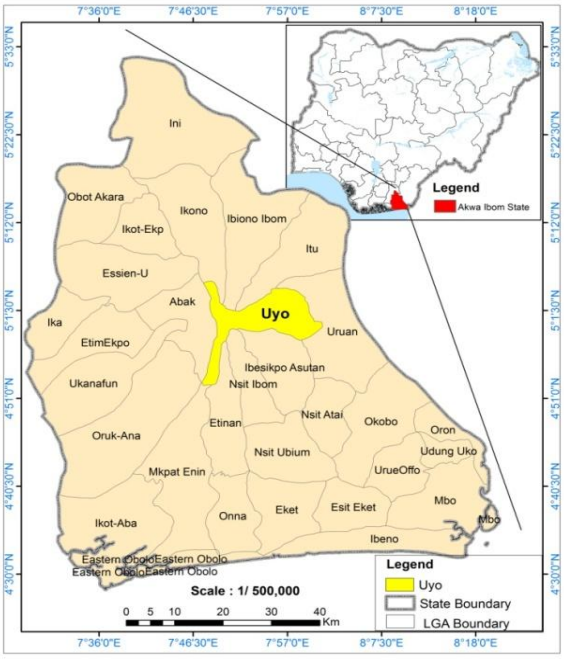
The leaves were dried under shaded conditions and pulverized with mortar and pestle. Chemo-microscopy was conducted on the powders to determine the presence of starch, calcium oxalate crystals, and lignified vessels. A judicious quantity of the sample was dropped on a glass slide. One drop of chloral hydrate was dropped and passed over a Bunsen burner repeatedly until bubbles formed. This signified the successful clearing of the tissues. The presence of lignin was tested by dropping phloroglucinol and concentrated hydrochloric acid (1:1) on a little quantity of the cleared powder on a glass slide, and glycerin was added to aid observation under a light Olympus Tokyo (Japan No.271961) microscope at ×100 magnifications. To test for starch, a drop of iodine was added to a little quantity of the cleared leaf powder on a glass slide and observed under a light Olympus Tokyo (Japan No.271961) microscope at ×400 magnification. A drop of Iodine solution and concentrated acetic acid (1:1) added to a little quantity of the cleared leaf powder on a glass slide revealed the presence or absence of calcium oxalate crystals, raphides, and cystoliths. Sudan IV reagent was used for fats and oil test, Ruthenium red for gum and mucilage, and Biuret reagent, ninhydrin for protein (Nwafor et al. 2019).

2.3 Phytochemical Studies

Qualitative phytochemical screening was carried out by following the standard methods. The presence of the alkaloid was tested by using Dragendorff's test (Sofowora 1993). Fehling's test was carried out for the presence of reducing sugars while the Frothing test was carried out for the estimation of Saponins, and Molisch's test was carried out for the presence of carbohydrates (Sofowora 1993). Liebermann-Buchard's test was carried out to evaluate the presence of Steroids and Terpenoids (Sofowora 1993). Ferric chloride was used for the estimation of the presence of Tannins, and a sodium hydroxide test was carried out for flavonoids (Trease and Evans 2002). Legal's Test was carried out for the estimation of glycosides, while the Ferric Chloride test was used for Phenols (Vijisaral and Subramanian 2013). Quantitative tests were also conducted on the phytochemicals that showed a



Nsukka in Enugu state, Nigeria



Uyo in Akwa Ibom State, Nigeria

Figure 1 Study locations

positive results. This was determined spectrophotometrically according to El-Olemyl et al. (1994) and Nwokonkwo (2009). AUv-Vis spectrophotometer (Shimadzu – UV 1800) was used to measure absorbance and values were calculated from the standard curves.

2.4 Statistical Analyses

Data obtained from the results were subjected to the Analysis of Variance (ANOVA) on the Statistical Package for Social Sciences (SPSS version 20) platform and a significant difference was tested

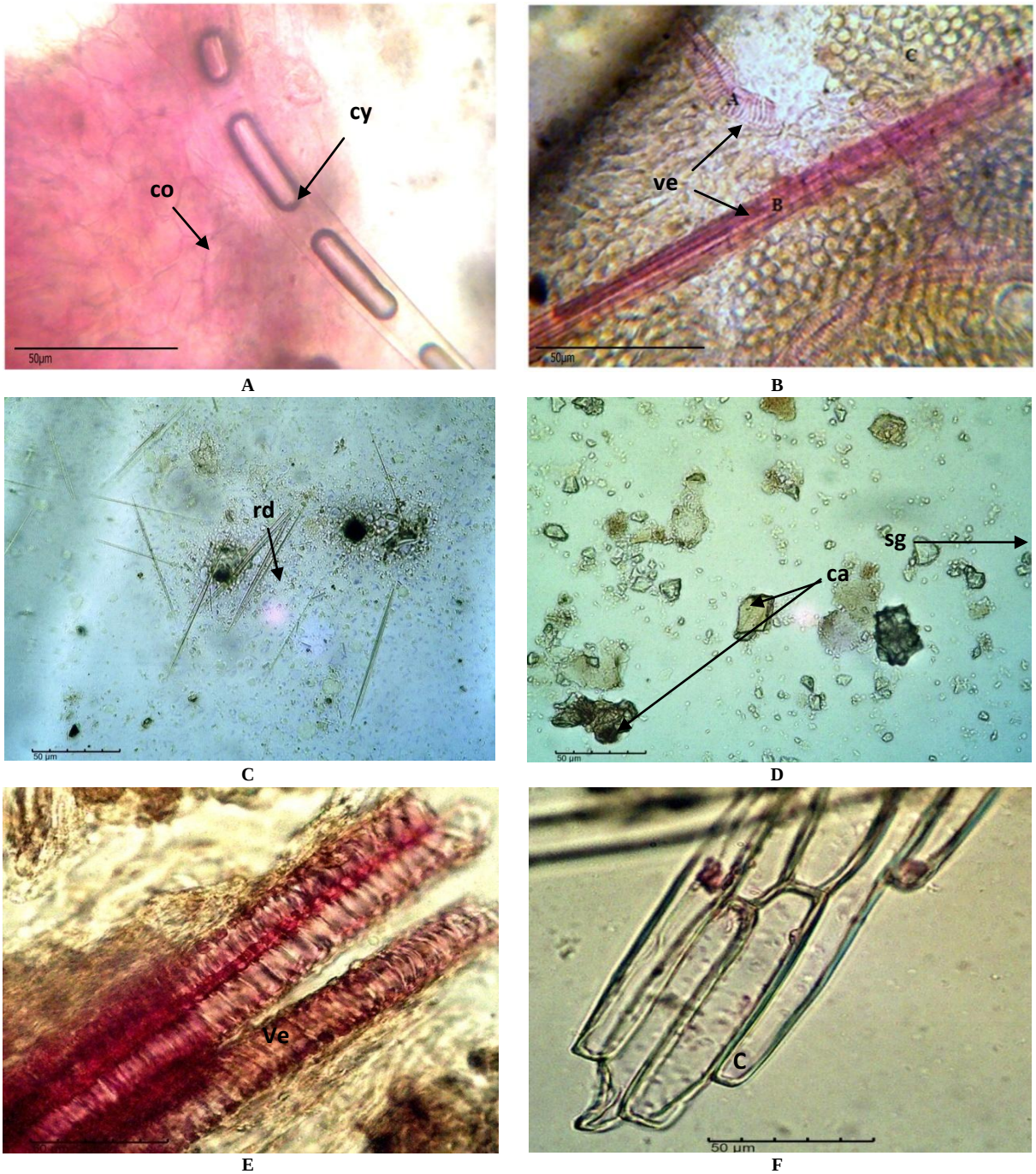


Figure 2 Chemomicrophotographs of the leaves of the *Mussaenda* taxa: A=co (lignified collenchyma cell) and cy (cystolith); B = ve (lignified vessel element) and c (cork cell); C = rd (raphide); D = ca (calcium oxalate crystal) and sg (starch grain); E = ve (lignified vessel element); F = c (cork cell)

at 95% probability (at $P \leq 0.05$) whereas Duncan's Multiple Range Test (DMRT) was used as post hoc test for separation of means. Students' independent sample T-test was used to test for significance (at $P \leq 0.05$) and to compare means of data from the two locations. Principal Component Analysis (PCA) was used to establish the taxonomical relationship among the taxa.

3 Results

3.1 Chemomicroscopy

The chemomicroscopy of the leaf powder of the four studied taxa of *Mussaenda* showed the presence of the various histochemicals (Table 1). The histochemicals were observed at different proportions across the studied four species. Among the studied taxa, lignin was reported at a higher concentration across all four taxa while fats, oil, and protein were absent. The presence of cystoliths was reported from the MEL and MER while it was absent in MDA and MDL. Further, Raphides were unique to MEL while gum and mucilage were reported from the MDA (Table 1). The chemomicrophotographs of the *Mussaenda* taxa are presented in Figure 2 and showed the presence of lignified collenchyma cell, cystolith, lignified vessel element, raphide, calcium oxalate crystal, starch grain, and cork cell.

3.2 Qualitative and Quantitative Estimation of Phytochemicals

The phytochemical analysis results of the four studies on taxa of *Mussaenda* showed the presence of various phytochemicals such as alkaloid, flavonoid, glycoside, hydrogen cyanide, phenol, and reducing sugar, saponin, soluble carbohydrate, steroid, tannin, and terpenoid in different proportions. MEL was unique with the high presence of HCN while MER was unique with the lower presence of reducing sugars and

Table 1 Histochemicals present in the leaves of the four taxa of *Mussaenda*

Histochemical	MEL	MER	MDA	MDL
Lignin	+++	+++	+++	+++
Starch	++	++	++	++
Calcium oxalate crystals	++	+	+	+
Raphides	+	-	-	-
Cystoliths	+	+	-	-
Fats and oil	-	-	-	-
Gum and mucilage	-	-	+	-
Protein	_	_	_	_

+++: Highly present; ++: moderately present; +: present; - : Absent

Table 2 Qualitative phytochemical constituents of the four *Mussaenda*taxa

Phytochemicals	MEL		MER		MDA		MDL	
	DS	RF	DS	RF	DS	RF	DS	RF
Alkaloids	++	++	++	++	++	++	++	++
Carbohydrates	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+
Glycosides	++	++	++	++	++	++	++	++
HCN	++	++	+	+	+	+	+	+
Phenols	++	++	++	++	++	++	++	++
Reducing sugars	++	++	+	+	++	++	++	++
Saponins	+	+	+	+	+	+	+	+
Steroids	++	++	+	+	++	++	+	+
Tannins	++	++	++	++	++	++	++	++
Terpenoids	++	++	+	+	++	++	++	++

+ = present; ++ = highly present

Table 3 Quantitative phytochemical constituents of the four *Mussaenda*taxa

Phytochemical (mg/100 g)	MEL	MER	MDA	MDL
Alkaloids	238.93 ± 7.92^{b}	230.55 ± 4.55^{b}	311.01 ± 0.37^{a}	223.33 ± 6.26^{b}
Flavonoids	34.13 ± 0.32^{c}	45.08 ± 0.04^{b}	21.17 ± 0.05^{d}	69.34 ± 0.15^{a}
Glycosides	321.25 ± 0.08^{b}	306.04 ± 0.38^{c}	278.89 ± 0.00^{d}	339.64 ± 0.22^{a}
HCN	0.61 ± 0.01^{c}	0.56 ± 0.01^{c}	1.00 ± 0.07^{b}	1.34 ± 0.18^{a}
Phenols	659.75 ± 0.45^{c}	649.82 ± 0.00^{d}	899.27 ± 0.04^{a}	843.07 ± 0.03^{b}
Reducing sugars	603.85 ± 0.22^{b}	478.14 ± 0.06^{d}	976.35 ± 0.21^{a}	584.05 ± 0.03^{c}
Saponins	0.60 ± 0.02^{d}	0.68 ± 0.00^{c}	0.82 ± 0.00^{a}	0.74 ± 0.01^{b}
Carbohydrates	12.86 ± 0.02^{d}	14.33 ± 0.00^{c}	27.68 ± 0.01^{a}	19.58 ± 0.00^{b}
Steroids	2.34 ± 0.01^{b}	0.76 ± 0.02^{d}	2.89 ± 0.03^{a}	0.83 ± 0.00^{c}
Tannins	304.11 ± 2.26^{d}	311.35 ± 2.24^{c}	393.16 ± 0.45^{a}	323.38 ± 0.06^{b}
Terpenoids	650.88 ± 0.36^{a}	178.53 ± 2.24^{d}	467.62 ± 0.25^{c}	524.65 ± 0.14^{b}

*Means with different superscript alphabets across each row differ significantly at $P \leq 0.05$

Table 4 Quantitative comparative phytochemical constituents of the four *Mussaenda* taxa across the study sites

Phytochemical (mg/100 g)	MEL		MER		MDA		MDL	
	DS	RF	DS	RF	DS	RF	DS	RF
Alkaloids	256.63 ± 0.10*	221.23 ± 0.15	238.22 ± 0.01*	222.89 ± 6.69	311.84 ± 0.01	310.18 ± 0.00	237.33 ± 0.03*	209.33 ± 0.03
Flavonoids	34.85 ± 0.01*	33.42 ± 0.06	45.05 ± 0.05	45.11 ± 0.05	21.28 ± 0.00	21.06 ± 0.02	69.67 ± 0.01	69.01 ± 0.00
Glycosides	321.43 ± 0.01	321.08 ± 0.01	306.89 ± 0.01*	305.18 ± 0.01	278.88 ± 0.01	278.88 ± 0.01	340.14 ± 0.01*	339.14 ± 0.01
HCN	0.63 ± 0.00*	0.60 ± 0.00	0.58 ± 0.03*	0.54 ± 0.01	1.15 ± 0.01*	0.85 ± 0.01	1.74 ± 0.00	0.94 ± 0.00*
Phenols	660.75 ± 0.00*	658.75 ± 0.00	649.82 ± 0.01	649.82 ± 0.01	899.33 ± 0.01	899.20 ± 0.07	843.14 ± 0.01	843.00 ± 0.00
Reducing sugars	604.35 ± 0.00*	603.35 ± 0.00	478.00 ± 0.00	478.27 ± 0.01	976.68 ± 0.01*	976.01 ± 0.33	584.09 ± 0.04	584.00 ± 0.00
Saponins	0.65 ± 0.00*	0.55 ± 0.00	0.68 ± 0.00	0.68 ± 0.00	0.83 ± 0.00*	0.81 ± 0.00	0.77 ± 0.00*	0.71 ± 0.00
Carbohydrates	12.89 ± 0.00*	12.82 ± 0.04	14.33 ± 0.00	14.33 ± 0.00	27.68 ±0.01	27.68 ± 0.01	19.58 ± 0.00	19.58 ± 0.00
Steroids	2.37 ± 0.00*	2.32 ± 0.00	0.75 ± 0.03	0.78 ± 0.00	2.96 ± 0.01*	2.82 ± 0.00	0.83 ± 0.01	0.83 ± 0.01
Tannins	309.17 ± 0.00*	299.05 ± 0.02	316. 35 ± 0.01*	306.35 ± 0.01	394.16 ± 0.01*	392.16 ± 0.01	323.52 ± 0.01	323.23 ± 0.02
Terpenoids	651.68 ± 0.00*	650.08 ± 0.00	183.53 ± 0.01*	173.53 ± 0.01	468.17 ± 0.00*	467.07 ± 0.03	524.35 ± 0.00	524.95 ± 0.02

* = significantly higher at $P \leq 0.05$

terpenoids. Also, the high presence of steroids in MDA differentiates it from MDL (Table 2). The amounts of the phytochemicals significantly ($P < 0.05$) varied across the different taxon (Table 2). MDA recorded significantly ($P < 0.05$) higher amounts of alkaloid, phenol, reducing sugar, saponin, soluble carbohydrate, steroid, and tannin (Table 3). Significant differences in the amounts of phytochemicals evaluated across the two study locations in each of the taxa were also recorded (Table 4). Generally, the phytochemicals were significantly higher in plants collected from the derived savannah as compared to the rainforest. *M. elegans* collected from the derived savannah had a significantly higher concentration of alkaloid, flavonoid, hydrogen cyanide, phenol, reducing sugar, saponin, soluble carbohydrate, steroid, tannin, and terpenoid contents as compared to those collected from the rain forest (Table 4).

3.3 Cluster analysis of the taxa

The cluster analysis which was based on correlations using Ward's method grouped the accessions into 3 clusters for histochemical and phytochemical attributes (Figure 3). MEL was in cluster 1, cluster 2 contained MDA and MDL while cluster 3 had MER. However, MDA and MDL were more related to MEL than MER. The low concentrations of reducing sugars and terpenoids could be the distinguishing factor of MER.

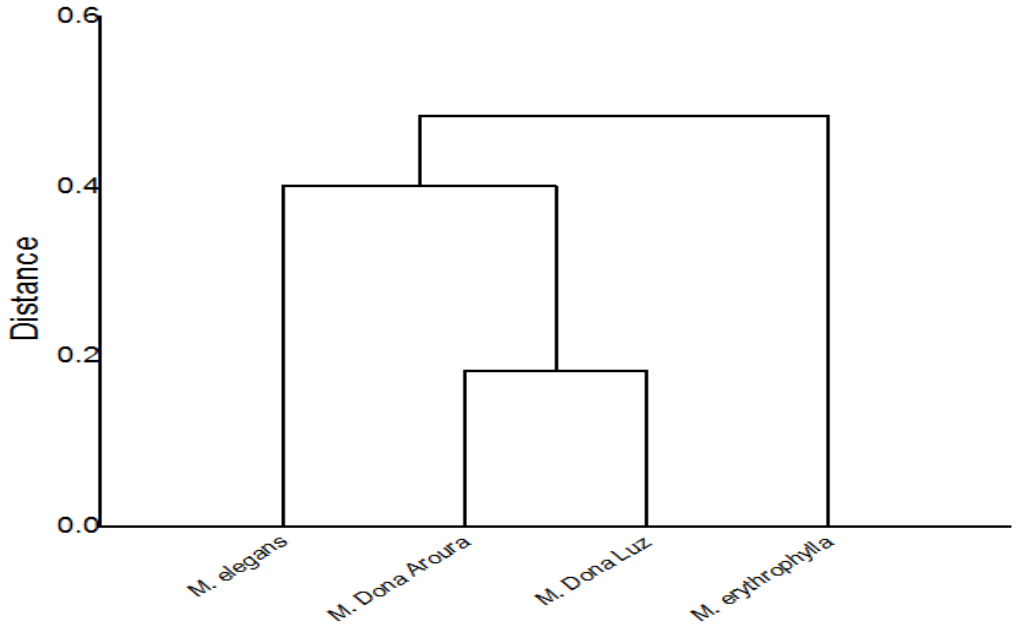


Figure 3 Cluster pattern of the histochemical and qualitative phytochemicals analysis of the four taxa of *Mussaenda* generated from Hierarchical cluster analysis using the ward's correlation method

4 Discussion

Results of the chemomicroscopy showed that lignified tissues, starch granules, calcium oxalate crystals, and cystoliths were present in all studied taxa. These histochemicals are important diagnostic features in the identification and standardization of crude drugs even when in powdered form (Sonibare and Adeniran 2014; Erst et al. 2021). *M. elegans* stood out by possessing rod-like calcium oxalate crystal forms raphides. Calcium oxalates and cystoliths are often found in plant tissues, especially in trichomes to protect them from harsh environmental conditions and herbivores. This could be a reason why the use of these plant species for grazing and animal feed has not been reported in the literature.

Further phytochemical screening revealed the presence of phenols, glycosides, tannins, and terpenoids like secondary metabolites in higher concentrations. These were closely followed by reducing sugars and alkaloids. The considerable quantities of flavonoids and phenolics reported here could an indication that the examined taxa could be investigated for potential anti-oxidative stress agents (Suksungworn and Duangsrisai 2021). The higher contents of alkaloid, tannin, and terpenoid as observed are indications of possible antiparasitic, antiviral and antimicrobial properties of the species (Akiyama et al. 2001; Kolodziej and Kiderlen 2005; Soladoye and Chukwuma 2012; Nweze and Nwafor 2014; Kolawole et al. 2017).

The steroid composition of these samples suggested their possible use in the development and production of sex hormone-related drugs (Okwu, 2001). Asl and Hossein (2008) emphasized the contribution of saponins in orally administered herbal formulations and their ability to hydrolyze glycosides from terpenoids. Interestingly, unhealthy phytoconstituents such as hydrogen cyanide and soluble carbohydrate were minutely present to prove the safety of these plants in the treatment and management of ailments as already been reported in the literature (Manandar and Manandar 2002; Vidyalakshmi et al. 2007).

Specific plant chemicals or groups of compounds have been proven good chemical markers for the taxonomic delineation of plants (Nwafor and Orabueze 2019). In this study, it was reported that the four taxa were successfully distinguished based on the differences and similarities in their phytochemical make-up. *M. elegans* was in cluster 1, cluster 2 contained *M.* "Dona Aurora" and *M.* "Dona Luz" while cluster 3 had *M. erythrophylla*. The two infraspecific and exotic taxa (MDA and MDL) were confirmed to be most closely related based on cluster analysis. It also shows that they are more related to MEL than MER (both of which are indigenous species) (Burkill 1985). The low concentrations of reducing sugars and terpenoids could be the distinguishing factor of MER.

Generally, the phytochemicals were significantly higher in plants collected from the derived savannah as compared to their counterparts collected from the rainforest (wetter) region. This could be explained by the fact that plants produce phytochemicals (secondary metabolites) in response to varying environmental stress conditions, to survive under harsh situations such as drought, diseases, pest attacks, etc. (Liu et al. 2016; Nwafor and Orabueze

2019). This also opens up the opportunity of employing plant metabolomics in ecological studies and environmental monitoring. Results of the study also reported the environmentally-influenced changes in the wood anatomical features of the same species (Nwafor et al. 2021).

Conclusion

The results of the study can be concluded that phytochemical content can be used as an important diagnostic tool in the delineation and identification of the studied taxa. For instance, only MEL had raphides while gum and mucilage were observed in MDA. Their evolutionary relationship was also established based on cluster analysis of data obtained from the qualitative and quantitative phytochemical studies. The evolutionary closeness of the two infraspecific and exotic species (MDA and MDL) were confirmed and their taxonomic relationship with the indigenous infrageneric taxa (MEL and MER) was established. The results also highlight the opportunity of employing plant metabolomics in ecological studies and environmental monitoring.

References

Akiyama, H., Fujii, K., Yamasaki, O., Oono, T., & Iwatsuki, K. (2001). Antibacterial action of several tannins against *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy, 48* (4), 487 – 491.

Asl, M. N., & Hossein, H. (2008). Review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. *Phytotherapy Research,22* (6),709 – 724.

Bhargava, V.V., Patel, S.C., & Desai, K.S. (2013). Importance of terpenoids and essential oils in chemotaxonomic approach. *International Journal of Herbal Medicine, 1*(2),14 – 21.

Burkill, H.M. (1985). *The Useful Plants of Tropical Africa*. Royal Botanic Gardens, Kew, Richmond, United Kingdom. 960 pp.

Ekeke, C., & Agbagwa, I. O. (2014). Ergastic substances (calcium oxalate crystals) in the leaf of *Combretum* Loefl. (Combretaceae) species in Nigeria. *American Journal of Plant Science, 5*, 2389 – 2401.

El-Olemyl, M.M., Muhtadi, F.J.A., & Afifi, A.A. (1994). *Experimental Phytochemistry: A Laboratory Manual*. College of Pharmacy, King Saud University, Saudi Arabia.

Erst, A.A., Petruk, A.A., Zibareva, L.N., & Erst, A.S. (2021). Morphological, histochemical and biochemical features of cultivated *Rhodiola rosea* (Altai mountains ecotype). *Contemporary Problems of Ecology*, *14*, 701 – 710.

Gott, B., Barton, H., Samuel, D., & Torrence, R. (2006). Biology of Starch. In: R. Torrence, & H. Barton, (eds.), *Ancient Starch Research* (pp 35 – 45), Left Coast Press, California.

Harvey, A. (2000). Strategies for discovering drugs from previously unexplored natural products. *Drug Discovery Today*, *5* (7),294 – 300.

Jayasinghe, U. L. B, Jayasooriya, C. P., & Bandara, B. M. R. (2002). Antimicrobial activity of some Sri Lankan Rubiaceae and Meliaceae. *Fitoterapia,73* (5),424 - 427.

Jing-Qiu, D., Zhong-Li, L., & Li, Y. (2002). Non-glycosidic iridoids from *Cymbaria mongolica. Phytochemistry*, *59*, 537–542.

Kim, N. C., Desjardins, A. E., Wu, C. D., & Kinghorn, A. D. (1999). Activity of triterpenoid glycosides from the root bark of *Mussaenda macrophylla* against oral pathogens. *Journal of Natural Products*, *62*,1379 – 1384.

Kolawole, O. S., Jimoh, M. A., Yakubu, F., & Chukwuma, E. C. (2017). Taxonomic value of the leaf micro-morphology and quantitative phytochemistry of *Jatropha integerrima* Jacq. And *Jatropha podagrica* Hook. (Euphorbiaceae) – known horticultural plants in Nigeria. *Anales de Biologia*, *39*, 55 – 62.

Kolodziej, H., & Kiderlen, A. F. (2005). Antileishmanial activity and immune modulatory effects of tannins and related compounds on Leishmania parasitised RAW 264.7 cells. *Phytochemistry*, *66* (17),2056 – 2071.

Liu, W., Yin, D., Li, N., Hou, X., Wang, D., Li, D., & Liu, J. (2016). Influence of environmental factors on the active substance production and antioxidant activity in *Potentilla fruticosa* L. and its quality assessment. *Scientific Reports*, *6*, 1-18.

Manandar, N. P., & Manandar, S. P. (2002). *Plants and People of Nepal*, Timber Press, 327 pp.

Nwafor, F.I., & Orabueze, C.I. (2019). Role of Phytochemistry in Plant Classification: Phytochemotaxonomy. In C. Egbuna, J.C. Ifemeje, S.C. Udedi, & S. Kumar, (eds.). *Phytochemistry, Volume 1: Fundamentals, Modern Techniques, and Applications.*Apple Academic Press, UK &Canada, pp 197 – 222.

Nwafor, F.I., Igwe, U., Ogbonna, C., Ajuziogu, G.C., & Nwosu, M.O. (2021). Wood anatomical studies reveal taxonomic relationships, environmental influence and pulp potential in four taxa of *Mussaenda* (Rubiaceae) grown in Nigeria. *Journal of Experimental Biology and Agricultural Sciences 9* (1),100-107.

Nwafor, F.I., Nwosu, M.O., & Nwafor, A.Z. (2019) Taxonomic and ecological significance of foliar epidermal characters in four

taxa of *Mussaenda* L. (Rubiaceae) in Nigeria. *Annual Research & Review in Biology*,*32*(5): 1-12.

Nweze, N.O., & Nwafor, F.I.(2014). Phytochemical, proximate and mineral composition of leaf extracts of *Moringa oleifera* Lam. from Nsukka, South-Eastern Nigeria. *IOSR Journal of Pharmacy and Biological Sciences, 9*(1): 99 – 103.

Nwokonkwo, D. C. (2009). Phytochemical constitution and antimicrobial activity of the stem bark extract of *Ficus Asperifolia* (sand paper) tree. *Journal of Chemical Society of Nigeria*, *34* (2):119 – 122.

Okwu, D. E. (2001). Evaluation of the chemical composition of indigenous spices and flavouring agents. *Global Journal of Pure and Applied Sciences*, *7* (3): 455 – 459.

Pandey, S. N., & Misra, S. P. (2008). *Taxonomy of Angiosperms*. Ane Books Pvt. Ltd.,New Delhi, India, 620 pp.

Salim, A. A., Chin, Y. W., & Kinghorn, A. D. (2008). Drug Discovery from Plants. In: K. G. Ramawat, and J. M. Merillon, (eds). *Bioactive Molecules and Medicinal Plants* (pp. 10 – 25). Springer, USA.

Singh, R. (2016). Chemotaxonomy: A tool for plant classification. *Journal of Medicinal Plants Studies*, *4*(2),90 – 93.

Sofowora, A. (1993). *Medicinal Plants and Traditional Medicinal in Africa* (2nd Ed). Spectrum Books Ltd, Sunshine House, Ibadan, Nigeria.

Soladoye, M. O., & Chukwuma, E. C. (2012). Phytochemical analysis of the stem and root of *Cissus populnea* (Vitaceae) - an important medicinal plant in Central Nigeria. *Phytologia Balcanica*, *18* (2),149-153.

Sonibare, M. A., & Adeniran, A. A. (2014). Comparative micromorphological study of wild and micropropagated *Dioscorea bulbifera*Linn. *Asian Pacific Journal of Tropical Biomedicine*, *4* (3),176 – 183.

Stuart, G. (2016). *Philippine Alternative Medicine*. Retrieved from http://stuartxchange.com/. Accessed on 27th June, 2016.

Suksungworn, R., & Duangsrisai, S. (2021). Phytochemical contents and antioxidant activity of medicinal plants from the Rubiaceae family in Thailand. *Plant Science Today*, *8*(1): 24 – 31.

Sunit, S., Kanjana, W., & Kanyawim, K. (2003). Iridoid glucosides from the sepals of *Barleria lupulina*. *Planta Medica*, *69*, 877 - 879.

Trease, G. E., & Evans, W. C. (2002). *Pharmacognosy* (15th Ed). Saunders Publishers, London.

Venkatesh, K., Rao, U. U., Kiranmayi, G. V. N., Naik, R. N., Mukharjee, N. S. V., Vinay, V. N. V., & Phanindra, K. (2013). Phytochemical screening and evaluation of diuretic activity of ethanolic and chloroform extracts of *Mussaenda erythrophylla* in rats. *International Journal of Biological and Pharmaceutical Research, 4* (1): 8 – 10.

Vidyalakshmi, K. S., Charles, D. A., & Hannah, R.V. (2007). Antimitotic and cytotoxic activity of *Mussaenda* "Queen Sirikit". *Journal of Pharmacology and Toxicology*, *2* (7), 660 – 665.

Vijisaral E. D., & Subramanian, A. (2013). Identification of Phytochemical Constituents and Antimicrobial Activity of Indigofera Suffruticosa Leaves. *International Journal of Current Biotechnology*, *1*(7),6-10.

Yaolan, L., Linda, S. M., Hua, W., Paul, P. H., & Vincent, E. C. O. (2004). Antiviral activities of medicinal herbs traditionally used in southern mainland China. *Phytotherapy Research*, *18* (9),718 – 722.

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Assessment of trace element accumulation in surface sediment of Sepang Besar river, Malaysia

Kumar Krishnan[1*], Nadia AS[1], Chong MY[2], Prakash Balu[3]

[1]Faculty of Health & Life Sciences, INTI International University, Negeri Sembilan, Malaysia
[2]Matrix Global School, Negeri Sembilan, Malaysia
[3]Department of Biotechnology, Vels Institute of Science, Technology and Advanced Studies (VISTAS) Tamil Nadu, India

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ICP-MS

ABSTRACT

Due to non-scientific industrial activity and urbanization, trace elements contamination has posed a threat to Malaysia's biodiversity-rich coastal wetlands, streams, estuaries, and mangroves. Commercialization has taken a toll on mangroves in backwater canals and along the banks of the Sepang River. As a result, a thorough examination of sediment quality from the Sepang River mangrove habitats is done with a focus on trace element pollution and pollution issues, taking into account the enormous ecological services that are offered to coastal communities and offering guidance for upcoming restoration efforts. The concentration of trace elements (Cr, As, Pb, Ni, Mo, Co, Cd, and Hg) in the sediment samples was measured using an induced plasma mass spectrometric (ICP-MS). Results of the study revealed that Arsenic (As) levels exceeded the Canadian range of low effects, indicating the possibility of deleterious biological consequences on mangrove plants and animals. In all sampling locations, the enrichment factor (EF) analysis revealed extraordinarily high enrichment of As (9.89–23.65) and Mo (4.74–12.03). The geo-accumulation index of As (1.83 – 3.04), Mo (1.40 – 2.74), and Cd (0.652 – 3.03) revealed that mangrove locations in the Sepang River have almost extreme pollution effects. Pearson's correlation, which deduced the anthropogenic influence of As, Cd, and Mo in mangroves, backed up this claim. Results of the study recommended that continue monitoring of pollutants released from anthropogenic sources is highly required and there is a strong need to take more stringent measures to protect the environment.

* Corresponding author
E-mail: kumar.krishnan@newinti.edu.my (Kumar Krishnan)

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1 Introduction

The term "trace elements" is also known as "common contaminants," which refers to a chemical element found in tiny amounts in a sample (soil, water, or air) or environment (Mishra et al. 2020). Metallic elements with a high atomic number (weight) > 100 grams or a relative density of more than five that are lower on the periodic table are known as trace elements (Malhi et al. 2004; Bhunia 2017). Living organisms only require trace elements in small amounts to grow normally. Some trace metals have a deleterious nutritional effect on plants, animals, and people, whereas others have a beneficial nutritional effect. Natural and man-made (human activity) sources of trace metals are usually found in the environment (Pandey et al. 2019; Islam 2021). The incorporation of heavy metals into agricultural and seafood products are a form of pollution (Ali and Khan 2019; Krishnan et al., 2022). Lead, cadmium, chromium, nickel, silver, and zinc are among the biologically active trace metals that are ground in soil (Zhang et al. 2019). Micronutrient levels are influenced by the amount of sewage sludge, industrial waste, and fertilizer pollutants that enters into the soil (Alloway 2013; Ozkara et al. 2016). Although plants and animals require less than 20 trace elements for optimum growth, high quantities of these elements can be phytotoxic and harm animal health (Garbisu et al. 2020; Zine et al. 2020). Data on trace elements in soils have been collected from a variety of worldwide habitats, including industrialized towns, highways, rural areas in former mining districts, roadsides, and agricultural land utilized for crops or grazing (Yuan et al. 2004; Burges et al. 2018).

Anthropogenic activities including aquaculture, mining, tourism, agriculture, and the industrial usage of metals have increased the concentrations of heavy metals in sediments in Malaysia, further degrading the ecosystem in the mangrove zone (Sericano et al. 1995; Pande and Nayak 2013). A deposit is a detritus-eating marine living organism, which can absorb metals from polluted ground and water. It then becomes a critical channel for toxins to reach its predators (including humans who consume the affected marine organisms) Due to huge economic expansion and activities, as well as the concentration of human population and farming in coastal areas, a series of multi-element studies have been conducted along the west coast of Peninsular Malaysia (Kamarudin et al. 2015). Surface sediments are the final resting place for all sorts of pollution generated by human activities, resulting in a wide range of environmental issues. Furthermore, surface sediment interacts regularly with suspended elements, affecting metal release into overlying water (Zvinowanda et al. 2009). This research focuses on sediments collected from the Sepang River on Peninsular Malaysia's west coast. The current degree of contamination, which is continually changing, is indicated by the top several inches of sediments. As a result, there is a need to examine the pollution level of elements using sediment samples since sediments can provide useful information on marine pollution. The degree of pollution in the sediment of the Sepang River was evaluated using the geoaccumulation index and enrichment factor.

2 Materials and Methods

2.1 Study sites and samples collection

The research area of this study is focused on the sediments collected from the Sepang Besar River (Sungai Besar Sepang) in 2019, which falls along the Straits of Malacca in the Sepang District, Selangor, West Malaysia. Figure 1 shows the GPS coordinate: 02◦ 36’7. 41’’ N and 101◦ 44’ 8.62’ E and the

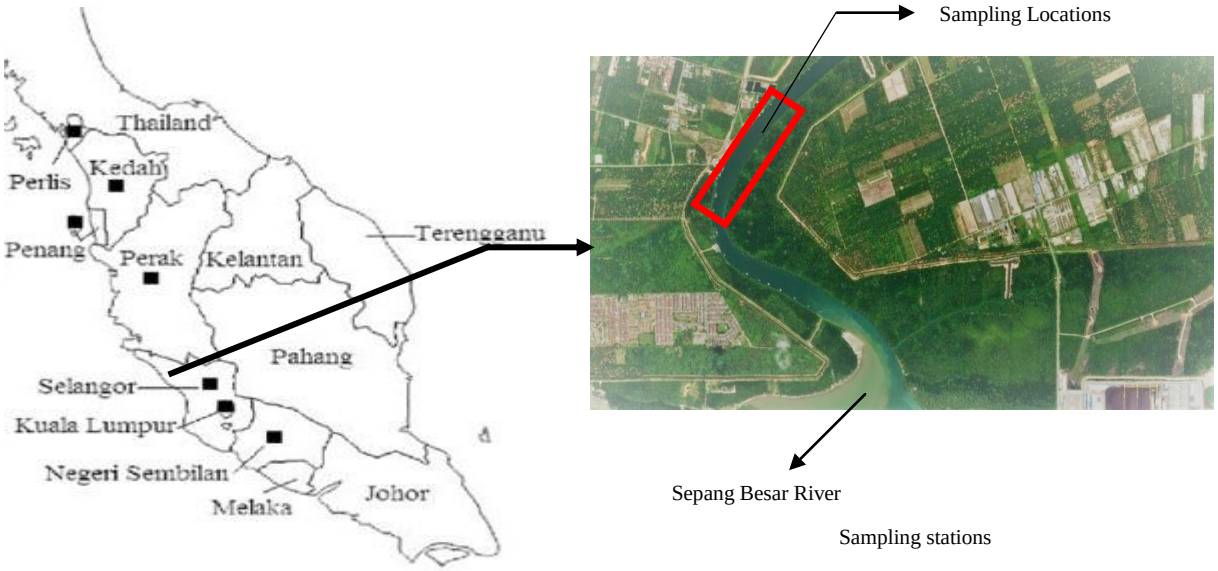

Figure 1 Map of the mangrove region sampling site along Sepang Besar River in Selangor, Malaysia.

Table 1 The description of the sampling site

Location ID	Nearby activity/Location	GPS Coordinate
Station 1	Shrimp pond, Bukit Pelanduk	02° 39`35.8'' N 101° 44'8.62' E
Station 2	Pig Farm, Bukit Pelanduk	2° 39'18.6' N 101° 44'7.85' E
Station 3	Sepang Fish Farm	2° 36'7.41' N 101° 42'39.4' E

nearby activities from the sampling station as also stated in Table 1. The study area's environment has year-round tropical conditions with temperatures ranging from 27°C to 34°C and a moderately humid atmosphere. The cloud cover is modest and November is the wettest month though rain rarely occurs. The Sepang district experienced rapid and significant growth from 1995 to 2015 because of its proximity to the administrative capital of Malaysia- Putrajaya. This expansion of urban growth has affected Sungai Sepang Besar's ecosystems. The forests and mangroves of the Sepang district cover about 546.7 hectares of reserved land corridors along with the Sungai Sepang Kecil and Sungai Sepang Besar rivers. As part of the Sepang 2025 Local Plan, mangrove forests are designated as Level 1 Environmentally Sensitive Areas (SEAs) (Yasin et al., 2019; Muhammad et al., 2020).

2.2 ICP-MS measurements

A plastic spoon was used to scrape the top layer of the mangrove sediments, which weighed roughly 600 grams and were then put into a labeled, clean plastic bag. The sediments were collected at random from a depth of 3.0-5.0 cm. In the laboratory, the sediments were dried for at least 72 hours at 80°C in a kiln until a dry weight was obtained. Using a glass mortar, each sediment sample is ground into a fine powder form. Then the powder was filtered through an opening made of stainless steel of 63 µm. Samples were stored in plastic pill boxes after being stirred vigorously and until ready for further analysis (Kumar et al. 2014).

From the collected sample, 200 mg of homogenized Sepang Besar River sediment samples were digested in a solution of 5 mL concentrated nitric acid (49 percent HF - analytical grade) and 2 mL concentrated hydrofluoric acid (67 percent HNO3 - Trace Metal Fisher brand) using a microwave. Each digestion batch contained duplicate samples, SRM (IAEA soil 7), and at least two blank acid reagents. The default settings of the microwave are 1200 watts, 200 ^{o}C, ramped for 20 minutes and maintained for 15 minutes at a constant pressure of 0.6 MPa. The sediment samples were digested for 15 minutes. The samples were withdrawn from the microwave oven after cooling for at least 30 minutes while maintained at a microwave temperature below 50^{o}C. The digestion process was repeated with the addition of 1 mL HNO_3 if there were any residues in the solutions to produce cleaning solutions. Following the transfer to a Teflon beaker, the solution was rinsed with 3mL of Milli-Q water. The digested sample was then put in a Teflon beaker, which was heated at 60 to 70 degrees Celsius until dry and then rinsed with 20 milliliters of Milli-Q water. After being filtered into a polythene container with filter paper, the mixture was diluted with Milli-Q water to a level of 50 mL for ICP-MS analysis (Whatman brand, diameter 125mm) (Marque et al. 2000, Suhaimi et al. 2018).

2.3 Statistical analysis

The data were analyzed by the calculation of average and standard deviation via Microsoft Office Excel 2010. Pearson correlation analysis was performed with SPSS 26.0 for Windows software. The two-tailed test was used to analyze the statistical significance of the correlation coefficient between trace elements in the sediment.

2.4 Tools to measure quality assurance and pollution level

In the analytical method analysis, the SRM (IAEASoil-7) was employed as a tool to measure quality control and quality assurance. The procedures to measure the SRM was similar to the procedures employed in sample analyses. Equation 1 shows how the recovery (percentage) is calculated (Alfian et al. 2020).

$$\% \text{ recovery} = \frac{C_{\text{measured}} - C_{\text{certified}}}{C_{\text{certified}}} \text{ x } 100\% \rightarrow \quad (1)$$

The z-score was also used to evaluate the precision and accuracy of the analytical techniques used. By calculating the standardized difference z while taking into account the uncertainties of both the certified value and the measured results of the SRM, the accuracy of the analysis method is determined. Equation 2 is used to calculate the z-score.

$$z = \frac{C_{measured} - C_{certified}}{\sqrt{\sigma_{measured}^{\ 2} + \sigma_{certified}^{\ 2}}} \rightarrow \quad (2)$$

If the Z-score falls between -3 and 3, it means that the certified value should lie within the 99 percent confidence interval of the SRM analysis results.

The pollution or contamination status of sediment can be obtained by evaluating the geo-accumulation index (Igeo) and the

enrichment factor (EF). The enrichment factor (EF) was developed to evaluate possible anthropogenic sources of hazardous elements as shown in Equation 3 (Kumar et al., 2014):

$$EF = [(X/Y)_{sample}]/[(X/Y)_{shale}] \rightarrow \quad (3)$$

Where X_{sample} is used to refer to the element concentration in the experimental sample, Y_{sample} is used to refer to the Fe concentration in the sample, X_{shale} is used to refer to the element concentration in the average shale, and Y_{shale} is used to refer to the element concentration in the average sale. According to Sutherland's (2000) version of the EF values, EF = 1 denotes no enrichment, minor enrichment if EF = 3-5, EF = 5–10 indicates moderately severe enrichment, severe enrichment if EF = 10–25, EF = 25–50 extremely severe enrichment, and if EF > 50 it indicating the sediment extremely severe enrichment.

The Geo-Accumulation Index (I_{geo}), developed by Muller (1969), is one of the most accurate tools for determining the contaminated state of a sample. It can be determined by equation (4):

$$I_{geo} = \log_2(X_n/1.5Y_n) \rightarrow \quad (4)$$

Whereas X_n denotes the amount of an element in the experiment sample, Y_n denotes the average quantity of an abundant and common element, and X_n denotes the amount of an element in the experiment sample (Turekian and Wedepohl 1961).

To reduce the variation caused by lithogenic influences, a background matrix correction factor of 1.5 is used. Muller (1969) divided the Igeo value into seven categories: virtually unpolluted if I_{geo}=0; 0-1, unpolluted to moderately polluted if I_{geo}=0-1, moderately polluted if I_{geo}=1-2, heavily polluted if I_{geo}=3-4, heavily to severely polluted if I_{geo}=4-5; and, extremely polluted if I_{geo} greater than 5.

3 Results and Discussion

Results presented in Table 2 show the certified and measured value, the percentage recovered, and the coefficient of variation (z-score). The SRM recovery and coefficient of variation percentages varied from 76 to 112 % and - 0.91 to 0.81, respectively. The recoveries between the measured and certified value in the ICP-MS method are within the acceptable range i.e. 100 ± 20% (Alfian et al. 2020). As shown in table 2, the calculated z-score for each element using the ICP-MS technique is acceptable and lies between -3 and +3 (Kumar et al. 2014).

Table 3 shows the concentration of trace elements in sediments collected at the three sampling locations. The average

Table 2 The recoveries and z-score between measured and certified values of Soil–7

Elements	Measured value Soil-7 (mg/kg)	Reference value Soil-7 (mg/kg)	Recoveries (%)	z-score
As	12.3 ± 0.9	13.4 ± 0.8	109	- 0.91
Cd	1.4 ± 0.4	1.3 ± 0.7	93	0.12
Co	9.2 ± 0.6	8.9 ± 1.2	97	0.22
Cr	66 ± 8	60 ± 14	91	0.37
Hg	0.038 ± 0.018	0.04 ± 0.03	105	- 0.06
Mo	3.3 ± 0.9	2.5 ± 1.6	76	0.70
Ni	23.2 ± 4.3	26 ± 5	112	- 0.42
Pb	72.1 ± 10.2	60 ± 11	83	0.81

Table 3 The concentrations of trace elements (mg/kg) at the three sampling location

Heavy metals	Station 1	Station 2	Station 3
As	16.77 ± 1.09	16.76 ± 1.16	7.258 ± 0.294
Cd	0.116 ± 0.006	0.092 ± 0.005	0.022 ± 0.006
Co	3.295 ± 0.142	3.174 ± 0.225	2.791 ± 0.117
Cr	17.32 ± 12.67	23.03 ± 0.68	10.85 ± 0.34
Hg	0.014 ± 0.002	0.018 ± 0.007	0.005 ± 0.001
Mo	5.688 ± 0.956	6.065 ± 0.652	2.391 ± 0.746
Ni	9.778 ± 1.14	10.72 ± 1.96	4.967 ± 0.136
Pb	16.44 ± 1.38	16.38 ± 1.23	10.60 ± 0.20

concentration of As, Cd, Co, Cr, Hg, Mo, Ni, and Pb (in mg/kg) from all stations are13.59, 0.077, 3.087, 17.07, 0.012, 4.715, 8.489 and 14.47, respectively. In general, trace element accumulation in sediments at Station 1, Station 2 and Station 3 were found in the order of Cr >As>Pb> Ni > Mo > Co > Cd > Hg, Cr > As >Pb> Ni > Mo > Co> Cd > Hg, and Cr >Pb> As > Ni > Co > Mo > Cd > Hg. The concentration of Cr was highest and Hg was lowest at all sampling locations. The mean concentrations of all trace elements were found highest at stations 1 and 2 than at station 3. Sediment particles are made up of biogenic and non-biogenic (lithogenic) components and are considered to be the final sinks for trace elements transferred to the mangrove environment. Water has a lower capacity for accumulating persistent hazardous chemicals than sediments (Rodriguez-Barroso et al. 2008; Yuan et al. 2011).

Results presented in table 4 revealed the comparisons between the hazard trace elements reported in this study with the MacDonald Canadian Freshwater Sediment Quality Guidelines (2000) and other studies on rivers in Malaysia and elsewhere. The deposits were considered clean to slightly contaminated if the trace element content in the sediment is less than the threshold effect levels TEL). No effect on the majority of sediment species is expected if the trace element concentration is lower than the TEL value. Sediments are classified as heavily polluted if the trace element concentrations are higher than the probable effect levels (PEL). If the concentration of trace elements exceeds the Freshwater Sediment Quality Guidelines (FSQGs – PEL) level, the consequences are negative impacts will be on the majority of sediment-dwelling species. The concentrations of Cd, Co, Cr, Ni, and Pb in the present study were lower than other studies of Malaysia river sediments except for As which in the present study is slightly higher than previous studies conducted in the Juru river for example (Krishnan et al. 2022). Furthermore, the concentration of Cd, Co, Cr, Ni, and Pb were found to be lower than the Canadian FSQGs, except for As, which were higher than the Canadian FSQGs' TEL values. The concentration of Asin Sepang River sediments was found to be 2.3 times higher than that of the Canadian -FSQGs – TEL value (MacDonald et al. 2000). These results show that the Sepang River sediments were contaminated with As, and could have a negative impact on the majority of sediment-dwelling species. Anthropogenic activities like dredging, the discharge of industrial and municipal garbage, etc. may be to blame for this. This research's findings are in line with earlier findings (Zulkifli et al. 2014, Yasin et al. 2019).

The enrichment factor (EF) is presented in Table 5. The EF analysis demonstrated an anthropogenic source of metal buildup in the Sepang River mangrove sediments. The EF of As, Cd, Co, Cr, Hg, Mo, Ni, and Pb exhibited enrichment from numerous sources, which might be attributable to industrial discharges, mangrove exploitation, aquaculture operations, agricultural inputs, or fertilizers (Nath et al. 2021). In all sampling locations, the EFs of As (EF values 9.89–23.65) and Mo (EF values 4.74–12.03) exhibit severe to excessive enrichment. On the other hand, Cd and Pb

Table 4 Comparison of trace elements in this study with other studies and Canadian - FSQGs in (mg/kg)

Various sites		As	Cd	Co	Cr	Hg	Mo	Ni	Pb	Ref
Sepang River Present study		6.93 -17.9 (13.6)	0.028 -0.122 (0.077)	2.66 – 3.41 (3.09)	2.77 - 25.9 (17.1)	0 – 0.713 (0.0123)	1.58 – 6.79 (4.71)	4.81 - 12.98 (8.49)	10.4 - 17.94 (14.5)	
Sepang River, Malaysia		na	1.15±0.04	na	na	na	na	26.05±0.08	86.89±0.13	Zulkifli et al. 2014
Juru River		9.15 – 11.47 (10.5)	0.572 – 0.696 (0.639)	7.70 – 8.89 (8.23)	78.85 – 89.04 (82.21)	na	na	na	27.64 – 45.25 (34.52)	Krishnan et al. 2022
Linggi River		3.6 – 65.9 (36.0)	0.09 – 1.10 (0.29)	na	1.8 – 105 (33.2)	na	na	1.8 – 29.7 (10.3)	8.2 – 52.3 (30.0)	Khan 1990
Langat River, Selangor		12.4 – 27.3 (17)	3.0 – 37.9 (12.1)	na	11 – 73 (29)	na	na	na	na	Sarmani 1989
Klang River, Selangor		na	0.57 – 2.19 (1.54)	na	na	na	na	5.9 – 24.5 (16.3)	24.2 – 64.1 (47.9)	Naji and Ismail 2016
Kerteh River, Terengganu		na	4.0 – 5.0 (4.3)	na	13 – 67 (33.7)	na	na	7 – 24 (11.2)	11 – 25 (15.5)	Rozaini et al. 2010
Han River, Korea		na	0.05- 1.02	na	na	na	na	9.16- 45.0	14.2- 96.6	Kim et al. (2011)
Hainan Island, China		na	0.11-0.13	na	na	na	na		15.0-19.0	Qiu et al. (2011)
Canadian - FSQGs	TEL	5.9	0.60	na	37.3	0.17	na	18	35.0	MacDonald et al. 2000
	PEL	17.0	3.50	na	90.0	0.49	na	36	91.3	MacDonald et al. 2000

Table 5 Enrichment factor for trace element in sediments of Sepang Besar River

Heavy Metal	Station 1	Station 2	Station 3
As	22.164 ± 1.44	22.154 ±1.53	9.595 ±0.37
Cd	1.843 ± 0.09	1.452 ± 0.07	0.354 ± 0.09
Co	0.314 ± 0.01	0.302 ± 0.02	0.266 ± 0.01
Cr	0.404 ± 0.27	0.537 ± 0.02	0.253 ± 0.01
Hg	0.401 ± 0.05	0.491± 0.18	0.073 ±0.07
Mo	11.280 ± 1.73	12.028 ±1.29	4.742 ±1.46
Ni	0.277 ± 0.03	0.304 ± 0.05	0.141 ± 0.01
Pb	2.794 ± 0.23	2.783 ± 0.21	1.801 ± 0.03

Table 6 Geo-accumulation index for trace elements in sediments of Sepang Besar River

Heavy Metal	Station 1	Station 2	Station 3
As	3.037 ± 0.09	3.037 ± 0.10	1.829 ± 0.06
Cd	3.034 ± 0.07	2.690 ± 0.07	0.652 ± 0.39
Co	-6.902 ± 0.06	-6.956 ± 0.10	-7.141 ± 0.06
Cr	-8.565 ± 1.72	-8.153 ± 0.04	-9.240 ± 0.04
Hg	1.654 ± 0.19	1.946 ± 0.50	-0.043 ± 0.53
Mo	2.648 ± 0.21	2.740 ± 0.16	1.397 ± 0.48
Ni	-8.829 ± 0.17	-8.696 ± 0.23	-9.806 ± 0.04
Pb	-2.910 ± 0.12	-2.915 ± 0.11	-3.543 ± 0.03

enrichment ranged from low to severe. Other trace elements including Cr, Co, Hg, and Ni did not exhibit any enrichment at any of the sampling sites. The EF of metals and heavy metals can be useful as an indirect indicator for assessing sediment pollution or toxicity. However, relying solely on the enrichment factor to assess sediment toxicity at a given location is insufficient. To determine the sediment's toxicity at a particular location, the degree of contamination in the sediment should be considered and compared to sediment guidelines. Examples of anthropogenic sources of trace elements, such as waste disposal, industry, and urbanization are the factors that contribute to the highest enrichment factor (EF) (Gao et al. 2010; Krishnan et al. 2022).

Based on the data presented in Table 6, the geo-accumulation index (Igeo) of Co, Cr, Pb, and Ni are below zero, which indicates that the examined mangrove sediment in the Sepang mangrove forest is unpolluted. Meanwhile, at station 3, the positive value of the Cd geoaccumulation index (Igeo) was recorded between 0 and 1, indicating that the mangrove area is significantly polluted. However, in Stations 1 and 2, the Igeo index was recorded between 3 and 4 suggests that the analyzed mangrove sediment in the Sepang mangrove forest is heavily contaminated with As and Cd. The geo-accumulation index of Mo and Hg at stations 1 and 2 falls in the class range between 2 and 3 indicating that the mangrove area is moderate to strongly polluted. Based on Igeo value, it can be concluded that the studied area enriched with As, Cd, Mo, and Hg. The presence of various metals in sediment may result from the interaction of elevated particulate matter with relatively unpolluted sediments (Singh et al. 2020).

The pollution of As, Cd, Mo, and Pb was assumed to have occurred as a result of anthropogenic activities nearby the sampling location as mentioned in Table 1. The use of phosphate fertilizers, lead-arsenate pesticides, and pesticides in agricultural activities is also a possible source of the elements As and Pb (Chen et al. 2004). At Sungai Besar Sepang, a noticeable alteration in mangrove vegetation was detected, indicating a transition from primary mangroves to mixed mangroves and second-rate mangroves. Another noteworthy shift was also observed at the river mouth of the Sungai Sepang Besar, where mangroves were reduced and degraded. The change in land use and land cover in the Sepang District has influenced river features, resulting in a dramatic shift in mangroves (Yasin et al. 2019).

Table 7 Pearson correlation coefficients (r) between trace elements (N = 12) concentrations in sediment samples

	As	Cd	Co	Cr	Hg	Mo	Ni	Pb
As	1							
Cd	.962**	1						
Co	.872**	.908**	1					
Cr	.549	.473	.395	1				
Hg	.864**	.805**	.648*	.579*	1			
Mo	.217	.405	.478	.264	.228	1		
Ni	.908**	.849**	.719**	.520	.778**	.062	1	
Pb	.992**	.946**	.874**	.463	.834**	.158	.881**	1

*: p<0.05; **: p<0.01

In this study, the relationships between element concentrations in surface sediments were investigated using a Pearson's correlation analysis. Table 7 shows the result of correlation coefficients between element concentrations in surface sediments. The relationship between As and Cd, As and Co, As and Hg, As and Ni, As and Pb, Cd and Co and Cd and Hg, Cd and Ni, Cd and Pb, Co and Pb, Hg and Pb, Ni and Pb, and Ni and Pb reveal high correlations between elements with r values of 0.962, 0.872, 0.864, 0.908, 0.982, 0.908, 0.805, 0.849 respectively. Based on the results of correlations analysis, the above trace elements are likely to have come from common sources (Landajo et al. 2004; Kumar et al. 2014). A tepidly positive correlation exists between As and Cr, Cd and Cr, Co and Mo, Cr and Hg, Cr and Ni, Hg and Ni with corresponding r values of 0.549, 0.473, 0.478, 0.579, 0.520, 0.778 respectively.

Conclusion

This study attempted to assess the status of several trace elements (Cr, As, Pb, Ni, Mo, Co, Cd, and Hg) in the surface sediments of the Sepang Besar River. The study showed that the total heavy metals concentrations in the sediment samples in the streams followed the order of Cr > As > Pb > Ni > Mo > Co > Cd > Hg. The values of EF and Igeo show that the sediments surrounding the Sepang River is contaminated with trace metals, especially As, Cd, Mo, and Pb. Pearson's correlation makes it clear that there is an anthropogenic influence of As, Cd, and Mo in the mangroves of the Sepang Besar River. Data collected from the sediments of this study indicate that there is a significant discharge of trace elements to the estuary from anthropogenic activities, though more research is needed to determine the true ecological impact of this pollution on the immediate environment to preserve its biodiversity and resources for human communities.

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References

Alfian, Yusuf, S., & Sutisna. (2020). Elemental analysis of SRM 1547 peach leaves, 1573a tomato leaves, and 1570a spinach leaves. *Journal of Physics*: *Conference Series, 1436*, 012044.

Ali, H., & Khan, E. (2019). Bioaccumulation of Cr, Ni, Cd and Pb in the economically important freshwater fish *Schizothorax plagiostomus* from three rivers of Malakand Division, Pakistan: risk assessment for human health. *Bulletin of Environmental Contamination and Toxicology, 102*, 77–83. https://doi.org/10.1007/s00128-018-2500-8.

Alloway, B. J. (2013). Sources of Heavy Metals and Metalloids in Soils. *Heavy Metals in Soils 22*, 11-50.https://doi.org/10.1007/978-94-007-4470-7

Bhunia, P. (2017). Environmental Toxicants and Hazardous Contaminants: Recent Advances in technologies for Sustainable Development. *Journal of Hazardous, Toxic, and Radioactive Waste*, *21*, 02017001.

Burges, A., Alkorta, I., Epelde, L., & Garbisu, C. (2018). From phytoremediation of soilcontaminants tophytomanagement of ecosystem services in metal contaminated sites. *International Journal of Phytoremediation*, *20*, 384–397.

Chen, Y.X., Lin, Q., He, Y.F., & Tian, G.M. (2004). Behavior of Cu and Zn under combined pollution of 2,4-dichlorophenol in the planted soil. *Plant and Soil*, *61*, 127–134.

Gao, X., Arthur Chen, C.T., Wang, G., Xue, Q., Tang, C., & Chen, S. (2010) Environmental status of Daya Bay surface sediments inferred from a sequential extraction technique. *Estuarine, Coastal and Shelf Science, 86*(3), 369–378.

Garbisu, C., Alkorta, I., Kidd, P., Epelde, L., & Mench, M. (2020). Keep and promote biodiversityat polluted sites under

phytomanagement. *Environmental Science and Pollution Research, 27*, 44820–44834.

Islam, M. S. (2021). Preliminary assessment of trace elements in surface and deep waters of anurbanriver (Korotoa) in Bangladesh and associated health risk. *Environmental Science and Pollution Research, 28*(23), 29287-29303, Doi:10.1007/ s11356-021-12541-5.

Kamarudin, M. K. A., Toriman, M. E., Rosli, M. H., Juahir, H., et al. (2015). Analysis of meander evolution studies on effect from land use and climate change at the upstream reach of the Pahang River, Malaysia. *Mitigation and Adaptation Strategies for Global Change, 20*, 1319-1334.

Khan, I.S.A.N. (1990). The mineralogy and trace element constituents of suspended streamsediments ofthe Linggi River Basin, Malaysia. *Earth, 4*, 133–139.

Kim, K.T., Ra, K., Kim, E.S., Yim, U.H., & Kim, J.K. (2011). Distribution of Heavy Metals in the Surface Sediments of the Han River and its Estuary, Korea, SI 64. *Proceedings of the 11th International Coastal Symposium*, pp. 903 – 907. Szczecin, Poland, ISSN 0749-0208.

Krishnan, K., Saion, E. B., CK, Y., Chong, M. Y., & Nadia, A. S. (2022). Determination of Trace Elements in Sediments Samples by Using Neutron Activation Analysis. *Journal of Experimental Biology and Agricultural Sciences*, *10*(1), 21–31. https://doi.org/10.18006/2022.10(1).21.31

Kumar, K., Saion, E., Halimah, M., Yap, C., & Hamzah, M.S. (2014). Rare earth element (REE) in surface mangrove sediment by instrumental neutron activation analysis. *Journal of Radioanalytical and Nuclear Chemistry, 301*, 667–676.

Landajo, A., Arana, G., de Diego, A., Etxebarria, N., Zuloaga, O., & Amouroux, D. (2004). Analysis of heavy metal distribution in superficial estuarine sediments (estuary of Bilbao, Basque Country) by open-focused microwave-assisted extraction and ICP-OES. *Chemosphere, 56*,1033–1041.

MacDonald, D.D., Ingersoll, C.G., & Berger, T.A. (2000). Development and evaluation of consensus-basedsediment quality guidelines for freshwater ecosystems. *Archives of Environmental Contamination and Toxicology, 39*, 20-31 Doi:http://dx.doi.org/ 10.1007/s002440010075.

Malhi, Y., Baker, T.R., Phillips, O.L., Almeida, S., et al. (2004). The above-ground coarse wood productivity of 104 neotropical forest plots. *Global Change Biology*, *10*, 563–591.

Marques, M.J., Salvador, A., & Morales-Rubio, A.E.M. (2000). Trace element determinationin sediments: a comparative study between neutron activation analysis (NAA) and inductively coupled plasma-mass spectrometry (ICP-MS), *Microchemical Journal, 65*, 177–187.

Mishra, A.K., Santos, R., & Hall-Spencer, J.M. (2020). Elevated trace elements in sediments andseagrasses at CO_2 seeps. *Environmental Research, 153*, 104810, https://doi.org/10.1016/ j.marenvres.2019.104810

Muhammad, Y.Y., Nisfariza, M.N., Mariney, M.Y., Jamalunlaili, A., & Norzailawati, M.N. (2020). SPOT Imagery Observation on Mangrove Changes Using NDVI Density Analysis: The Case of Sepang Besar River, Malaysia. *The Arab World Geographer / Le Géographe du monde arabe*, *23*(2-3),217-228. https://doi.org/ 10.5555/1480-6800.23.2.217.

Muller, G. (1969). Index of geoaccumulation in sediments of the Rhine River. *Geojournal, 2*(3), 108-118.

Naji, A., & Ismail, A. (2016). Assessment of Metals Contamination in Klang River Surface Sediments by using Different Indexes. *Environmental Asia, 4*, 30–38, *DOI:10.14456/ea.2011.5.*

Nath, A., Samanta, S., Banerjee, S., et al. (2021).Threat of arsenic contamination, salinity and waterpollution in agricultural practices of Sundarban Delta, India, and mitigation strategies. *SN Applied Sciences General, 3*, 560. https://doi.org/10.1007/s42452-021-04544-1

Özkara, A., Akyıl, D., & Konuk, M. (2016). Pesticides, environmental pollution, and health. In M.L. Larramendy & S. Soloneski (Eds.) *Environmental Health Risk-Hazardous Factors to Living Species*; IntechOpen: london, UK. DOI: 10.5772/63094

Pande, N., & Nayak, G.N. (2013). Understanding distribution and abundance of metals with space andtime in estuarine mudflat sedimentary environment. *Environmental Earth Sciences, 70*, 2561–2575. https://doi.org/10.1007/s12665-013-2298-y.

Pandey, L. K., Park, J., Son, D. H., Kim, W., et al. (2019). Assessment of metal contamination in water and sediments from major rivers in South Korea from 2008 to 2015. *Science of The Total Environment, 51*, 323–33. doi:10.1016/j.scitotenv.2018.09.057.

Qiu, Y.W., Yu, K.F., Zhang, G. & Wang, W. X. (2011). Accumulation and portioning of seven trace metals in mangroves and sediment cores from three estuaries wetlands of Hainan Island, *China Journal of Materials*, *190*, 631-638.

Rodríguez-Barroso, M. R., Ramírez-del Solar, M., Blanco, E., Quiroga, J. M., & García-Morales, J. L. (2008). Qualitative

estimation of heavy metals in marine sediment using thermal analysis. *Soil and Sediment Contamination*, *17*, 107–120.

Rozaini, M.Z.H., Ramli, A.N., & Jaafar, M. (2010). The determination of heavy metal concentration in sediment from Kerteh River, Terengganu. *Journal of Sustainability Science and Management*, *5*, 1–11.

Sarmani, S.B. (1989). The determination of heavy metals in water, suspended materials and sediments from Langat River, Malaysia. *Hydrobiologia*, *176*, 233–238, doi:http://dx.doi.org/10.1007/BF00026558.

Sericano, J.L, Wade, T.L, & Jackson, T.J. (1995). Trace organic contamination in the Americas: An overview of the US national status and trends and the international mussel watch progammes. *Marine Pollution Bulletin*, *31*, 214-225.

Singh, J.K., Kumar, P., & Kumar, R. (2020). Ecological risk assessment of heavy metal contamination in mangrove forest sediment of Gulf of Khambhat region, West Coast of India. *SN Applied Sciences*, *2*, 2027. https://doi.org/10.1007/s42452-020-03890-w.

Suhaimi Elias, M., Shariff, I., Kamarudin, S., Shamsiah, A.R., & Yii, M.W. (2018). Assessment of toxic elements in sediments of Linggi River using NAA and ICP-MS techniques. *Methods X*, *5*, 454–465.

Sutherland, R. A. (2000). Bed sediment-associated trace metals in an urban stream, Oahu, Hawaii. *Environmental Geology*, *39*, 611–627.

Turekian, K.K., & Wedepohl, K.H. (1961). Distribution of the elements in some major units of the earth's crust. *Bulletin of Geological Society of America*, *72*, 175–92.

Yasin, M. Y., Yusoff, M. M., & Noor, N. M. (2019). Urban sprawl assessment using time seriesLULC and NDVI variation: a case study of Sepang Malaysia. *Applied Ecology and Environmental Research*, *17*(3). 5583-5602. DOI: 10.15666/aeer/1703_55835602.

Yuan, C.G., Shi, J.B., He, B., Liu, J.F., Liang, L.N., & Jiang, G.B. (2004). Speciation of heavy metals in marine sediments from the East China Sea by ICP-MS with sequential extraction. *Environment International*, *30*, 769—783. DOI: http://dx.doi.org/10.1016/j.envint.2004.01.001.

Yuan, X., Huang, H., Zeng, G., Li, H., et al. (2011). Totalconcentrations and chemical speciation of heavy metals in liquefaction residues of sewage sludge. *Bioresource Technology*, *102*, 4104–4110.

Zhang, X., Yan, L., Liu, J., Zhang, Z., & Tan, C. (2019). Removal of different kinds of heavy metalsbynovel PPG-nZVI beads and their application in simulated stormwater infiltration facility. *Applied Sciences*, *9*, 4213.

Zine, H., Midhat, L., Hakkou, R., El Adnani, M., & Ouhammou, A. (2020). Guidelines for a phytomanagement plan by the phytostabilization of mining wastes. *Scientific African*, *10*, 2468–2476.

Zulkifli, S. Z., Siti, A. R., Ferdaus, M. Y., & Ahmad, I. (2014). Geochemical Fractionations of HeavyMetals in Sediments of Sepang Besar River, Malaysia. *Acta Biologica Malaysiana*, *3*(1): 1-9. DOI: http://dx.doi.org/10.7593/abm/3.1.1.

Zvinowanda, C. M., Okonkwo, J. O., Agyei, N. M., Sekhula, M. M., & Sadiku, R. (2009). Application of maize tassel for the removal of Pb, Se, Sr, U and V from borehole water contaminated with mine wastewater in the presence of alkaline metals. *Journal of Hazardous Materials*, *164* (2-3), 884-891.

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Stress Management Programme on the Stress of Chiang Mai University Students: A Pilot Study

Natthanit Joompathong, Wannipa Bunrayong, Supat Chupradit*

Department of Occupational therapy, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand

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ABSTRACT

Stress is the problem that is often found in students due to their higher expectations and the changes happening at the personal, social and environmental levels. The main objective of the present study was to develop and analyze the results of a stress management programme conducted for students at Chiang Mai University, Chiang Mai, Thailand. This was quasi-experimental research, conducted according to the one-group plan. The student initiatives included five Chiang Mai University students, selected through purposive sampling after passing the initial screening. They were evaluated by completing the basic information questionnaire of the Suanprung Stress Test-20 (SPST-20) and the Suanprung Stress Test-60 (SPST-60) before and after the programme. The tools used in this study were developed from the conceptual framework according to the Canadian Model of Occupational Performance and Engagement (CMOP-E) model. The researcher used the breathing technique using the diaphragm, and muscle relaxing technique. The concept of emotional awareness was conducted according to the four foundations of mindfulness, and cognitive behavioral therapy (CBT) with group process in occupational therapy. The results of the study revealed that the sample population had significantly lower stress levels after joining the stress management programme. However, it was observed that there was no difference in the susceptibility to stress levels before or after joining the programme. In general, this stress management programme can be undertaken by other students of Chiang Mai University to monitor their stress levels.

* Corresponding author
E-mail: supat.c@cmu.ac.th (Supat Chupradit)

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1 Introduction

Nowadays, students and young people are more prone to stress and stress-related problems due to different kinds of internal and external expectations they encounter in everyday life. Moreover, it has been pointed out that young people were particularly sensitive towards their studies, owing to the changes in personnel and social levels (Reddy et al. 2018). Stress-related problems arise from more work responsibilities, classroom participation, punctuality, financial stability, and maintaining a balanced life. This in turn would create more risks resulting in negative stress levels that might impact their academic achievements as well (Portoghese et al. 2019). The mental health service from Thailand reported that the majority of the people who consulted regarding mental health problems were the youth of aged between 20 and 25 years (20.1%), who rendered 70,534 times advice services via telephone (Ministry of Public Health 2020). Additionally, in 2019, the Child and Adolescent Mental Health Rajanagarindra Institute (CAMRI) reported that 51.36% of youth called to receive guidance on stress-related issues leading to mental illness (Ministry of Public Health 2020). These evidences supported that chronic stress could also have an important role in initiating severe psychiatric symptoms and defects such as depression, and bipolar disorder, after experiencing a catastrophic event (Davis et al. 2017). Therefore, to prevent mental health problems that will occur in students, Universities should promote stress management skills for students.

On analyzing the literature data, there were about 24 studies focused merely on stress management programme with 1,431 university students. Regehr et al. (2013) highlighted the efficiency of cognitive-behavioral therapy and efficient mindfulness could reduce stress in university students. The participants of these programmes were more female students from western countries like the USA, Switzerland, Scotland, etc. Similarly, in Thailand, various techniques such as progressive muscle relaxation training, Anapanasati meditation (Leungprawat 2004), yoga, (Chidduan 2013), breath training, muscle relaxing technique, meditation, (Singhchada et al. 2016), provision of advice in an integrated focus group, aromatherapy, massage, (Yooiam et al. 2017), and Buddhist psychology (Chiangkoontod 2018) were followed by the students to manage stress. These entire studies highlight that stress management can be achieved by following the aforementioned techniques. Previous studies have shown that stress management programme only use certain techniques. Very few stress management programme incorporate stress relaxation techniques and promote stress-management skills together. Therefore, the researchers were interested to understand the techniques related to managing stress by analyzing the symptoms of stress, the emotions caused by stress, and the management of stressful thoughts, among the students of Chiang Mai University.

The researcher analyzed and selected stress management techniques to create an appropriate stress management programme for university students. The stress relaxation techniques included the breathing technique using the diaphragm and muscles relaxing technique, and stress management skills with the concept of emotional awareness and cognitive behavioral therapy. Diaphragmatic breathing is a deep breathing, integrated mind-body training that is effective for managing stress and mental states, also effective in improving one's ability to manage emotions as well as reducing stress, anxiety, and depression (Ma et al. 2017; Stromberg et al. 2015; Brown and Gerbarg 2005). The diaphragmatic breathing technique is a technique that enables one to relax and manage the occurring stress. This is an adjustment of the sympathetic nerve system (Hopper et al. 2019) which increases the capacity of managing emotions (Hamasaki 2020). Muscle relaxation techniques bring adequate muscle relaxation which in turn can reduce stress. Deep muscle relaxation reduces physiological tension (La and John 2002; Carmody et al. 2008; Dhyani et al. 2015; Merakou et al. 2019; Silveira et al. 2020). Thus, this is a practiced method for relaxing the stress symptoms, which can be done by anybody, even in a working environment, and this has a positive effect on the development of personnel activity (Silveira et al. 2020). Emotional awareness is a type of mindfulness meditation that aims to make an individual able to deal with emotions by being mindful or directing the emotions, thoughts, feelings, and internal states that arise within oneself. Mindfulness can reduce stress, and help to improve a person's physical and mental health which contributed to the reduction of mental and physical symptoms of stress (Grossman et al. 2004; Carmody et al. 2008; Sedaghatb et al. 2011; Lia et al. 2020). Cognitive-behavioral therapy helps to change a person's irrational thinking about himself and the outside world. Using cognitive behavioral therapy students can reduce their stress and adjust stress responses (Mennuti et al. 2006; Regehr et al. 2013; Jafar et al. 2016).

Hence, through the present study a stress management programme was developed and evaluated its effect on students' activities at Chiang Mai University. The students who were selected to join a group called the sample population evaluated themselves on the form of those stress that affected their life activity. The programme which was developed for this study maintains an adjustment between stress relaxation, and stress management skills. The programme focused on managing the physical symptoms of the stress, the occurring emotions from the stress, adjustment of the thinking that leads to stress, and creating comprehensive stress management. The main objective of this study is to develop and analyze the results of a stress management programme conducted for students at Chiang Mai University, Chiang Mai, Thailand.

2 Materials and Methods

This study was quasi-experimental research, wherein, an experiment was conducted according to the one-group plan that evaluated the results before and after the respondents joined the stress-releasing programme developed for the respondents. Herein, the results of the sample population were measured for the period before and after programme (one-group pretest-post-test design).

2.1 Participants

The sample population in this study was five undergraduate students in the normal academic semester of Chiang Mai University comprising two male students (40%) and three female students (60%). They were screened by using purposive sampling via the initial screening that evaluated their stress from a medium to violent level, which in turn would affect their life activity.

2.2 Stress Releasing programme

The main tool used in this experiment was a stress management programme. This allowed the researcher to conduct the experiment and develop the programme from the conceptual framework according to the CMOP-E model. The sample population participated in the programme one time per week for 90 minutes per session for eight weeks. The programme was separated into two periods i.e. (1) the stress management skills and (2) the stress relaxation techniques (Regehr et al. 2013; Jafar et al. 2016; Singhchada et al. 2016). The programme was designed to assist in relaxing the occurring symptoms of stress with diaphragmatic breathing techniques and the muscle relaxing method as the stress relaxing techniques. For managing issues related to emotions, the Vipassana-Kammatthana principle and the same meaning as the four foundations of mindfulness were utilized (Regehr et al.2013). For the part of managing issues related to the thinking method of creating stress, the cognitive behavioral therapy technique including the group process in occupational therapy as the skill of managing the stress periods was used (Cole 2018).

The programme passed the content validity test conducted by qualified experts in mental health and psychology and the cognitive behavioral therapy process and the behaviour. The experts examined the validity of the stress management programme's content by calculating the conformance index with a checklist. The overall program consistency consisted of 5 aspects, namely, the appropriateness of the theory of program name suitability, the suitability of activities order, the content suitability following the objectives, the suitability of the programme duration, and the suitability of the used equipment. In the program, the correspondence between the assessment and the objectives, the correspondence between the activities and the programme, the correspondence between the knowledge and the objectives, and the correspondence between the work and the objectives were also noted. The expert confirmed that the stress-management programme had a conformance index that demonstrated the programme's compliance across all aspects of the checklist.

For gathering the required data, the tools consisted of the basic information questionnaire of the sample population from the Suanprung Stress Test-20 (SPST-20) and the Suanprung Stress Test-60 (SPST-60). These tests had been developed by Mahatnirunkul et al. (1997), with details as follows:

1) First part of the questionnaire has been developed for the extraction of basic information like gender, age, class year, average revenue per month, and the parents' status of the sample population.

2) The SPST-20 stress test was created to measure the stress level suitable for the Thai people and this comprises five levels of the estimation scale. The interpretation of the results would segregate the overall stress level of the sample population into one of the following four levels, such as low level (score of 0-23), medium level (score of 24-41), high level (score of 42-61) and violent level (score of 62 or more) (Mahatnirunkul et al. 1997).

3) The SPST-60 test questionnaire comprised of three parts which determine the level of stress (Mahatnirunkul et al. 1997) and for this the questions were developed to measure the susceptibility to stress level (survey question was based on the daily life of the student, which might affect the stress level), the sensitivity of the sources of stress (this part of the questionnaire tries to explore the cause of the stress), and the symptoms of stress (this would be a result of a personal desire or high pressure on his/her mind). The interpretation of the above results of the SPST-60 could separate the score into three levels as follows:

The stress lever	Low	Medium	High	Violent
Part 1: Susceptibility to stress level	0-20	21-26	27-33	33+
Part 2: Sources of Stress	0-36	37-57	58-79	79+
Part 3: Symptoms of stress	0-17	18-36	37-57	57+

2.3 Ethical approval

The research work carried out in the present study passed consideration of human research ethical requirements and received approval from the Ethical Committee of the Research, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand (AMSEC-64EX-019). The researcher provided information about the details of the research, including the right and decision to

participate or not to participate, to the respondents. Moreover, the personal information of the participants was kept confidential and was not revealed in any matter.

2.4 Procedure

A stress management programme was developed or outlined by reviewing the available literature, past concepts, and theories related to the stress management programme. Further, a tool was developed and used in the operational experiment to check for the content validity found in the index of item-objective congruence (IOC) by five qualified people related to mental health and psychology. During the study, the process and progress of the cognitive behavioral therapy group was monitored by the psychotherapist to develop suitable results and recommendations. For data extractions, a well-structured questionnaire (SPST-20 and SPST-60) based on the basic information related to the before and after joining the programme was circulated to the selected respondents.

The experiment of the stress management programme was used with the sample population once a week for 90 minutes per session for eight weeks. The researcher initiated the programme by building a relationship to bring it into the programme each session. During the stress management skills period, the researchers provided information on stress management techniques and allowed the subjects to comment on what they had learned together and their experiences in managing stress. In the stress relaxation period, the researchers taught members to use diaphragmatic breathing and muscle relaxation.

2.5 Data Analysis

The analysis of the general data of the sample population using description statistics comprised the percentage, mean, and standard deviation. In addition, for the comparison of different levels of stress, the sensitivity of the creation of different stress levels, sources of stress, and the symptoms of stress on the respondents before and after joining the stress management programme was analyzed by the Wilcoxon signed-rank test.

3 Results and Discussion

General information related to the respondent's gender, age, faculty of study, the students' class year, average revenue per month, and the parents' status were collected by a well-developed questionnaire. The sample population of five people is composed of two males (40%) and three females (60%), with an average age of 21 years. Four students were from the Faculty of Health Sciences (80%), and one was from the Faculty of Humanities and Social Sciences (20%), where three people were in their fourth year (60%), one student in the first year and one in the second year. In most cases, the respondents lived with their parents (80%) (Table 1).

From the SPST-20 test results, the overall stress level could be interpreted by the measurement taken before and after the stress management programme. It was observed that the stress of the sample population decreased from a violent level to a high level after the therapy process. In comparison, the stress level of the sample population before joining the programme had an average stress score ($\bar{x}$) of 66 scores, which was interpreted as being the violent stress level. However, after joining the programme, the average stress score was decreased to be 52.4 scores, which was interpreted as being a high-stress level. The SPST-60 test results showed that after participating in the stress management programme, the sample population had lower levels of stress as measured by sources of stress and symptom of stress. After the programme sources of stress level dropped to a high average stress score ($\bar{x}$) of 60 scores, and symptoms of stress dropped to a high average stress score ($\bar{x}$) of 55. However, no change in the susceptibility to stress levels was found before and after joining by the programme (Table 2).

Table 1 General information of the sample population

Gender	2 males (40%)
	3 females (60%)
Age	Average of 21 years old (Min. 19; Max. 22)
Faculty	Faculty of Science and Technology: 0 people (0%). Faculty of Science and Health: 4 students (80%). Faculty of Humanities and Social Sciences: 1 student (20%).
Class year	1st year: 1 student (20%) 2nd year: 1 student (20%) 4th year: 3 students (60%)
Average revenue per month	≤ THB 5,000: 1 student (20%) THB 5,001 - THB 15,000: 4 students (80%)
Parents' status	Parents live together: 4 students (80%) Parents have separated: 1 student (20%)

Table 2 The average stress of the sample population before and after the stress management programme

Instrument	Stress Level	Pretest (n=5)		Post-test (n=5)		t	p-value
		$\bar{x}$	S.D.	$\bar{x}$	S.D.		
The SPST-20 test	Overall stress level	66.00	12.410	52.40	12.973	2.023	0.043
The SPST-60 test	The susceptibility to stress level	26.80	1.643	25.00	4.528	1.633	0.102
	Sources of stress	80.60	4.561	60.60	5.727	2.060	0.039
	Symptoms of stress	79.00	18.480	55.00	11.136	-2.032	0.042

*p < 0.05

Results presented in table 2 revealed the overall comparison of the stress level, the susceptibility to the stress level, the sources of stress, and the symptoms of stress. It was found that after joining the stress management programme, the sample population had a low-stress level with a statistical significance level ($p \geq 0.05$). The stress level from the sources of stress and the symptoms of stress after joining the programme was found significantly lower ($p \geq 0.05$) after joining the programme.

Overall, from the analysis, it was found that the students had differences in stress levels before and after participating in the stress management programme, indicating that the stress management programme was able to help the students to manage their stress. Using the group process to create a stress management programme helped group members learn better stress-management skills and methods as the sample populations had similar problems with the academic stress of university students (Yooiam et al. 2017). In addition, physically this programme included stress relaxation and stress management skills, which enabled the sample populations to comprehensively manage their stress (Singhchada et al. 2016; Hopper et al. 2019) emotionally this program increased the capacity of managing their emotions (Hamasaki 2020). Further, mindfulness as a base can reduce stress, help in the improving person's physical and mental health and contribute to the reduction of mental and physical stress symptoms (Grossman et al. 2004; Carmody et al. 2008; Sedaghatb et al. 2011; Regehr et al. 2013; Chiangkoontod 2018; Lia et al. 2020). Cognitive behavioral therapy can help to reduce student stress and adjust their stress response (Brown and Gerbarg 2005; Mennuti et al. 2006; Regehr et al. 2013; Stromberg et al. 2015; Jafar et al. 2016; Ma et al. 2017). When the sample populations were aware of the stress-induced changes, they were able to choose appropriate stress management methods that resulted in a decrease in their overall stress level. Finally, the group process helped in learning the skills and making strategies for managing stress. Moreover, this used the psycho-education group of cognitive-behavioral principles based on the appropriateness and specifically with stress for the stress management skills training. This encouraged the sample population to create their assessment in managing their stress.

Conclusion and Future Research

In general, the present study was focused on the results of the stress management programme for the students of Chiang Mai University. Results of the study suggested that this programme enables the respondents to manage their stress through various stress relaxation techniques and stress management skills. Hence, this stress management programme was found successful in managing the stress of Chiang Mai University students.

This study is a pilot study; therefore, the number of respondents kept less and the results of this study cannot be applied to larger populations, but these results provide the possibility of applying stress management programs to larger samples. Additionally, this research was designed as a one-group form, which had an evaluation of the results related to the before and after the respondents joined the programme. Hence, future studies could design two or more groups for research.

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Conflicts of Interest

The authors declare no conflict of interest.

References

Brown, R.P., & Gerbarg, P.L. (2005). Sudarshan Kriya Yogic breathing in the treatment of stress, anxiety, and depression: Part II-clinical applications and guidelines. *The Journal of Alternative and Complementary Medicine, 11*(4), 711-717.

Carmody, J., Reed, G., Kristeller, J., & Merriam, P. (2008). Mindfulness, spirituality, and health-related symptoms. *Journal of Psychosomatic Research, 64(4), 393-403.*

Chiangkoontod, S. (2018). The development of the stress management program according to the Buddhist Psychology for

students in Faculty of Nursing, Siam University. Unpublished PhD thesis submitted to the Maha Chulalongkorn Rajavidyalaya University, Bangkok.

Chidduan Y. (2013). The result of using the program to reduce the stress of Children and the youth of the children and the youth center of observation and protection, Nakhon Pathom Province. *Radio Journal*, *24*(4), 65-15.

Cole, M.B. (2018). *Group dynamics in occupational therapy: The theoretical basis and practice application of group intervention* (5thed.). USA: SLACK Incorporated.

Davis, M.T., Holmes, S.E., Pietrzak, R.H., & Esterlis,I. (2017). Neurobiology of chronic stress-related psychiatric disorders: Evidence from molecular imaging studies. *Chronic Stress*, *1*(1), 1-21.

Dhyani, D., Sen, S., & Raghumahanti, R. **(**2015**)**. Effect of progressive muscular relaxation on stress and disability in subjects with chronic low back pain. *IOSR Journal of Nursing and Health Science*, *4*(1), 40-45.

Grossman, P., Niemann, L., Schmidt, S., & Walach, H. (2004). Mindfulness based stress reduction and health benefits: A meta-analysis. *Journal of Psychosomatic Research*, *57*(1), 35-43.

Hamasaki, H. **(**2020**)**. Effects of diaphragmatic breathing on health: A narrative review. *Medicines*, *7*(65), 1-19.

Hopper, S.I., Murray, S.L., Ferrara, L.R., & Singleton, J.K. (2019). Effectiveness of diaphragmatic breathing for reducing physiological and psychological stress in adults: A quantitative systematic review. *Joanna Briggs Institute Database of Systematic Reviews & Implementation Reports*, *17*(9), 1855-1876.

Jafar, H.M., Salabifard, S., Mousavi, S.M., & Sobhani, Z. (2016). The effectiveness of group training of CBT-based stress management on anxiety, psychological hardiness and general self-efficacy among university students. *Global Journal of Health Science*,*8*(6), 47-54.

La, P., & John, J.E. (2002). The impact of abbreviated progressive muscle relaxation on salivary cortisol. *Biological Psychology Journal*, *60*(1), 1-16.

Leungprawat, N. (2004). The result of using the method of managing with the stress by the meditation, Four Noble Truths and the tensing and the relaxing of muscles training for managing with the stress of Srinakharinwirot University students. Unpublished M.Sc. dissertation submitted to the Srinakharinwirot University, Bangkok.

Lia, Y., Suna, W., Sunb, X., Suna, J. et al. **(**2020**)**. Effects of mindfulness meditation on anxiety, depression, stress, and mindfulness in nursing students: A meta-analysis and trial sequential analysis of randomized controlled trials. *Frontiers of Nursing*,*7*(1), 59-69.

Ma, X., Yue, Z., Gong, Z., Zhang, H., et al. (2017**).** The effect of diaphragmatic breathing on attention, negative affect and stress in healthy adults. *Frontier in Psycholo*gy, *8*(874), 1-12.

Mahatnirunkul, W., Poomphisalchai, W., & Tapunya, P. (1997). *The research report in the topic of the creation of the Suanprung Stress Test*. Chiang Mai: Suanprung Hospital, Chiang Mai Province.

Mennuti, B.R., Freeman, A., & Christner, W.R. (2006). *Cognitive behavioral intervention in educational settings, a handbook for practice*. New York: Taylor & Francis Group

Merakou, K., Tsoukas, K., Stavrinos, G., Amanaki, E., et al. (2019). The effect of progressive muscle relaxation on emotion competence: depression, anxiety, stress, sense of coherence, health-related quality of life, and well-being of unemployed people in Greece: An intervention study. *Explore*, *15*(1), 38-46.

Ministry of Public Health (2020). Department of Mental Health revealed that Thai adolescents consulted the mental health hotline 1323 in 2019, found the most problem is the stress problem and suggest the stress management techniques Retrieved from https://www.dmh.go.th/news-dmh/view.asp?id=30188

Portoghese, I., Galletta, M., Porru, F., Burdorf, A., et al. (2019). Stress among university students: factorial structure and measurement invariance of the Italian version of the Effort-Reward Imbalance student questionnaire. *BMC Psychology*, *7*(68), 1-7.

Reddy, K.J., Menon, K.R., & Thattil, A. (2018). Academic Stress and its Sources among University Students. *Biomedical & Pharmacology Journal*, *11*(1), 531-537.

Regehr, C., Glancy, D., & Pitts, A. (2013). Interventions to reduce stress in university students: A review and meta-analysis. *Journal of Affective Disorders*, *148*(1), 1-11.

Sedaghatb, M., Mohammadia, R., Alizadeha, K., & Imania, A.H. (2011**).** The effect of mindfulness-based stress reduction on mindfulness, stress level, psychological and emotional well-being in Iranian sample. *Procedia Social and Behavioral Science*, *30*(11), 929-934.

Silveira, E.A., Batista, K.M., Grazziano, E.S., Bringuete, M.E.O., & Lima, E.F.A. (2020) Effect of progressive muscle relaxation on stress and workplace well-being of hospital nurses. *Enfermería Global*, *58*(1), 486-493.

Singhchada, A., Rojphisalkij, K., & Nokdee, S. (2016). The result of the stress management program on the stress of elderly persons. *Academic journal of Huachiew Chalermprakiet University*, *19*(38), 49-60.

Stromberg, S.E., Russell, M.E., & Carlson, C.R. (2015). Diaphragmatic breathing and its effectiveness for the management of motion sickness. *Aerospace Medicine and Human Performance*, *86*(5), 452-457.

Yooiam, S., Srisawad, P., Sooknaisit, A., & Kullanapadol, P.(2017). The development of the stress management program with the giving of the advice in the integrated form together with the alternative medicine. *Journal of the Police Nurse*, *9*(2), 139-152.

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Studies on NF-κB Docking with Common Bioactive Compounds in *Punica granatum* peel and *Vitis vinifera* Seeds

Ashok Kumar Krishna Kumar[1*], Vijayalakshmi Krishnamurthi[2], Saruniyadevi Moorthy[3], Jayanthi Malaiyandi[1]

[1]Department of Biotechnology, School of Life Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, 600 117, Chennai, Tamil Nadu, India
[2]Department of Biochemistry, Bharathi Womens College (Autonomous), Chennai 600 108, Tamil Nadu, India
[3]MTA Infotech, Pratap Nagar Colony, Taktatpur, Varanasi, India

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Palmitic acid

ABSTRACT

Plant-based products have long been utilized as traditional remedies throughout the world. Higher plants serve as a "reservoir" of phytochemicals known as bioactive compounds, which are used as valuable medicines to fight a variety of diseases across the world. The materials that are considered waste in plants possess bioactive components with potential medicinal properties due to the presence of important secondary metabolites known as phytochemicals. In this study, the interaction of phytochemicals that are present in both *Punica granatum* peel and *Viti vinifera* seeds was analyzed on protein NF-κB. Compounds 2,3- dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), α-tocopherol-β-D-mannoside, gamma-sitosterol, glycerine, guanidine, pyrogallol, palmitic acid, and ethyl palmitate were the eight phytoconstituents which are present in both the selected plant materials and further investigated for *in-silico* analysis. The 3D protein structure of NF-κB was retrieved from the protein data bank. The structures of bioactive compounds were obtained from Chemspider and drawn using Chemsketch software. This study clearly shows that α-tocopherol-β.-D-mannoside interacts with target protein NF-κB with an energy level of -10.88 kcal/mol (2 hydrogen bonds). The interaction of α-tocopherol-β-D-mannoside with NF-κB may play a major role in anti-oxidant and anti-cancer potential and provide chemopreventive property for both *P. granatum* peel and *V. vinifera* seeds.

* Corresponding author
E-mail: bioashok2002@gmail.com (Ashok Kumar Krishna Kumar)

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1 Introduction

Plants have been used for therapeutic purposes long before the early civilization, and these are the basis of much contemporary medicine. Many traditional medications are derived from plants. The fruit of the pomegranate (*P. granatum*) is a rich source of bioactive chemicals with numerous medicinal values. Pomegranate extracts have also been shown to lower blood fat levels and have potent anticancerous, antiviral, and anti-inflammatory properties (Li et al. 2006; Hossin 2009; Lin et al. 2013; Bassiri-Jahromi 2018). Pomegranate polyphenols may enhance the effectiveness of cancer therapy by protecting normal cells from cancer-related toxicity (Mukherjee et al. 2021). It is also utilized in various herbal therapies to treat ailments, including flu and upper respiratory infections. Further, fruits of pomegranate are considered a dietary medicine due to its high nutritional content, health advantages, and antioxidant components. The peels of pomegranate fruits are one of the most common by-products of pomegranate food processing. All the waste components of the pomegranate fruit, such as the peel and seeds, may be processed into value-added products with industrial, medical, and cosmetic applications (Dhumal et al. 2014).

Grapes (*V. vinifera*) are also one of the world's most valuable traditional fruits (Zhu et al. 2015). Many kinds of research have shown that grapes may be associated with illness prevention and health promotion activities, which has piqued people's curiosity in recent years. Grape seeds are becoming more popular as a source of functional food components, including natural antioxidants and nutritional supplements (Girard and Mazza 1998; Ferrer-Gallego et al. 2010). The effects of *V. vinifera* stem extracts on cancer cells resulted in a decrease in cancer cell growth, death by apoptosis, and a decrease in the antioxidant enzyme TrxR1, which increases cellular levels of ROS capable of inhibiting NF-kB binding to the nucleus and causing proteasome upregulation (Quero et al. 2021).

Nuclear factor-B (NF-κB) is a family of inducible transcription factors that controls several genes involved in immunological and inflammatory response pathways (Oeckinghaus and Ghosh 2009). Akt/PKB stimulates the NF-κB survival pathway by phosphorylation of IκB kinase α (IKK α), and it suppresses p53 pro-apoptotic signaling by phosphorylation of the oncogene *mdm2* and activated the inhibition of p53 (Mayo and Donner 2002). In the transformation process, both NF-κB and mdm2 are activated inappropriately or over-expressed (Orlowski and Baldwin 2002; Chene 2003). The signaling pathways are less controlled during carcinogenesis (Zhang et al. 2017; Sun et al. 2017). In cancer, abnormal regulation of cell-signal-transduction pathways plays a crucial role and obstruction or anomalies in signaling pathways which can lead to excessive cell proliferation, angiogenesis, apoptotic resistance, invasion, and metastasis all of which can lead to cancer development and progression (Chen et al. 2006).

NF-κB is a crucial signaling pathway involved in the genesis and development of cancer. The NF-κB pathway is involved in both inflammatory and programmed cell death processes, and, predictably, it is also involved in necroptosis (Verzella et al. 2020). NFκB regulates tumor cell proliferation, survival, and angiogenesis by modulating the expression of target genes such as TNFA, IL6, BCLXL, BCL2, BCLXS, XIAP, and VEGF (Baud and Karin 2009). NF-kB can be activated by cytokines (TNF-, IL-1), growth factors, bacterial and viral products (lipopolysaccharide (LPS), dsRNA, UV, and ionizing radiation, reactive oxygen species (ROS), DNA damage, and oncogenic stress from inside the cells. Exploring the naturopathic formulations may play an important role in identifying new NF-κB inhibitors considering the multi-target effects of phytochemicals, on various components of the NF-κB transduction pathway (Chauhan et al. 2021).

The advent of immune-informatics has made it feasible to design novel compounds *"in-silico"* and predict their functionality. This technique aids in the selection of better compounds for *in-vitro* or *in-vivo* testing. Using an immune-informatics approach, many research groups have created a variety of *in-silico* drugs (Kaliamurthi et al. 2018; Kar and Srivastava 2018).

Inflammation, cancer, and other human disorders are linked to the hyperactivation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) in the cellular system (Cheemanapalli et al. 2019) but, the *in-silico* docking studies of NF-κB and its interaction with bioactive compounds are yet to be studied in detail. Therefore, the purpose of this study was to analyze the interaction of NF-κB protein against bioactive compounds present in *P. granatum* peel and *V. vinifera* seeds using molecular docking. This study may pave the way to find whether any anti-cancer, anti-oxidant, and anti-inflammatory agents present in selected plant materials interacts with the NF-κB protein and play a role in its regulation. The inhibition of NF-κB may provide insights for future treatment strategies

2 Materials and Methods

2.1 Database

2.1.1 Protein Data Bank (Pdb)

The 3D structure of the target protein NF-κB of Homo sapiens was elucidated by X-ray diffraction at a resolution of 2.60Å (1SVC) (Space fill Model - Figure 1) (https://www.rcsb.org/structure/1SVC) a repository for the 3-D structural data of proteins and nucleic acids. The NF-κB molecule possesses two chains (Chain D and Chain P), with Chain D containing 76 polydeoxyribonucleotides and Chain P comprising 365 amino acids.

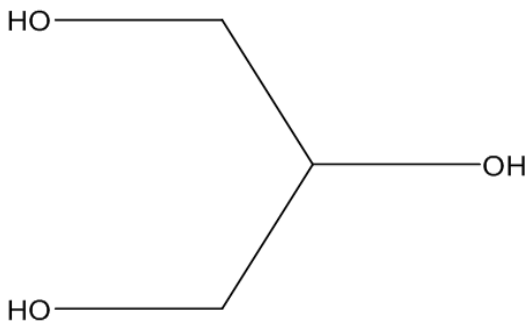

Figure 1 Structure of glycerine

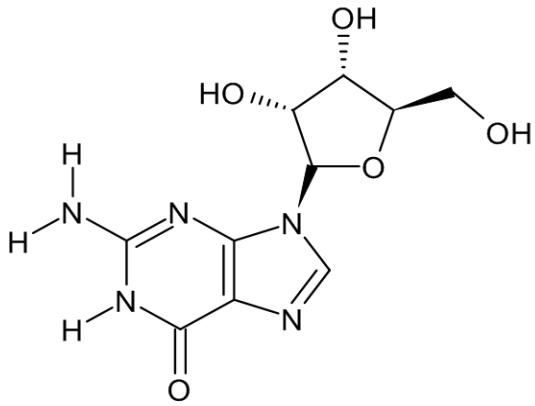

Figure 2 Structure of guanosine

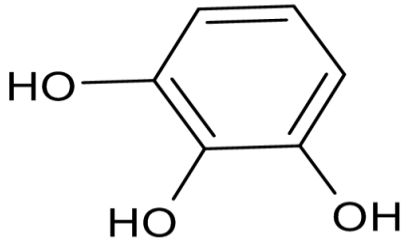

Figure 3 Structure of pyrogallol

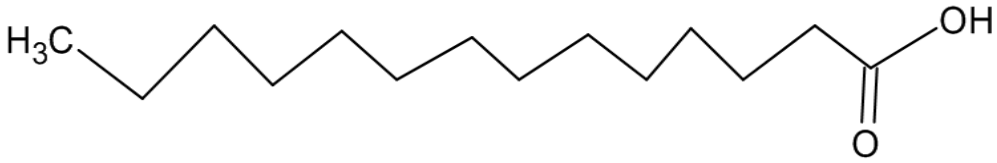

Figure 4 Structure of palmitic acid

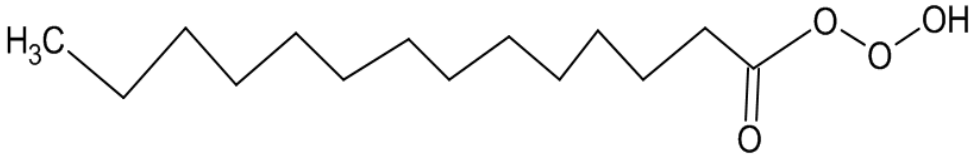

Figure 5 Structure of ethyl palmitate

2.1.2 Chemspider

The structures of bioactive compounds 2,3- dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one, α-tocopherol- β.-D-mannoside, and gamma-Sitosterol used for this study were obtained from ChemSpider. (http://www.chemspider.com/).

2.2 Tools Used for the Studies

2.2.1 Chemsketch

Chemsketch is a web-based drawing tool used in this study to design the structures for the five chemicals found in both the selected extracts: glycerine (Figure 1), guanosine (Figure 2), pyrogallol (Figure 3), palmitic acid (Figure 4), and ethyl palmitate (Figure 5). The structure of the selected ligands is provided in the following diagrams.

2.2.2 Argus Lab

Argus Lab is a molecular modeling, graphics, and drug design application that is available online and provides a rapid and reliable way of binding site optimization, which implies that the program can automatically discover binding sites and accelerate the docking process (Tangyuenyongwatana and Jongkon 2016). Many researchers utilize ArgusLab to execute their molecular

docking since it eliminates the requirement for blind docking, which takes a lot of time to calculate and often results in an erroneous binding site (Naz et al. 2009; Oda and Takahashi 2009). In this study, Argus Lab 4.0 is used for docking of the selected target protein NF-kB with eight selected ligands.

2.2.3 Pymol

The interaction between selected ligands and the target protein is visualized using PyMOL2.1 (Yuan et al. 2017), an open-source molecular visualization toolkit. PyMOL is used to visualize the docking in this study. PyMOL display Protein Residue Networks, results of numerous analyses, indicating the size and location of the binding site which may be modified interactively (Seeliger and de Groot 2010; Sladek et al. 2021).

2.3 Molecular Docking

The 3D structure of the target (p53), was obtained from PDB. Ligand structure was determined using Chemspider or Chemsketch. The interactions of target proteins with various ligands were analyzed using Argus Lab. Pymol 2.1 (2018) software was used to conduct the docking investigation and to predict the docking of a specific ligand with target proteins. The interactions with the lowest energy and the number of atoms involved in docking the ligand with a target protein are criteria to select the best docking. The docking technique involves the extrapolation of ligand/inhibitor conformation and orientation inside a specific binding site or active site. The promising posture with greater binding energy, ligand efficiency, and intermolecular Hydrogen-bonds was retained for extensive intermolecular interaction study based on docking simulations. Hydrogen bonds are required for molecular recognition as well as a protein's and its complex's overall stability. The protein-inhibitor complex system's intermolecular hydrogen bonds were investigated (Mukund et al. 2019).

3 Results

The eight compounds commonly present in both the fruit materials were selected for docking studies. The results of docking are displayed in Table 1, showing the details of the interaction between eight selected ligands from the two plant materials and the target protein NF-κB. (Figure 6)

The hydrogen bond formation between the amino acid residues of the target protein and the atoms of ligands selected for this study are tabulated in table 1. The table also provides details on the number of hydrogen bonds formed and the hydrogen bond distance between the target protein and the ligands. The energy value for the formation of the hydrogen bond is provided in table 1 which is a criterion to assess the better docking ligand with the target proteins. The interaction of the ligands with the target proteins visualized through Pymol was presented in Figures 7 – 11. In this *in-silico* study, the following ligands pyrogallol, guanosine, α-tocopherol-beta.-D-mannoside, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one, and glycerin interaction with NF-κB via hydrogen bonds. Other ligands selected for the docking didn't show any interaction with NF-κB. Out of the above five ligands showing interaction, α -tocopherol-beta-D-mannoside forms interaction with an energy value of -10.88 kcal/mol with two hydrogen bonds, and pyrogallol forms two hydrogen bonds with an energy value of-7.07 kcal/mol.

Table 1 Results of NF-κB docking with the selected ligands

S.No	Ligand name	Protein residue atom	Ligand atom	Hydrogen bond distance (^{0}A)	No. of Hydrogen bonds	Energy value (Kcal/ mol)
1	alpha.-tocopherol-beta.-D-mannoside	– H – N Gly 294 B – H – N Gly 294 B	O O	2.91 2.97	2	-10.88
2	Pyrogallol	Gly 296 N – H – Gly 296 O-H	O H	2.6 2.6	2	-7.07
3	Guanosine	Thr 313 OG1 – H Thr 313 OG1 – H Thr 313 OG1 – H Thr 316 N – H Thr 316 N – H	H N O O O	2.8 3.0 2.9 3.0 2.8	5	-6.40
4	2,3- dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one	Arg 164 N – H Tyr 166 O – H Lys 95 N – H Thr 125 OG1 – H	O O O O	2.76 2.22 2.80 2.52	4	-6.2
5	Glycerin	Thr 313 O – H	H	1.8	1	-5.65
6	gamma.-Sitosterol	No Interaction	No Interaction	Nil	Nil	-10.73
7	Palmitic acid	No Interaction	No Interaction	Nil	Nil	-8.56
8	Ethyl palmitate	No Interaction	No Interaction	Nil	Nil	-7.96

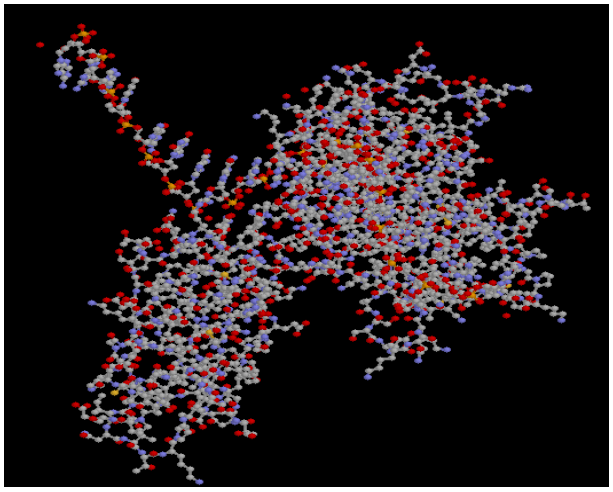

Figure 6 - 3D structure of the NF-κB protein (1SVC) Homo sapiens (2.60Å)

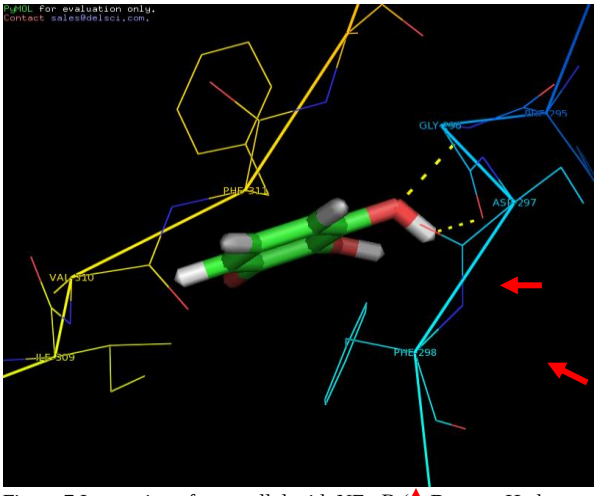

Figure 7 Interaction of pyrogallol with NF-κB (▲ Denotes Hydrogen bond)

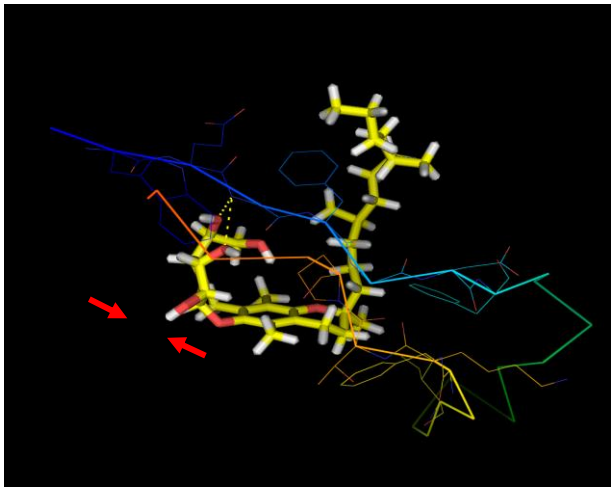

Figure 8 Interaction of α-tocopherol-beta.-D-mannoside with NF-κB (▲ Denotes Hydrogen bond)

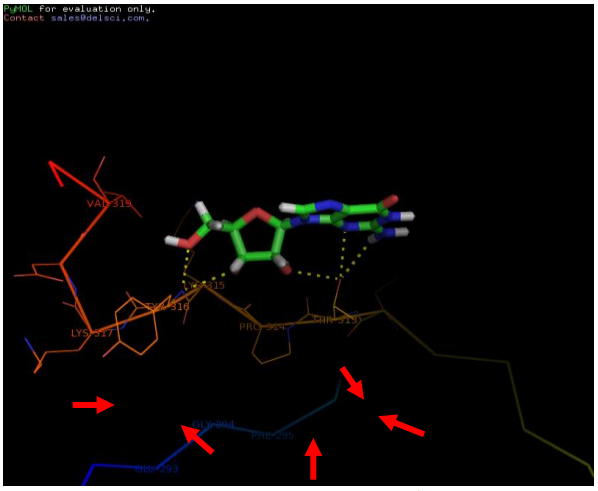

Figure 9 Interaction of guanosine with NF-κB (▲ Denotes Hydrogen bond)

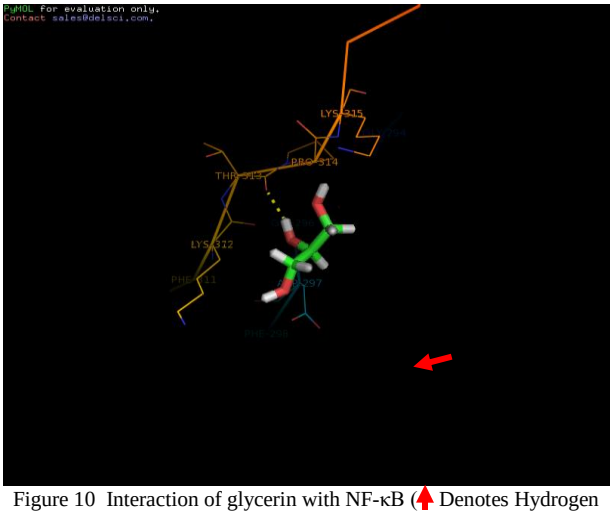

Figure 10 Interaction of glycerin with NF-κB (▲ Denotes Hydrogen bond)

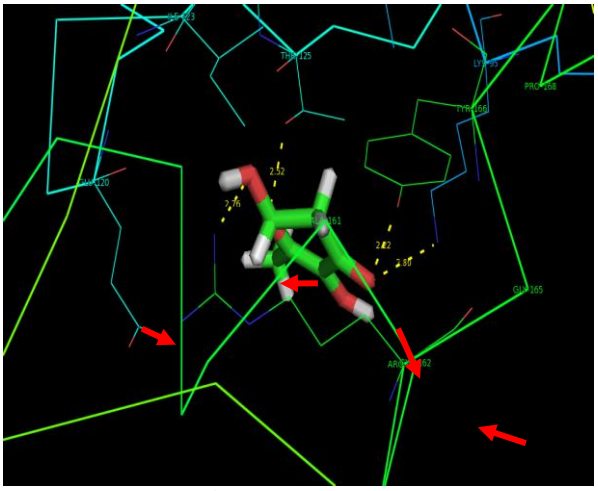

Figure 11 Interaction of 2, 3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one with NF-κB (▲ Denotes Hydrogen bond)

The guanosine forms five hydrogen bonds with an energy value of -6.40 kcal/mol and 2,3- dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one interacts with an energy value of -6.2 kcal/mol with four hydrogen bonds. The Glycerin interacts with an energy value of -5.65 kcal/mol with one hydrogen bond. The docking study clearly shows that α-Tocopherol-beta.-D-mannoside and pyrogallol forms interaction with the NF-κB target proteins at the lower energy level.

4 Discussion

Several natural chemopreventive compounds, such as curcumin, resveratrol, and lycopene are effective inhibitors of NF-κB (Aggarwal and Shishir 2006). The transcription factor NF-κB is a newly discovered target molecule that helps develop anti-tumor, anti-inflammatory, and pro-apoptotic medicines (Piccagli et al. 2008). Carcinogens, tumor promoters, and inflammatory drugs all activate NF-κB. NF-κB regulates apoptosis suppression, and chemopreventive drugs might inhibit NF-κB activity (Aggarwal and Shishodia 2004). *Withania somnifera's* with anolides beneficial chemicals interact with the NF-κB protein to modulate its activity and may be employed in medication development (Nithya et al. 2009). In comparison to pulp extract, peel extract contains more total phenolics, flavonoids, and proanthocyanidins. Peel extract's high phenolic content may account for its potent antioxidant activities (Li et al. 2006).

Pyrogallol and α-tocopherol-beta-D-mannoside found in the EPGP and EVVS showed interaction with NF-kB protein in this study. The α-tocopherol-beta-D-mannoside has anti-oxidant, anti-mutagenic, and anti-proliferative properties (Duke's Phytochemical and Ethnobotanical Databases database 2016). Pyrogallol shows anti-bacterial, anti-oxidant, and anti-tumor activities. The pomegranate peel and grape seeds possess chemopreventive potential (Ashok Kumar and Vijayalakshmi 2015) and, the interaction of these two ligands with the chosen target protein may provide the specific property to these plant materials. In the current *in-silico* study, five phytochemicals of the selected plant extracts interact with the target protein via hydrogen bonds. The α -tocopherol-beta -D-mannoside and Pyrogllol show the highest docking with NF-κB protein with the energy value of -10.88 kcal/mol and -7.07 kcal/mol. The docking studies of resveratrol, a naturally occurring antioxidant with NF-κB, showed strong interaction with the NF-κB and might prevent its binding ability with DNA (Banagalapalli et al. 2013). This result is similar to our study as the antioxidants pyrogallol and α-tocopherol-beta-D-mannoside present in selected plant materials show interaction with NF-κB and may inhibit its activity.

Similarly, the epigallocatechin-gallate (EGCG), which possesses protective action against inflammatory colitis showed a high binding profile against NF-κB in docking studies which might play a role in its anti-inflammatory activity (Varthya et al. 2020). The genistein which, possesses anti-cancer potential against NF-κB activated breast cancer, showed a strong binding affinity with NF-κB with four hydrogen bonds similar to our results. This interaction might play an important role in its anti-cancer potential against breast cancer (Mukund et al. 2019). The α.-tocopherol-beta-D-mannoside interacts with amino acid glycine at 294 positions of NF-κB via two hydrogen bonds with hydrogen bond lengths of 2.91^{0}A and 2.97^{0}A**,** respectively. The pyrogallol interacts with NF-κB at amino acid glycine in position 296 via two hydrogen bonds with the same bond length of 2.6 ^{0}A.

Conclusion

This *in-silico* docking study was carried out to show the interaction of bioactive constituents present in *P. granatum* peel and *V. vinifera* seeds with the selected target protein NF-κB. The five phytochemicals in selected plant materials showed a binding affinity with the NF-κB via Hydrogen bonds. The α-tocopherol-beta-D-mannoside and pyrogallol show higher interaction with NF-κB than other selected ligands. These two bioactive constituents with antioxidant and anti-cancer properties might play a role in providing anti-cancer potential for the selected plant materials by inhibiting NF-κB activity. This study provides insights for *in-vitro*, *in-vivo*, and simulation studies, which might pave the way for future personalized treatment strategies against cancer and inflammation activated by NF-κB and the signaling pathways involved in the inhibition of NF-κB.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Aggarwal, B. B., & Shishir, S. (2006). Molecular target of dietary agents for prevention and therapy of cancers. *Biochemical Pharmacology, 71*, 1397-1421.

Aggarwal, B. B., & Shishodia, S. (2004). Suppression of the nuclear factor kappa B activation pathway by spice derived phytochemicals: reasoning for seasoning. *Annals of the New York Academy of Sciences, 1030*, 434 - 441.

Ashok Kumar, K., & Vijayalakshmi, K. (2015). Protective Effect of *Punica granatum* Peel and *Vitis vinifera* Seeds on DEN-Induced Oxidative Stress and Hepatocellular Damage in Rats. *Applied Biochemistry and Biotechnology, 175*, 410-420.

Banaganapalli, B., Mulakayala, C. D. G., Gowsia, D., Mulakayala, N., et al. (2013). Synthesis and biological activity of new resveratrol derivative and molecular docking: dynamics studies on NFkB. *Applied Biochemistry and Biotechnology, 171*, 1639-1657.

Bassiri-Jahromi, S. (2018) *Punica granatum* (Pomegranate) activity in health promotion and cancer prevention. *Oncology Review*, *12*, 345-349.

Baud, V., & Karin, M. (2009). Is NF-κB a good target for cancer therapy? Hopes and pit falls. *Nature reviews drug discovery*, *8*,33–40.

Chauhan, A., Islam, A., Prakash, H., & Singh, S. (2021). Phytochemicals targeting NF-κB signaling: Potential anti-cancer interventions, *Journal of Pharmaceutical Analysis*, 2021, https://doi.org/10.1016/j.jpha.2021.07.002.

Cheemanapalli, S., Chinthakunta, N., & Shaikh, N. M. (2019) Comparative binding studies of curcumin and tangeretin on up-stream elements of NF-kB cascade: a combined molecular docking approach. *Network Modeling Analysis in Health Informatics and Bioinformatics*, *8***,** 15-19.

Chen, H., Huang, Q., Dong, J., & Lan, Q. (2006). Cancer initiating cell theory: popularity and controversies. *AiZheng*, *25*, 779–784.

Chene, P. (2003). Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. *Nature review cancer*, *3*, 102-109.

Dhumal, S. S., Karale, A. R., Jadhav, S. B., & Kad, V. P. (2014). Recent advances and the developments in the pomegranate processing and utilization: A review. *Journal of Agriculture Crop Science*, *1*, 1-17.

Duke's Phytochemical and Ethnobotanical Databases database, 2016.

Ferrer-Gallego, R., García-Marino, M., Hernández-Hierro, J. M., Rivas-Gonzalo, J. C., et al. (2010). Statistical correlation between flavanolic composition, colour and sensorial parameters in grape seed during ripening. *Analytica Chimica Acta*, *660*, 22-28.

Girard, B., & Mazza, G. (1998) Functional grape and citrus products. In Mazza, *Functional Foods* (pp.139-154).Technomic Publishing, PA, USA.

Hossin, F. L. A. (2009). Effect of pomegranate (*Punica granatum*) peels and it's extract on obese hypercholesterolemic rats. *Pakisthan Journal of Nutrition*, *8*, 1251–1257.

Kaliamurthi, S., Selvaraj, G., & Junaid, M. (2018). Cancer Immunoinformatics: a promising era in the development of peptide vaccines for human papillomavirus-induced cervical cancer. *Current Pharmaceutical Design*, *24*, 3791–3817.

Kar, P. P., & Srivastava, A. (2018). Immuno-informatics analysis to identify novel vaccine candidates and design of a multi-epitope based vaccine candidate against theileria parasites. *Frontiers in Immunology*, *9*, 2213-2218.

Li, Y., Guo, C., Yang, J., Wei, J., et al. (2006) Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chemistry*, *96*, 254-260.

Lin, L. T., Chen, T. Y., Lin, S. C., Chung, C. Y., et al. (2013). Broad-spectrum antiviral activity of chebulagic acid and punicalagin against viruses that use glycosaminoglycans for entry. *BMC Microbiology*, *13*, 187.

Mayo L. D. & Donner D. B., (2002). The PTEN, Mdm2, p53 tumor suppressor oncoprotein network. *Trends in Biochemical Sciences*, *27*, 462-467.

Mukherjee, S., Ghosh. S., Choudhury. S., Gupta, P., et al. (2021). Pomegranate Polyphenols Attenuate Inflammation and Hepatic Damage in Tumor-Bearing Mice: Crucial Role of NF-κB and the Nrf2/GSH Axis. *Journal of Nutritional Biochemistry*, *97*, 108812. doi: 10.1016/j.jnutbio.2021.108812.

Mukund, V., Behera, S. K., Alam, A., & Nagaraju, G. P. (2019). Molecular docking analysis of nuclear factor-κB and genistein interaction in the context of breast cancer. *Bioinformation*, *15*(1), 11-17.

Naz, A., Bano, K., Bano, F., Ghafoor, N. A., et al. (2009). Conformational analysis (geometry optimization) of nucleosidic antitumor antibiotic show domycin by Argus Lab 4 software. *Pakistan Journal of Pharmacy*, *22*,78-82.

Nithya, K., Shanthi, N., & Kalaiselvi, K. (2009). Molecular docking of Withanolides against the transcription factor Nuclear Factor kappa B (NFkB) Using Glide. *Advanced Biotechnology*, *9*, 23-27.

Oda, A., & Takahashi, O. (2009). Validation of ArgusLab efficiencies for binding free energy calculations. *Chem-Bio informatics Journal*, *9*, 52-61

Oeckinghaus, A., & Ghosh, S. (2009). The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harbor Perspectives in Biology*, *1*, 1-14

Orlowski, R. Z., & Baldwin, A. S. (2002). NF-kappaB as a therapeutic target in cancer. *Trends Molecular Medicine*, *8*, 385-389.

Piccagli, L., Fabbri, E., Birgatti, M., Bezzerri, V., et al. (2008). Docking of molecule identified bioactive medicinal plant extract into the p50 NF-kappa B transcription factor: Correlation with inhibition of NFkappa B/ DNA interactions and Inhibitory effects on IL- 8 gene expression. *BMC Structural Biology*, *8*, 1-13.

Quero. J., Moreno. N., Esparza. I., Osada. J., et al. (2021). Grape Stem Extracts with Potential Anticancer and Antioxidant Properties. *Antioxidants, 10,* 243. https:// doi.org/10.3390/antiox10020243

Seeliger, D., & de Groot, B. L. (2010). Ligand docking and binding site analysis with PyMOL and Autodock/Vina. *Journal of Computer-aided Molecular Design, 24*, 417-422.

Sladek, V., Yamamoto, Y., Harada, R., Shoji, M., et al. (2021). pyProGA—A PyMOL plugin for protein residue network analysis. *PLoS ONE, 16*, 1-17.

Sun, Y., Fan, X., Zhang, Q., Xu, G., et al. (2017). Cancer-associated fibroblasts secrete FGF-1 to promote ovarian proliferation, migration, and invasion through the activation of FGF-1/FGFR4 signaling. *Tumour Biology, 39*, 1-9.

Tangyuenyongwatana, P., & Jongkon, N. (2016). Molecular docking study of tyrosinase inhibitors using ArgusLab 4.0.1: A comparative study. *Thai Journal of Pharmaceutical Sciences, 401*, 1-53.

Varthya, S. B., Sarma, P., Bhatia, A., Shekhar, N., et al. (2020). Efficacy of green tea, its polyphenols and nanoformulation in experimental colitis and the role of non-canonical and canonical nuclear factor kappa beta (NF-kB) pathway: a preclinical in-vivo and in-silico exploratory study. *Journal of Biomolecular Structure Dynamics*, *39*, 5314-5326.

Verzella, D., Pescatore, A., Capece, D., Vecchiotti, D., *et al.* (2020). Life, death, and autophagy in cancer: NF-κB turns up everywhere. *Cell Death and Disease, 11***,** 210 https://doi.org/ 10.1038/s41419-020-2399-y.

Yuan, S., Chan, H. C. S., & Hu, Z. (2017). Using PyMOL as a platform for computational drug design. *WIREs Computational Molecular Science, 7*, e1298.

Zhang, W., Yin, G., & Dai, J. (2017). Chemoprevention by quercetin of oral squamous cell carcinoma by suppression of the NF-κB signaling pathway in DMBA-treated hamsters. *Anticancer Research, 37*, 4041–4049.

Zhu, F., Du, B., Zheng, L., & Li, J. (2015). Advance on the bioactivity and possible uses of dietary fiber from grape pomace. *Food Chemistry, 186*, 207-212.

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Preliminary assessment of *Polytrichum commune* extract as an antimicrobial soap ingredient

Chan Kai Rol[1], Tam Yew Joon[1,2*], Chong Mee Yoke[1], Tan Joo Shun[3], Sahar Abbasiliasi[4], Wong Kok Kee[1], Ong Ghim Hock[1*]

[1]INTI International University, Persiaran Perdana BBN, Nilai, Negeri Sembilan, Malaysia
[2]Biogenes Technologies, Technology Incubation Centre, Unipark Suria Jalan Ikram-Uniten, Kajang, Selangor Darul Ehsan, Malaysia
[3]Bioprocess Technology, School of Industrial Technology, Universiti Sains Malaysia, Gelugor, Malaysia
[4]Halal Products Research Institute, Universiti Putra Malaysia, Selangor, Malaysia

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ABSTRACT

Mosses have long been used in traditional Chinese medicine due to the presence of secondary metabolites which have shown high biological activities. In particular, these secondary metabolites have demonstrated effective antibacterial activity against pathogenic microorganisms. In this study, the influence of different extraction solvents on the antibacterial activities of the *Polytrichum commune* was carried out using the disc diffusion method. Results showed that both 12.5 mg/mL of methanol moss extract and 6.25 mg/mL of ethanol moss extract were the most effective concentrations against *Bacillus cereus* and *Pseudomonas aeruginosa*. Additionally, the *P. commune* extracts were included as an added ingredient in soap bases to produce antibacterial soap prototypes where the effectiveness of the soaps containing the extracts in removing microorganisms from actual test individuals was carried out. Results of the thumb impression test of test individuals showed that the growth of microbial reduced after washing hands with the usage of both liquid and solid soap with the addition of *P. commune* extracts. Moreover, the antibacterial soaps performed better in eliminating microorganisms in comparison to control soaps without *P. commune* extracts. Taken together, *P. commune* extract could be a good candidate as a value-added ingredient utilized to produce antibacterial soaps due to its antibacterial properties.

* Corresponding author
E-mail: yjtam77@gmail.com (Tam Yew Joon);
ghimhock.ong@newinti.edu.my (Ong Ghim Hock)

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1 Introduction

Soap is an essential daily product that is mainly used for cleaning purposes, particularly for hand washing. Handwashing with soap and water is necessary for one's hygiene to avoid getting sick and spreading infections to others for generations (Yawson and Hesse 2013). However, washing hands with plain soap alone is often not enough. It has been shown that hand washing with plain soap resulted in almost triple the percentage of bacterial infectious diseases transmitted to food as compared with antibacterial soap (Sajed et al. 2014). In addition, the antibacterial activities of the soap can be enhanced by adding some natural active ingredients from botanicals to reduce the negative effect on human skin (Riaz et al. 2009).

Bryophytes are essential in pharmaceutical uses as they are the source of many biologically active compounds (Mishra et al. 2014; Hanif et al. 2014). Many moss species contain unique secondary metabolites such as terpenoids, alkaloids, flavonoids, phenols, and other aromatic compounds with therapeutic potential (Greeshma and Murugan 2018). These secondary metabolites have been reported to possess effective antibacterial and antifungal activities against various pathogenic microorganisms (Chauhan et al. 2014). Among the Bryophytes, *Polytrichum commune* (also known as common haircap, great golden maidenhair, great goldilocks, common haircap moss, or common hair moss) extract carries antimicrobial potential capabilities to inhibit the proliferation of pathogenic cells (Klavina et al. 2015). In addition, *P. commune* extract was demonstrated to have antioxidation potential that was strongly correlated with total phenolic contents found in the extract (Hanif et al. 2014). The antioxidants present in *P. Commune* were able to neutralize the reactive oxygen species and prevent oxidative damage to human cells and tissues, which in turn were able to help in the treatment of skin diseases (Addor 2017). There are a few reports on the antibacterial, cytotoxicity, and antimicrobial activities of solvent extract of *P. commune* grown in different parts of the world including Turkey (Klavina et al. 2015; Nikolajeva et al. 2012; Sevim et al. 2017).

Pseudomonas aeruginosa is an opportunistic pathogen that can colonize a healthy person, causing severe infections, especially in those who have weak immune systems or hospitalized patients (Nguyen et al. 2018). Additionally, *Bacillus cereus* can quickly spread to food products that cause food-borne diseases, emetic or diarrheal syndrome (Savini 2016). These bacteria strains are commonly found in the environment such as water, soil, plants, and animals (including human beings) (Savini 2016; Nguyen et al. 2018), serving as a continuous threat to human health. As such, eliminating these harmful bacteria through the use of antibacterial soap can reduce the chances of infections. The most crucial element in preventing nosocomial infections by preventing touch and fecal-oral transfer of pathogens is hand hygiene, which is frequently equated with handwashing (Boyce and Pittet 2002). An essential public health measure is hand washing and it has long been acknowledged as a practical, useful, and economical method of preventing infectious infections (Burton et al. 2011; Tao et al. 2013). In this research, the antimicrobial activity of *P. commune* extract with methanol and ethanol against *P. aeruginosa* and *B. cereus* was evaluated to assess the potential of methanol and ethanol extract of *P. commune* as an ingredient of antibacterial soap.

2 Materials and Methods

2.1 Plant Material

P. commune moss sample was brought from Terra Living Gallery & Farm House, Malaysia. Nutrient agar, nutrient broth, Petri dishes, 100% methanol, and 95% ethanol were bought from Sigma-Aldrich, USA.

2.2 Preparation of Plant Extract

Fresh leaves of *P.commune* were cleaned and air-dried under room temperature until a consistent weight was achieved and ground using a blender to obtain a fine homogenous powder. Thereafter, the extraction process was carried out by mixing 1 g of the dried *P. commune* powder with 100mL of 95% ethanol and 100% methanol, respectively. The mixed samples were then further incubated in a shaking incubator for 48 h at 150rpm at 37°C (Oyesiku and Caleb 2015). After the incubation period, the extracts were centrifuged (4,696 × g, 20 min, 4°C) and filtered using Whatman filter paper. The filtrates were concentrated over a hot plate stirrer at 60 °C until a concentration of 100 mg/mL was attained and stored at 4 °C for further use (Dulger et al. 2009; Sharma et al. 2013).

2.3 Preparation of Bacterial Standard Inoculum

B. cereus and *P. aeruginosa* were streaked on a fresh nutrient agar (NA) plate and incubated for 48 h at 37°C (Kazemian et al. 2015). At the end of the incubation period, single colonies of both the bacteria were picked from the respective plates and inoculated into the fresh nutrient broth (NB) then incubated for an additional 24 hrs, at 150 rpm and 37°C temperature in a shaking incubator. Subsequently, the broth cultures were centrifuged at 2,860 ×g, for 10min at 4 °C and the bacterial pellet was further rinsed thrice using 0.85% NaCl to remove the residual of NB and finally resuspended in 0.85% NaCl. The cell densities of the final suspensions were determined using a spectrophotometer at OD_{600nm} to obtain a reading of approximately OD of 0.5, which corresponds to $\times 10^8$ CFU/mL. The bacterial cell suspensions were used as standard inoculum for the anti-microbial activity assays (Modarresi et al. 2015).

2.4 Antibacterial Assay of P. commune Extracts

A 2-fold serial dilution method was carried out for both extracted solutions. The *P. Commune* extracts in both ethanol and methanol solutions were diluted in a range from 0 to 100mg/mL.

2.5 Disc Diffusion Assay

Six filter paper discs (about 6 mm in diameter) were soaked with *P. Commune* extracts of different diluted concentrations and the control solutions (95% ethanol and 100% methanol) for 15 min. The soaked discs with *P. Commune* extracts and a Ciprofloxacin disc (10 µg/mL) used as control were then placed on nutrient agar plates lawn with 30 µL of the standard inoculum bacteria (OD_{600nm} = 0.5) for *B. cereus* and *P. Aeruginosa*in triplicates for each of the bacteria. The plates were then incubated for 48 hrs at 37 °C (Balouiri et al. 2016). Followed by the measurement of the diameter of the clearing zones.

2.6 Preparation of Soap with the Plant Extracts

Liquid and solid soaps were prepared with added *P. commune* extracts. For liquid soap, the optimal concentration of *P. commune* extracts which depicts the highest antibacterial effect shown from the diffusion assays was chosen in making liquid soap by diluting the *P. commune* stock extract with a liquid soap base (Craftiviti, Selangor, Malaysia). A liquid soap base without any addition of *P. commune* extract was used as a control. For the solid soap, a solid soap base (Craftiviti, Selangor, Malaysia) was used by melting the solid soap base in a water bath at 70 °C before mixing with the *P. commune* extract stock to achieve the desired concentration. Thereafter, the mixture was poured into a silicon mold and placed at room temperature for 24 hrs to solidify. The solid soap base without any addition of *P. commune* extract was used as control.

2.7 Antimicrobial Assay of P. commune Extract Soaps

An antimicrobial assay was carried out with six test subjects. All the test subjects were requested to homogenize the microbes on both hands by rubbing them together before the test. For each test subject, nutrient agar plates were used to evaluate the soaps' effectiveness by which each plate was separated into four compartments with two compartments allocated for 'before washing' as control replicates while the remainder of the two compartments were then used for the washing with control soap (right hand) and washing with *P. commune* extract soaps (left hand). For each of the compartments, the subjects performed thumb impressions on the agar plate. Thereafter, the plates were incubated at 37 °C for 24 hrs (Wijetunge and Perera 2016). Subsequently, the amount of microbial growth obtained after incubation was recorded and differentiated using qualitative indicators of 0, +, ++, and +++ (Strigáč et al. 2018) as shown in Figure 1, to compare and evaluate the efficacy testing of handwashing and antimicrobial handwash.

2.8 Statistical Analysis

All the data collected from the antibacterial assay of moss extract was analyzed using a T-test with a 95% confidence level.

3 Results and Discussion

3.1 Disc Diffusion Assay

From the assay conducted, the results showed that the methanol and ethanol extracts of *P. commune* demonstrated clear zones of inhibition for *B. cereus* and *P. aeruginosa* for all the measured concentrations. On the other hand, there was no clear zone of growth inhibition observed for *B. cereus* and *P. aeruginosa* when control soap was used. Additionally, with *B. cereus*, methanol extraction of the *P. commune* showed the largest clear zone of inhibition with a diameter of 11.3 mm at a concentration of 12.5 mg/mL as compared to ethanol extraction, where the clear zone of inhibition demonstrated a diameter of 10.7 mm at a concentration of 6.25 mg/mL. Both methanol and ethanol extraction solutions were able to produce larger clear zones when compared to the control antibiotic, Ciprofloxacin, at 10 µg/mL (Figure 2) which has a clear zone diameter of 7.0 mm.

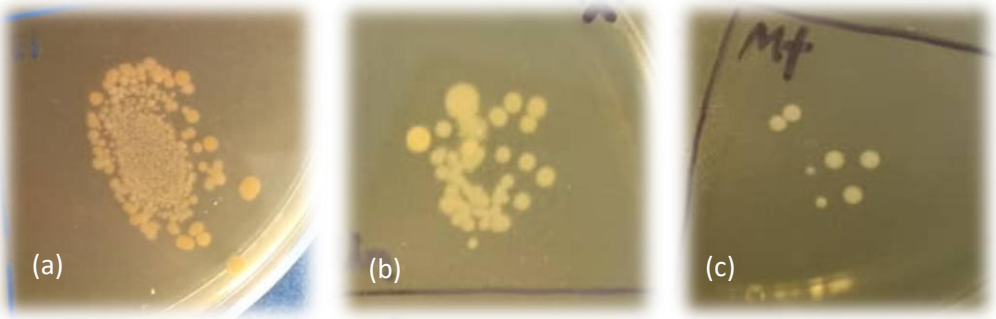

Figure 1 Microbial growth indicators representing their respective growth sizes as a form of qualitative measurement for the thumb impression test used as a form of an antimicrobial assay (a) High microbes growth was represented as + + +, (b) medium microbes growth was represented as + + while (c) low microbes growth was represented as +

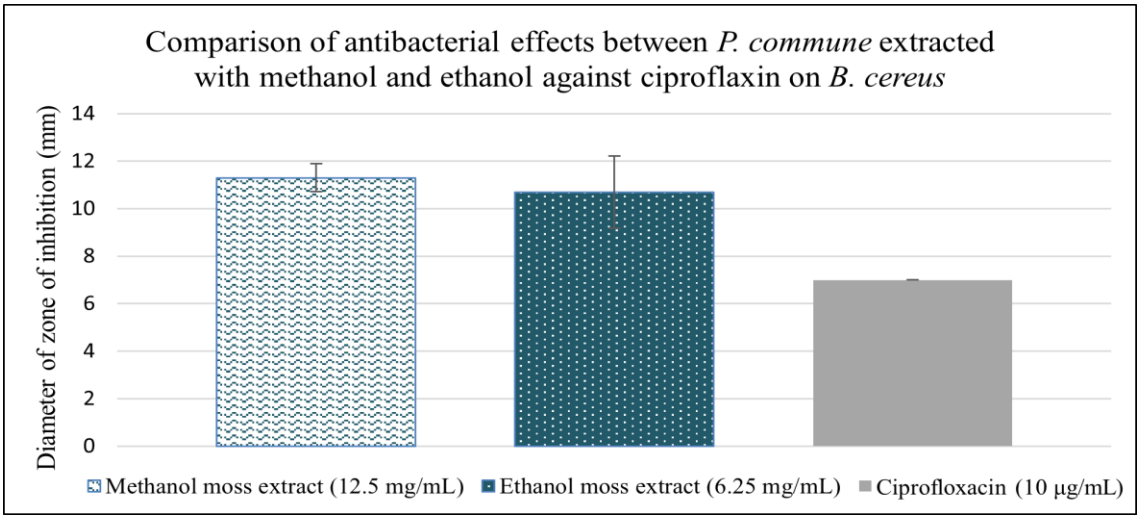

Figure 2 The comparison between the highest antibacterial effects of *P. commune* extracted with methanol at 12.5 mg/mL, *P. commune* extracted with ethanol at 6.25 mg/mL and ciprofloxacin at 10 µg/mL against *B. cereus* using disc diffusion assay.

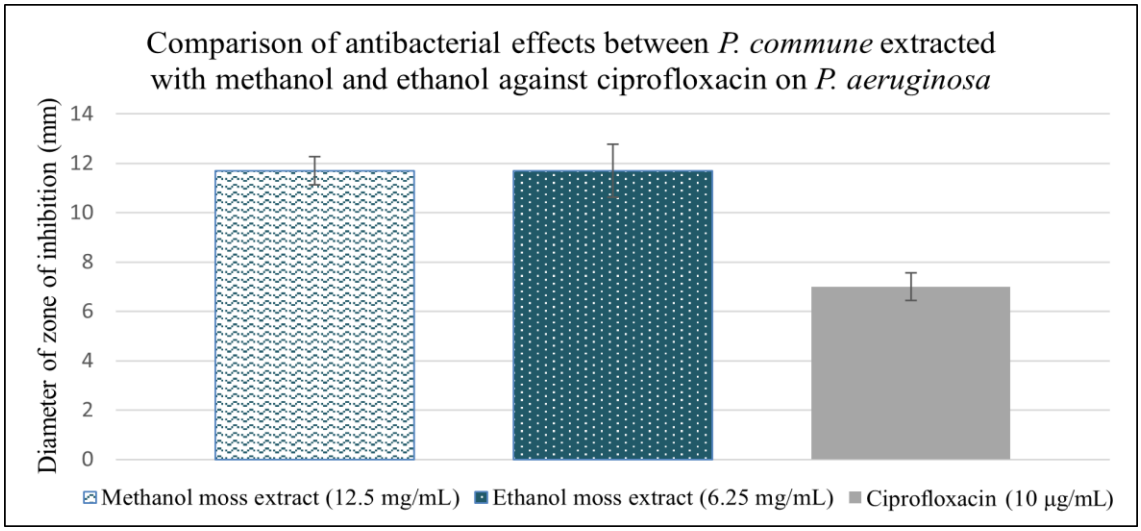

Figure 3 Comparison between the highest antibacterial effects obtained by *P. commune* extracted with methanol at 12.5 mg/mL, ethanol at 6.25 mg/mL and ciprofloxacin at 10 µg/mL on *P. aeruginosa* using disc diffusion assay

From the results obtained, this study demonstrated that the highest antibacterial effect against *B.cereus* and *P.aeruginosa* was seen with methanol extract of *P. commune* at a concentration of 12.5 mg/mL and for ethanol extract, this was reported highest at a concentration of 6.25 mg/mL. Although higher concentrations of methanol and ethanol extract of *P. commune* were also introduced into the agar medium, these concentrations failed to show a larger diameter of the growth inhibition zone, which suggests that an increase in concentration was unable to promote an additional antimicrobial effect. This may be because the amount of phenolic compounds that are present in the extraction is limited; further research is needed to confirm. The polarity of the compounds extracted under different solvents might affect their intrinsic bioactivity (Do et al. 2014). Thus, the polar solvent can be used to increase the solubility of the phenolic compound which increases the concentration of phenolic compounds that are present in *P. commune* extract to enhance the antimicrobial effect. In addition, the ability of extracted compounds to diffuse in different media that are used might also lead to differences in antibacterial efficiency (Do et al. 2014).

Contrarily, Figure 3 demonstrated that both methanol and ethanol extract of *P. commune* at a concentration of 12.5 mg/mL and 6.25 mg/mL, respectively, showed similar clear zones of inhibition against *P. aeruginosa* which were with a diameter of 11.7 mm. Notably, both clear zones were found to be larger than the diameter of the clear zone of inhibition developed by ciprofloxacin (10 µg/mL) which was with a diameter of 7.0 mm. In this study, the

antibacterial effect of *P. commune* methanol extract at the optimal concentration of 12.5 mg/mL was better than the optimal concentration of *P. commune* ethanolic extract at the optimal concentration of 6.25 mg/mL. Greater diameters were seen from the distinct zones of inhibition formed against *B. cereus* and *P. aeruginosa* with methanol extraction. This could be attributed to the higher extraction capability of methanol in generating a higher extraction yield of the *P. commune* than ethanol due to the higher polarity of the solvent (Sultana et al. 2009; Bouarab-Chibane et al. 2019). In this context, a higher extraction yield would lead to an additional increase in the concentration of secondary metabolites such as phenolic and flavonoid compounds, which in turn could promote the antibacterial effect of the *P. commune* extract. The presence of the phenolic compounds will change the permeability of cell membranes and modify the rigidity of cell walls through different interactions, which can disrupt bacterial cell membranes and cause the loss of cell integrity (Kim et al. 2015). Moreover, the extracted flavonoid compounds would also demonstrate the ability to disrupt the bacteria cell membranes and inhibit both energy metabolism and DNA synthesis of the bacteria which consecutively inhibit their growth (Kim et al. 2015).

3.2 Antimicrobial Assay of Soaps containing P. commune Extract

3.2.1 Liquid Soap

Results obtained from the thumb impression tests showed that the use of liquid soap containing *P.commune* methanol extract produced a significantly higher reduction in microbial growth as compared to the liquid control soap (Table 1). Interestingly, only subject 3, showed a reduction of microbial growth from high to medium (unwashed to washed) using liquid control soap while other subjects remained unchanged, which can be deduced that the liquid control soap can eliminate microbial load but with low efficiency as compared to the liquid soap containing methanol extract of *P. commune*, subjects 1, 2, 3, 4, and 6 demonstrated a reduction of microbial growth while only subject 5 remained unchanged, which indicated that the liquid soap containing methanolic extract of *P. commune* has higher efficiency in eliminating microbial load.

Meanwhile, liquid soap containing the ethanol extract of *P.commune* demonstrated that subjects 1, 2, 3, 5, and 6 had a reduction in the amount of microbial growth after washing their hands (Table 2). The reduction in microbial growth indicated that the liquid soap containing *P. comuune* ethanolic extract possesses antimicrobial properties. Other than this, it can be deduced that the effectiveness of liquid soap containing ethanol extract of *P. commune* was similar to liquid soap containing *P. commune* extract with methanol. This is a result of a decrease (either reduced from high to medium or from medium to low) in microbial growth following hand washing with both liquid soaps.

3.2.2 Solid Soap

In the second experiment of hand washing by using the solid control soap, only subjects 1 and 3 showed a reduction in the amount of microbial from high to medium growth while other subjects remained relatively unchanged. Thus, it can be deduced that the solid control soap can eliminate microbial load but with low efficiency which is similar to the liquid control soap. On the other hand, subjects 1, 2, 3, 4, and 5 showed a notable reduction in the amount of microbial growth after all of them washed their

Table 1 Qualitative indicator data showing the amount of microbial growth on unwashed, liquid control soap-washed, and liquid soap-washed hands with *P. commune* extracted with methanol

Treatments	Subjects					
	1	2	3	4	5	6
Unwashed hands	+ + +	+ +	+ + +	+ +	+ +	+ + +
Washed hand with liquid control soap	+ + +	+ +	+ +	+ +	+ +	+ + +
Washed hand with liquid soap containing *P. commune* extracted with methanol	+ +	+	+ +	+	+ +	+ +

Table 2 Qualitative indicator data showing the amount of microbial growth on unwashed, liquid control soap-washed, and liquid soap-washed with *P. commune* extracted with ethanol-washed hands

Treatments	Subjects					
	1	2	3	4	5	6
Unwashed hands	+ + +	+ +	+ + +	+ + +	+ +	+ + +
Washed hand with liquid control soap	+ +	+ +	+ + +	+ + +	+ +	+ +
Washed hand with liquid soap containing *P. commune* extracted with ethanol	+ +	+	+ +	+ + +	+	+ +

Table 3 Qualitative indicator data showing the amount of microbial growth on unwashed, solid control soap-washed, and solid soap-washed with *P. commune* extracted with methanol-washed hands

Treatments	Subjects					
	1	2	3	4	5	6
Unwashed hands	+ + +	+ + +	+ + +	+ +	+ + +	+ +
Washed hand with solid control soap	+ +	+ + +	+ +	+ +	+ + +	+ +
Washed hand with solid soap containing *P. commune* extracted with methanol	+	+ +	+	+	+ +	+ +

Table 4 Qualitative indicator data showing the amount of microbial growth on unwashed, solid control soap-washed, and solid soap-washed with *P. commune* extracted with ethanol-washed hands

Treatments	Subjects					
	1	2	3	4	5	6
Unwashed hands	+ + +	+ +	+ + +	+ +	+ +	+ +
Washed hand with solid control soap	+ + +	+ +	+ +	+ +	+ +	+ +
Washed hand with solid soap containing *P. commune* extracted with methanol	+ +	+	+	+	+	+ +

hands using the solid soap containing *P. commune* methanolic extracted. Moreover, for subjects 1 and 3, the reduction in microbial growth was reduced from high to low (Table 3). These results indicated that the soap containing *P. commune* methanolic extract contained antimicrobial properties and showed higher efficiency in eliminating microbial load by comparing to solid control soap, liquid control soap, as well as liquid soap containing *P. commune* extracted with methanol and ethanol.

From the thumb impression test, subjects 1, 2, 3, 4, and 5 showed a reduction in microbial growth with the usage of solid soap containing *P. commune* ethanolic extracted (Table 4), which indicated that the solid soap contained antimicrobial properties due to the addition of the *P. commune* extract. By comparing between subjects, only subject 3 showed a reduction of microbial growth from high to low. Therefore, it can be deduced that the efficiency of solid soap containing *P. commune* ethanolic extract has lower microbial elimination efficiency as compared to the methanolic extract of *P. commune* containing solid soap.

Based on the results from the thumb impression tests, the efficiencies of both solid and liquid control soaps in eliminating microbes were not as effective as the solid and liquid soaps containing *P. commune* extracts. As the control soaps used in this study did not have any known antimicrobial agent, our results have indicated that the improved reduction in microbial growth was due to the addition of *P. commune* extract as an ingredient that carries active antimicrobial activity (Lima et al. 2013). The outcomes of this study are in agreement with previous studies where it was found that the addition of herbal extract into soap has a higher antimicrobial effect than the soap without any additional herbal extract (Kareru et al. 2010; Blenkharn and Smales 2017). Meanwhile, by comparing the thumbprint test results between liquid and solid *P. commune* extract soaps, the solid soap was seen to have eliminated more microbial than the liquid soap. Similarly, other studies also revealed that using solid soap for hand wash was more effective than liquid soap and this was due to the mechanical movements involved in removing microbes that were transient on our hands (Sheikh 2018).

Interestingly, when compared, the antimicrobial effect between *P. commune* extracted using methanol and ethanol in solid soaps demonstrated that methanol extracted *P. commune* solid soap was able to eliminate more microbes through hand washing. This might be due to the higher amount of the secondary metabolites present as antimicrobial agents attributed to the higher extraction efficiency of methanol (Sultana et al. 2009). On this basis, it can be hypothesized that the solid soap containing methanolic extract of *P. commune* could eliminate more microbes on our hands than the solid soap containing ethanolic extract of *P. commune*.

Conclusion

In this study, it was demonstrated that *P. commune* extract was effective in inhibiting the growth of *B. cereus* and *P. aeruginosa* in which the optimal concentrations for *P.commune* extracted with methanol and ethanol extract was 12.5 mg/mL and 6.25 mg/mL, respectively. In addition, from the thumb impression tests, the results obtained showed that hand washing performed with both the solid and liquid soaps containing the *P. commune* extract can effectively reduce the presence of microbes in the hands. More specifically, it was demonstrated that the antimicrobial activity of soap containing *P. commune* extract was better in the solid form when compared with the liquid form. Taken altogether, the

addition of *P. commune* extract as an antimicrobial ingredient demonstrated the potential to increase the efficiency of microbial removal in hand washing.

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Conflict of Interest

There are no conflicts of interest.

References

Addor, F. A. S. A. (2017). Antioxidants in dermatology. *Anais Brasileiros De Dermatologia*, *92*(3), 356-362.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71-79.

Blenkharn, J. & Smales, C. (2017). Handwash product and handwash technique are equally important. *Journal of Hospital Infection*, *96*, 298-299.

Bouarab-Chibane, L., Forquet, V., Lantéri, P., Clément, Y., et al. (2019). Antibacterial properties of polyphenols: Characterization and QSAR (Quantitative Structure–Activity Relationship) models. *Frontiers in Microbiology*, *10*, 829.

Boyce, J. M., & Pittet, D. (2002). Guideline for hand hygiene in health-care settings: recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/ APIC/IDSA Hand Hygiene Task Force. *Infection Control & Hospital Epidemiology*, *23*(S12), S3-S40.

Burton, M., Cobb, E., Donachie, P., Judah, G., et al. (2011). The effect of handwashing with water or soap on bacterial contamination of hands. *International Journal of Environmental Research and Public Health*, *8*(1), 97-104.

Chauhan, R., Navlekar, A., Ghosh, E., & Abraham, J. (2014). Screening and evaluation of antimicrobial agents from *Funaria* sp. against various pathogens. *Asian Journal of Pharmaceutical and Clinical Research, 7*(2): 84-87.

Do, Q., Angkawijaya, A., Tran-Nguyen, P., Huynh, L., Soetaredjo, F., Ismadji, S., & Ju, Y. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *Journal of Food and Drug Analysis*, *22*(3): 296-302.

Dulger, B., Hacioglu, N., & Uyar, G. (2009). Evaluation of antimicrobial activity of some mosses from Turkey. *Asian Journal of Chemistry*, *21*(5): 4093-4096.

Greeshma, G. M., & Murugan, K. (2018). Comparison of antimicrobial potentiality of the purified terpenoids from two moss species *Thuidium tamariscellum* (C. Muell.) Bosch. & Sande-Lac and Brachythecium buchananii (Hook.) *A. Jaeger*. *Journal of Analytical and Pharmaceutical Research*, *7*(5), 530-538.

Hanif, U., Ali, H. A., Shahwar, D., Farid, S., et al. (2014). Evaluation of Two Bryophytes (*Funaria hygrometrica* and *Polytrichum commune*) as a Source of Natural Antioxidant. *Asian Journal of Chemistry*, *26*(14), 4339-4343..

Kareru, P., Keriko, J., Kenji, G., Thiong'o, G., et al. (2010). Antimicrobial activities of skincare preparations from plant extracts. *African Journal of Traditional, Complementary and Alternative Medicines, 7*(3), 214–218.

Kazemian, H., Ghafourian, S., Heidari, H., Amiri, P., et al. (2015). Antibacterial, anti-swarming and anti-biofilm formation activities of *Chamaemelum nobile* against *Pseudomonas aeruginosa*. *Revista da Sociedade Brasileira de Medicina Tropical*, *48*(4), 432-436.

Kim, S. A., Moon, H., Lee, K., & Rhee, M. S. (2015). Bactericidal effects of triclosan in soap both in vitro and in vivo. *Journal of Antimicrobial Chemotherapy*, *70*(12), 3345-3352.

Klavina, L., Springe, G., Nikolajeva, V., Martsinkevich, I., et al. (2015). Chemical composition analysis, antimicrobial activity and cytotoxicity screening of moss extracts (moss phytochemistry). *Molecules*, *20*(9), 17221-17243.

Lima, S., Diaz, G., & Diaz, M. A. N. (2013). Antibacterial chemical constituent and antiseptic herbal soap from *Salvinia auriculata* Aubl. *Evidence-Based Complementary and Alternative Medicine*, *480509*, 1-5.

Mishra, R., Pandey, V. K., & Chandra, R. (2014). Potential of bryophytes as therapeutics. *International Journal of Pharmaceutical Sciences and Research*, *5*(9), 3584-3593.

Modarresi, F., Azizi, O., Shakibaie, M. R., Motamedifar, M., et al. (2015). Iron limitation enhances acyl homoserine lactone (AHL) production and biofilm formation in clinical isolates of *Acinetobacter baumannii*. *Virulence*, *6*(2), 152-161.

Nguyen, L, Garcia, J., Gruenberg K., & MacDougall, C. (2018). Multidrug-resistant *Pseudomonas* infections: hard to treat, but hope on the horizon? *Current Infectious Disease Reports*, *20*, 23.

Nikolajeva V., Liepina, L., Petrina, Z., Krumina, G., et al. (2012). Antibacterial activity of extracts from some bryophytes. *Advances in Microbiology*, *2*(3), 345-353.

Oyesiku, O. O., & Caleb, O. J. (2015). Antimicrobial activity of three mosses, *Calympere*s *erosum* Müll. Hal., *Racopilum africanum* Mitt., *Cyclodictyon* Mitt. from Southwest Nigeria. *IOSR Journal of Pharmacy and Biological Sciences*, *10*(2), 1-5.

Riaz, S., Ahmad, A., & Hasnain, S. (2009). Antibacterial activity of soaps against daily encountered bacteria. *African Journal of Biotechnology*, *8*(8), 1431-1436.

Sajed, A. N., Shagufta, S. H., Yousaf, N. W., Ali, I. A. S., et al. (2014). Antibacterial activity of liquid hand washes against daily encounter bacteria. *IOSR Journal of Pharmacy*, *4*(2), 19-23.

Savini, V. (2016). *The diverse faces of Bacillus cereus*. Academic Press, The Netherland.

Sevim, E., Baş, Y., Çelik, G., Pınarbaş, M., et al. (2017). Antibacterial activity of bryophyte species against Paenibacillus larvae isolates. *Turkish Journal of Veterinary & Animal Sciences*, *41*(4), 521-531.

Sharma, D., Bhatia, V. K., Patil, S., & Sharma, P. C. (2013). Antimicrobial activity of selected cryptogams from Solan region. *International Journal of Biological and Pharmaceutical Research*, *4*(6), 448-454.

Sheikh, K. (2018). Is there any reason to use liquid soap instead of bar soap? Retrieved from https://www.vice.com/en_us/article/yw4bvb/is-there-any-reason-to-use-liquid-soap-instead-of-bar-soap accessed on 17 November 2019.

Strigáč, J., Števulová, N., Mikušinec, J., Varečka, L., et al. (2018). Antimicrobial efficiency of metallurgical slags for application in building materials and products. *Buildings*, *8*(2), 33.

Sultana, B., Anwar, F., & Ashraf, M. (2009). Effect of Extraction Solvent/Technique on the Antioxidant Activity of Selected Medicinal Plant Extracts. *Molecules*, *14*(6), 2167-2180.

Tao, S. Y., Cheng, Y. L., Lu, Y., Hu, Y. H., et al. (2013). Handwashing behaviour among Chinese adults: a cross-sectional study in five provinces. *Public health*, *127*(7), 620-628.

Wijetunge, W., & Perera, B. (2016). Preparation of medicinal soap products using the leaf extracts of *Punica granatum* (pomegranate). *International Journal of Pharmacy and Biological Sciences*, *6*(2), 7-16.

Yawson, A. E., & Hesse, A. A. (2013). Hand hygiene practices and resources in a teaching hospital in Ghana. *The journal of infection in developing countries*, *7*(04), 338-347.

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Studies on the feeding habit and digestive enzyme activities in three small indigenous fish species from Assam, India

Soumita Roy[1], Sanraja Muchahary[1], Heikham Dayami[2], Bichitra Narzary[1], Bronson Kumar Khangembam[1*]

[1]Department of Zoology, Bodoland University, Kokrajhar, Assam-783370, India
[2]Department of Life Sciences, Manipur University, Canchipur, Manipur-795003, India

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ABSTRACT

Knowledge of the feeding habit and the digestive physiology of a fish is important in making appropriate strategies for feed development and successful culture. Nutrient-rich small indigenous fish species (SIFs) are abundant in Assam, India. *Puntius sophore*, *Mystus tengara*, and *Trichogaster fasciata* of Gossaigaon, Assam are important SIFs for the local rural population, and also potential candidates for ornamental fish culture. The present study aims to evaluate the feeding habit and digestive enzyme activities of these species. Data obtained from the relative gut length and gut content analysis suggested that *M. tengara* is a carnivorous fish and the rest two fishes are omnivorous in habit. Further, the relative gut length was highest in *T. fasciata* (4.20±0.45) and lowest in *M. tengara* (0.55±0.11). Digestive enzyme activity indicates a correlation with the dietary habit of the fish. Further, total protease, trypsin, and amylase activity was reported highest in *P. sophore*. Acid protease pepsin was found to be significantly higher in *M. tengara* complementing its carnivorous habit and gut anatomy. The present study has established some important information on the digestive enzyme characteristics and feeding habits of the three fish species. This information might be useful in the development of suitable feed for the fish species for their culture.

* Corresponding author
E-mail: kbronson173@gmail.com (Bronson Kumar Khangembam)

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1 Introduction

Small indigenous fishes are an important group of fish with immense potential for culture both as food and ornamental fish. These are usually small with a maximum length of 25 cm to 30 cm and is considered a cheap source of proteins, vitamins, and minerals, especially for the rural population (Bhutia et al. 2021). In India maximum diversity of SIFs has been recorded from the northeast region (Duarah and Das 2019). SIFs are a high source of macro and micronutrients which are vital for human nutrition. Considered 'weed fishes', this category of fish has not been explored enough for mass culture and propagation. Although the majority (90%) of the population in Assam, India consumes fish, the increased fish production is yet to meet the growing population's demand (Yadav et al. 2020). Local indigenous fish species are good candidates for aquaculture species diversification and expansion as they are nutrient-rich and readily accepted by the people. But their culture is not very popular because of a poor understanding of their biology especially feeding habits and digestive physiology. Proper knowledge about the food and feeding habits of fish is essential for understanding the nutritional requirement of the species (Emmanuel et al. 2019) required for the successful development of an artificial culture system (Khan et al. 2022), and for the production and exploitation of the fish stocks (Meshram et al. 2022). The true natural feeding habit of a fish species can be better understood by in vivo studies (Khabade 2015). Information on the food and feeding pattern of a species is important to understand its nutritional requirement, distribution, and interaction with other organisms, and also for proper management (Gogoi et al. 2020; Kumar et al. 2022). Generally, fish are known to have high flexibility in digestive processes, and it depends on many factors. Digestive enzymes and their relationship with the composition of ingested food are essential to comprehending the feeding biology of a fish species (Almeida et al. 2018) and developing appropriate nutritional strategies.

Puntius sophore (Cypriniformes, Cyprinidae), *Mystus tengara* (Siluriformes, Bagridae), and *Trichogaster fasciata* (Anabantiformes, Osphronemidae) are freshwater fish species widely distributed throughout the Indian subcontinent in habiting small ponds, wetlands, lakes, and slow-flowing streams and rivers (Rahman et al. 2019; Mitu et al. 2019; Kumar et al. 2021). These species are important food fish, especially for the rural poor people. *M. tengara* is known to have good taste and a high nutrient profile. It is also reported to have good protein content (Ahmed et al. 2012) and is an indigenous ornamental fish with good export value (Gupta and Banerjee 2014). *T. fasciata* is an important species for small fish catchers with high commercial values both as ornamental and food fish (Rahman et al. 2019)

Food and feeding habits have been reported for several fish species (Alam et al. 2020; Dutta et al. 2020; Jewel et al. 2020; Kumar et al. 2022; Velasco-Reyes et al. 2022). But very few works have been reported on fishes from freshwater bodies in Assam, India especially in lower Assam (Gogoi et al. 2020). There is a scarcity of work on the digestive enzyme activity and its correlation with the feeding habit of SIFs of the region. The present investigation aimed to study the feeding habit and digestive enzyme activity of three important SIFs, *P. sophore*, *M. tengara*, and *T. fasciata*, found in the natural water bodies of the Gossaigaon, Assam, India.

2 Materials and Methods

2.1 Collection of fish

Adults of three small indigenous fish species (40 individuals for each species) from different families, *P. sophore* (length: 7.40±1.15 cm, weight: 2.84±1.25 g), *M. tengara* (Length: 9.23±0.84 cm, weight: 2.95±0.66 g) and *T. fasciata* (Length:8.69±0.64 cm, weight: 5.33±1.06 g) were collected randomly from the different natural water bodies and landing sites of the local river, Haraputa, in the Gossaigaon, Kokrajhar district of lower Assam (26°26'42.7" N, 89°56'39.4" E). The fish were identified with the help of standard keys and literature (Talwar and Jhingran 1991; Vishwanath et al. 2007; Froese and Pauly 2021). For digestive enzyme study, live fish samples were collected and transported to the laboratory facility at the Department of Zoology, Bodoland University, Assam, India. Representatives of each species were stored separately in 10% formalin to preserve their morphological structure for identification.

2.2 Relative gut length (RGL) and Gastro-somatic index (Ga.SI)

The individual fish samples were washed and digestive tracts were dissected on an ice-cold platform. The length and weight of the dissected gastrointestinal tract were recorded. The characterization of different fish as carnivores, herbivores, and omnivores was done by using the method of RGL as a morphological variable while the feeding intensity was done by calculating the Ga.SI. The RGL (Al-Hussani 1949) and the Ga.SI (Bhatnagar and Karamchandani 1970) were calculated by using the following formulae.

$$\text{RGL} = \text{Total length of gut}/\text{Total length of fish}$$

$$\text{Ga.SI.} = (\text{Weight of gut} \times 100)/\text{Weight of fish}$$

2.3 Gut content analysis

For gut content analysis, the gut (preserved in 5% formalin to prevent further breakdown of food particles) of individual fish was dissected. The gut contents of each fish were then carefully emptied into a petri dish and observed under a compound microscope. The contents were analyzed qualitatively for identification into groups of plant or animal-derived food. These

were further identified into respective major groups like macrophytic plant parts, algae, diatoms, phytoplankton, zooplanktons, rotifers, cladocerans, and crustaceans, insects and their larvae, molluscs, small fish, detritus, and some as unidentified organic or inorganic matter. Most of the food contents were partially or significantly digested and hence identification up to species was not possible. Therefore, they were grouped under this broad classification by observing specific visible characteristics of each group.

2.4 Preparation of crude enzyme extract

Ten fish of each species were anesthetized with MS-222 (Tricaine methanesulfonate) and dissected on a cold platform maintained at 0-4°C. The digestive tracts were separated, cleaned, and weighed. The dissected digestive tract of each species of fish was homogenized in cold distilled water (1:10 w/v, tissue: water) and centrifuged (Eppendorf 5425R, Germany) at 10,000 g for 30 min at 4°C. The supernatant was separated, labeled, and used for the estimation of digestive enzyme activity.

2.5 Digestive enzyme Activity

Amylase activity was assayed by following Bernfeld's (1955) method. Briefly, starch solution (1% w/v) was used as a substrate, and it was incubated with the crude enzyme extract, 1% starch in phosphate buffer (0.1 M, pH 7.0), and NaCl for 1 hour at 37°C. The reaction was stopped by adding 3,5- DNSA (3,5-dinitro salicylic acid) and the absorbance was measured at 540nm using UV-Visible Spectrophotometer (Shimadzu 1900i, Japan). Specific amylase activity was expressed as milligram of maltose liberated per milligram protein in reaction mixture per hour at 37°C.

Trypsin activity was measured following the method of Erlanger et al. (1961). N*α*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA, SRL, Mumbai India) was taken as substrate and incubated with the crude extract. The change in absorbance of the reaction mixture was recorded after 15 mins at 410nm in a UV-visible Spectrophotometer (Shimadzu 1900i, Japan). Specific trypsin activity was expressed as units per milligram protein per minute using the following formula:

$$\text{Activity units} = \frac{(\Delta\text{Abs } 410 \text{ nm/min} \times 1000 \times \text{mL of reaction mixture})}{(8800 \times \text{mg protein in reaction mixture})}$$

Pepsin activity was measured according to the method described by Anson (1938) using hemoglobin as the substrate. The activity was assayed using 500µl of 2% hemoglobin as a substrate. The reaction was started by adding 100 µl of crude extract and incubating at 37°C for 10 minutes. The reaction was stopped by adding 1000 µl Tricholoacetic acid (TCA, 5% w/v). The reaction mixture was centrifuged (12,000rpm, 5 min at room temperature) to separate the supernatant and its absorbance was measured at 280nm. Specific pepsin activity was calculated using the formula: Activity = Abs (test-blank)×1000/mg protein/min. The activity was expressed as Units/mg protein/min.

Total protease activity was measured following the method described by Garcia-Carreno (1992) and azocasein was taken as the substrate. Tris-HCl (SRL, India) was used as a buffer (pH 7.5). The reaction mixture consisting of crude extract, buffer, and azocasein was incubated at 25°C for 15 minutes, and thereafter, the reaction was stopped by adding 20% TCA. The samples were then centrifuged (10,000 rpm, 5 min at room temperature) to obtain the supernatant, and its absorbance was recorded at 366nm. The specific total protease activity was expressed as Abs (test-control)/mg protein in reaction mixture/min.

2.6 Protein estimation

Total soluble protein was measured according to Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard against the sample protein (1mg/ml).

2.7 Statistical Analysis

Data values are represented as Mean±S.D. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used to find out the significant difference between the means in SPSS 23.0. Statistical significance was accepted at $P<0.05$.

3 Results

3.1 Relative gut length and gastro somatic index

The RGL and Ga.SI values of three different fish species are represented in Figures 1a and 1b, respectively. The RGL value was found to be significantly ($P<0.05$) higher in *T. fasciata* (4.20±0.45), and lowest in *M. tengara* (0.55±0.11) among the three species. The Ga.SI indicates the fullness of the stomach. The highest Ga.SI value was recorded in *T. fasciata* (5.61±1.22), whereas this value was 4.69±1.28 and 4.95±1.12 for *P. sophore* and *M. tengara*, respectively, which indicates good feeding intensity of the three species in the study.

3.2 Gut content analysis

The different food items observed in the gut of *T. fasciata, P. sophore,* and *M. tengara* fishes are shown in Table 1. All the observations on the three fishes revealed that food particles in the gut continuously lost their morphology and identity due to digestion as they passed down the digestive tract. In the posterior portion of the digestive tract, food was completely digested, and the undigested portion of the food started forming fecal matter. The gut content of *M. tengara* was observed to mainly consist of small crustaceans, rotifers, copepods, partially digested small invertebrates like head parts of shrimp, parts of insect larvae, appendages, wings,

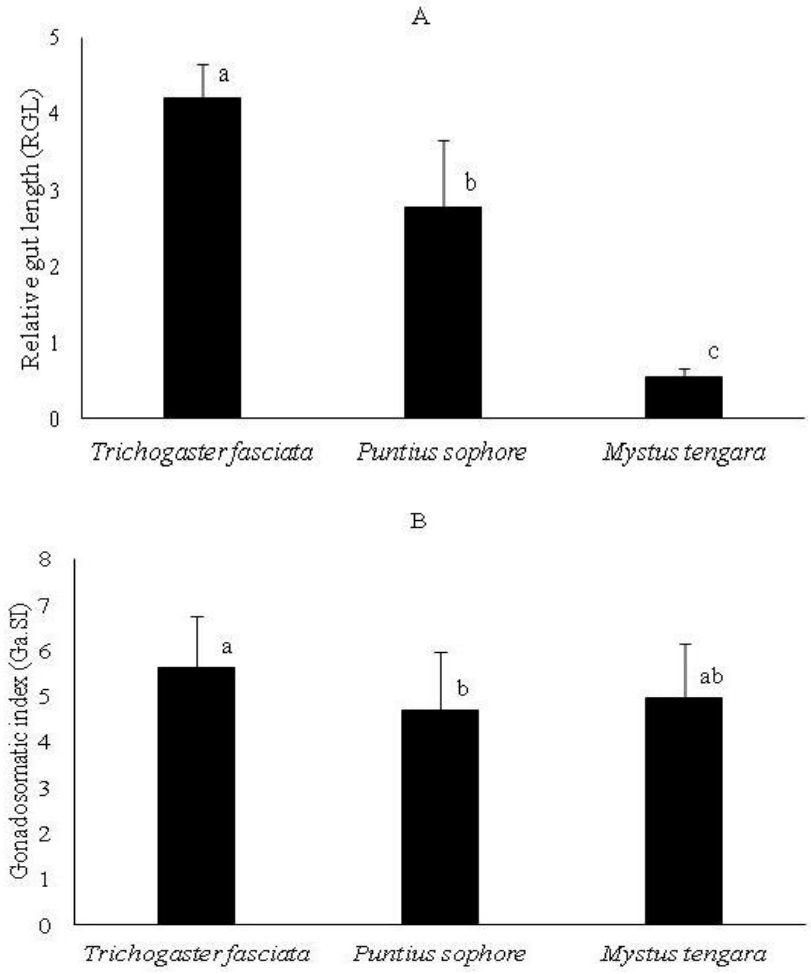


Figure 1 Relative gut length (RGL) and Gastro-somatic Index (Ga.SI) values of the three fish species studied. A: RGL. B: Ga.SI values. Values are represented as mean values ± SD (n=20). Means with different superscripts are significantly different (P<0.05).

Table 1 Composition of the gut content of the three fish species studied

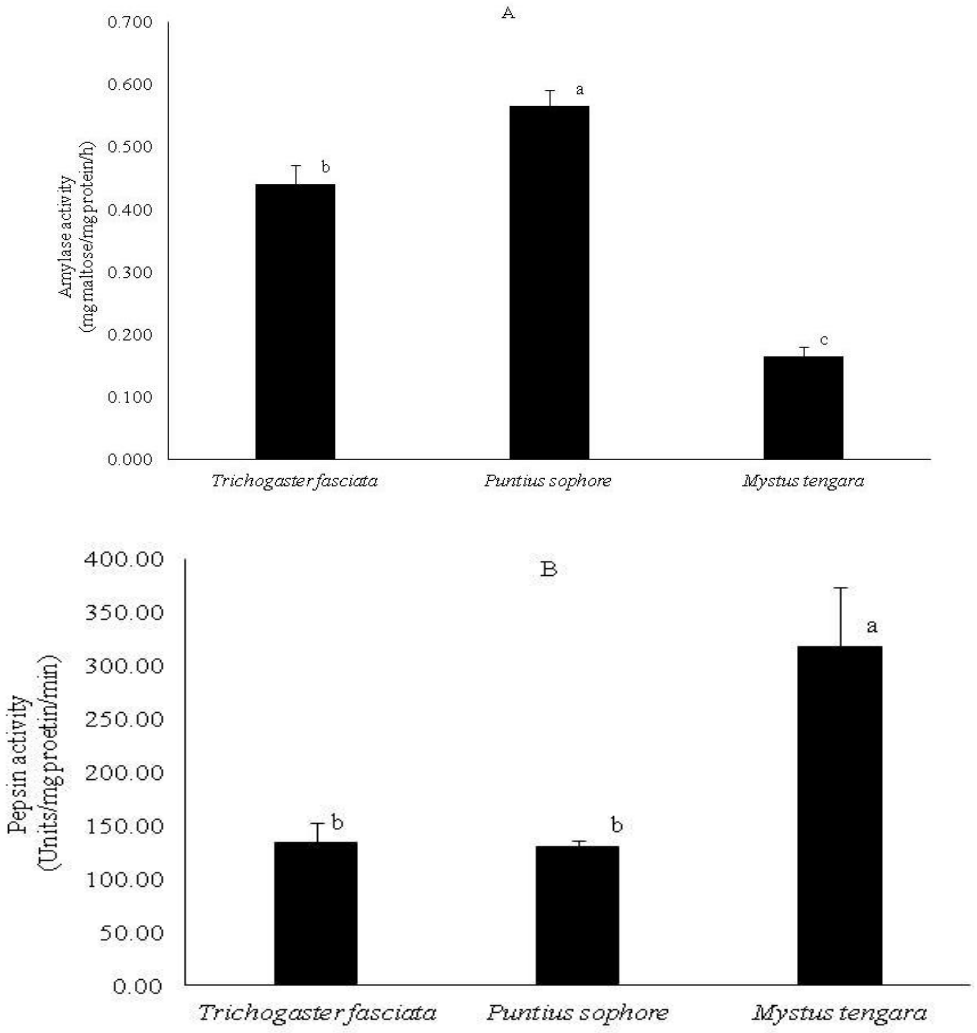
Sl. No.	Feed Item	*T. fasciata*	*M. tengara*	*P. sophore*
1.	Phytoplankton (*Oscillatoria spp., Spirulina spp., Rivularia spp., Achnanthes spp., Cymbella spp., Navicula spp., Tabellaria spp., Chlorella spp., Ulothrix spp., Oedogonium spp., Zygema spp.*)	+	-	+
2.	Zooplankton (Crustaceans and their larval forms, *Daphnia spp.*, *Ceriodaphnia spp.*,)	+	+	+
	Cyclops spp.,	+	-	+
	Rotifers, *Brachionus spp.*, *Asplanchna spp*,)	+	-	+
3.	Insects (Larvae, Pupa, Nymph, Adult, Exoskeleton, Appendages, etc.)	+	+	+
4.	Macrophytes	-	-	-
5.	Small fish and its parts	-	+	-
6.	Unidentified material	+	+	+
7.	Nematodes	-	-	+

+ indicates presence and – indicates absence

and exoskeleton. No recognizable phytoplankton or plant-derived material was observed in its gut. The gut contents of *T. fasciata* consist of diatoms, phytoplankton, zooplankton, insect exoskeleton, and insect larvae, and therefore, the fish may be described as an omnivore in nature. Similar observations were also made in the gut content of *P. sophore* where phytoplanktons, zooplanktons, and small insect larvae were observed. Small nematodes were also observed in some of the specimens. The presence of unrecognizable organic and inorganic matter was detected in both the sample, however, it was more prominent in the case of *P. sophore*.

3.3 Digestive enzyme activities

Digestive enzymes showed species-specific variation in our study. The amylase activities in the three species were estimated, and the activity was significantly (P<0.05) higher in *P. sophore* (0.57±0.03 mg maltose/mg protein/h) among the three species (Figure 2a). *M. tengara* was found to have the lowest amylase activity (0.17±0.02 mg maltose/mg protein/h) among the three species studied, while *T. fasciata* recorded intermediate amylase activity between the other two species. The acid protease pepsin activity was found to be significantly (P<0.05) higher in *M. tengara* (317.45±55.95 Units/mg protein/min) compared to the other two species (Figure 2b). Pepsin activity was lower in *P. sophore* and *T. fasciata* compared to *M. tengara* but it did not differ significantly (P>0.05) between the two species. The highest total protease activity was observed in *M. tengara* (2.23±0.23 Units/mg protein/min) among three species. The activity in *P. sophore* and *T. fasciata* were 2.13±0.02 Units/mg protein/min and 1.23±0.08 Units/mg protein/min, respectively (Figure 2c). The activity of serine protease trypsin was significantly (P<0.05) higher in *P. sophore* (1.89±0.17 Units/mg protein/min) compared to the other species (Figure 2d). The lowest trypsin activity was recorded in *T. fasciata* (0.78±0.03 Units/mg protein/min) and the activity was intermediate in *M. tengara*.



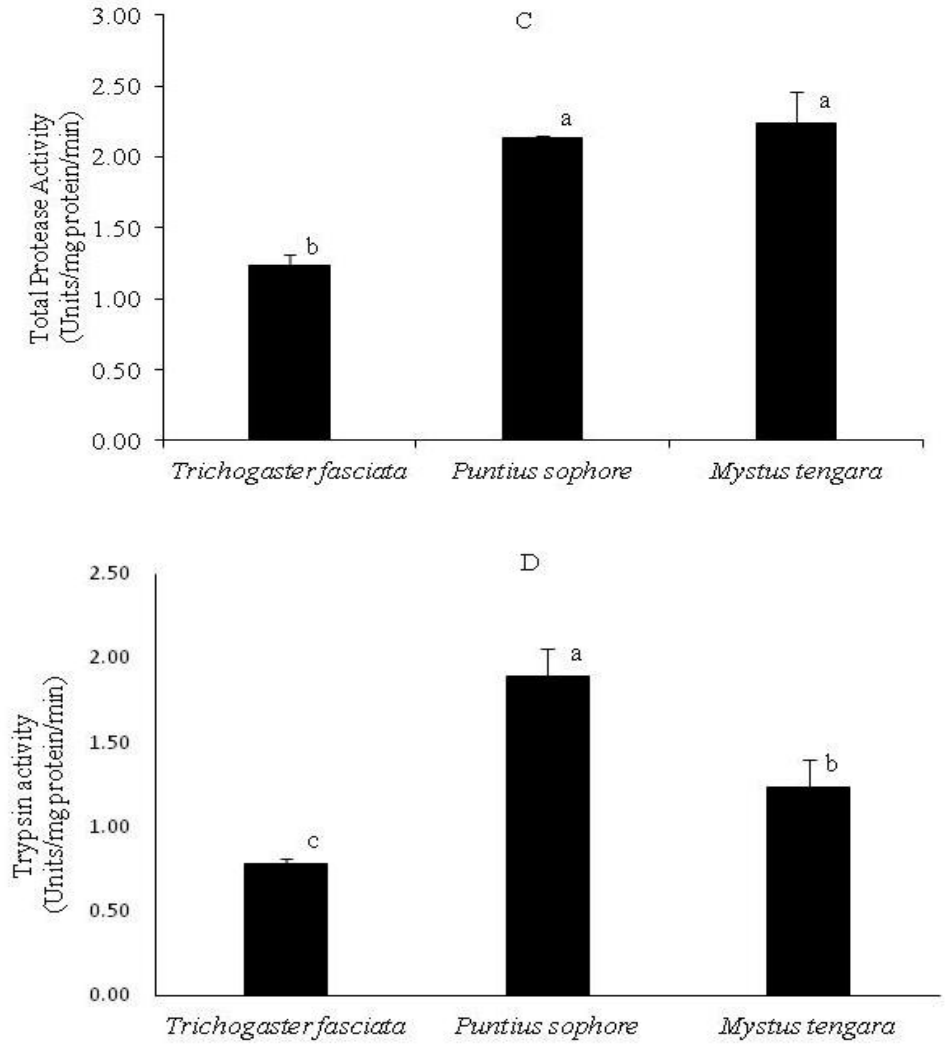


Figure 2 Digestive enzyme activity observed in the three fish species, *T. fasciata*, *P.sophore*, and *M. tengara*. A: Amylase activity. B: Pepsin activity. C: Total protease activity. D: Trypsin activity. Values are represented as mean±SD (n=10). Means with different lower case letters are significantly different (P<0.05).

4 Discussions

4.1 RGL and GaSI

Fish species have been classified as carnivores, herbivores, and omnivores by using the RGL as a main morphological variable while Ga.SI indicates feeding intensity (Manorama and Ramanujan 2017). Ga.SI values of all the three species in the present study indicate a good feeding intensity and these results were found to agree with earlier reports (Khongngain et al. 2017). Generally, when a fish species has the values of RGL less than 1, it is considered carnivorous in food habit, while 1-3 can be considered omnivore in nature. Herbivorous fishes having a plant or detritus-based diet are known to show RGL above 3 (Alam et al. 2019). In the present study, the higher RGL in *T. fasciata* and *P. sophore* reflects the presence of a relatively longer digestive tract compared to its body length. This allows the food to stay in the gut for a longer period, probably resulting in a more efficient mechanism for digestion and absorption, usually observed in animals with a diet rich in plant materials. The gut content analysis of the two species showed the presence of plant-derived materials in the present study. *M. tengara* with an RGL value of less than 1 indicates a highly carnivorous and predaceous feeding habit, which was also observed in its gut content analysis. Similar observations were made by Gupta (2004) where the RGL value was found to be 0.7, 3.7, and 4.7 for carnivorous, planktivorous, and herbivorous fishes, respectively, showing an increase with the increase in plant matter and a decrease with animal matter in the gut of the fish. RGL is closely related to the nature of food present in the fish gut (Khongngain et al. 2017). Plasticity of RGL under influence of diet

has been reported in some fish. RGL was low in younger *P. Ticto* and higher in adult individuals indicating their carni-omnivorous and herbi-omnivorous feeding nature, respectively (Koundal et al. 2012). Lanthaimeilu and Bhattacharjee (2018) also reported an increase in the RGL of *T. fasciata* with increasing size of the fish, indicating a shift in the feeding habit of the fish from carni-omnivore to herbi-omnivore.

4.2 Gut content analysis

The gut content analysis revealed the different feeding habits of the three species. The presence of exclusive animal-derived food and the absence of plant materials indicates the carnivorous and predatory nature of *M. tengara*. Similar results were reported by Gupta and Banerjee (2014), where zooplankton and rotifers were found as the most preferred food of *M. tengara* making it a carnivorous fish. In two related catfish species *M. seenghala* and *Wallago attu*, it was also observed that about 80-90% of animal food matter contributed to their gut content (Babare et al. 2013). However, a euryphagus omnivore feeding habit was also reported in *M. tengara* (Rao 2017) and a related species *M. gulio* (Begum et al. 2009; Sabbir et al. 2017) where the fish were found to feed on a wide range of food organisms.

The presence of a mixture of both plant-derived and animal-derived food materials in *T. fasciata* and *P. sophore* in the present study indicates their natural preference and also their plasticity in feeding habits. Such plasticity and flexibility in the diet preference may be an important reason for the wide distribution and success of these species. Similar plasticity in feeding habits was observed in an invasive mosquitofish (*Gambusia holbrooki*) from Italy and Spain (Pirroni et al. 2021). The omnivorous nature of *T. fasciata* and *P. sophore* has also been reported by previous researchers (Gupta 2004; Das and Kalita 2006; Khongngain et al. 2017). Our results of the gut content analysis of *P. sophore* agree with some earlier studies (Das et al. 2013; Risal et al. 2019) and with a related species *P. sarana* (Hossain et al. 2012). The results of the gut content analysis and that of RGL indicate that *T. fasciata* can be classified as an omnivore, while *M. tengara* is a carnivore. *P. sophore* with RGL less than 3 was found to have a highly omnivorous feeding habit. Studies on the gut content revealed a valuable information about the nature of food and feeding habits of the fish species, and also the type of food material available to the animals in the food chain. Further, present study revealed different feed compositions and feeding habits of the three species which may partially be due to differences in morphology (Velasco-Reyes et al. 2022), feed availability, and size differences of the studied species (Alam et al. 2020). Identifying the gut content is necessary to understand the availability of food in the natural environment of the fish, which may be useful in fisheries management (Al-Zibdah and Odat 2007). This may be useful in exploring the potential of the fish for culture and also for developing conservation strategies.

4.3 Digestive enzyme activities

Food and feeding habits along with the gut functional morphology influence the absence or presence of the digestive enzyme in fish. Our study indicates high amylase activity in *P. sophore* and this may be due to the significant contribution of phytoplankton and plant-based food component in its diet. Gioda et al. (2017) also reported higher amylase and maltase activities in the herbivorous species and intermediate activities in omnivorous species. A close relationship between herbivorous or omnivorous feeding habits and higher amylase activity was also reported by Hidalgo et al. (1999). In the present study, low amylase activity in *M. tengara* may be associated with its high carnivore diet based on animal-derived food. However, higher activities of carbohydrases, proteolytic, and lipases have been reported in detritivores species compared to the omnivorous and carnivorous fishes in some studies (López-Vásquez et al. 2009; Odedeyi and Fagbenro 2010).

Proteases are proteolytic enzymes that catalyze the breakdown of the larger protein molecules into smaller fragments and eventually to their component amino acid. Lower protease activities are generally reported in herbivorous and omnivorous fish species compared to carnivorous species (Chan et al. 2004; Chaudhuri et al. 2012). In the current study, high protease activity was observed in *M. tengara* compared to others. Similarly lower activity of amylase and higher alkaline protease activities in carnivorous fish than in the omnivorous and herbivorous fish species was reported by Champasri et al. (2021). High proteases and trypsin activities observed in *M. tengara* in the present study are in agreement with findings in other carnivorous fish species (Gioda et al. 2017; Weinrauch et al. 2019). Pepsin is an acid protease normally associated with the stomach region. The high pepsin activity observed in *M. tengara* indicates the differentiation of a stomach region and the presence of high animal protein in its diet. Trypsin is a serine protease active in alkaline conditions and its activity in omnivorous fish species are generally reported higher compared to the carnivores (López-Vásquez et al. 2009).

Results of the current study in the three SIFs suggest that the digestive enzyme activity in fish is influenced by the ingested diet or feeding habits which agrees with earlier reports (Chan et al. 2004; Langeland et al. 2013; Solovyev et al. 2014; Almeida et al. 2018). Digestive enzyme activities are thought to reflect the feeding habits of the fish and its diet preference (Langeland et al. 2013). Knowledge of the feeding biology of species is incomplete without knowing the relation between food habits and digestive enzyme activities (Almeida et al. 2018). Our study of the three species is significant in understanding the natural feeding pattern of the three species. Variations in the digestive enzyme activity are usually reported among different fish species and similar observations were also made in our study. The combined information on the feeding habits and the activities of the digestive

enzymes may be vital in formulating an appropriate diet for the successful culture of the three fish species.

Conclusions

In conclusion, the present study has established useful information on the activity of the digestive enzymes, and feeding habits of the three SIFs (*P. sophore, T. fasciata*, and *M. tengara*) in their natural habitat in lower Assam. Results of our study indicate that *P. sophore* and *T. fasciata* are omnivores, whereas *M. tengara* showed carnivore-type feeding habits. The digestive enzyme activities in the three species were found to be influenced by the food and feeding habit of each species. Diet-specific variations in the activities of amylase and proteases were observed in the study. High amylase and protease including trypsin activity was observed in *P. sophore* corresponding to the presence of both plants and animal food in its diet. Low amylase activity and high proteases activity in *M. tengara* corresponds to a highly carnivore-type feeding habitat and a minimal plant-based diet. Pepsin activity was seen to be highest in *M. tengara*, indicating the differentiation of a stomach. The results from this study may be useful in a better understanding of the feeding habit, and the digestive physiology of the three fish species in their natural habitat. This information may be useful in developing a suitable feed formulation required for the mass culture and production of the three species.

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Conflict of interest

All the authors declare no conflict of interest.

References

Ahmed, S., Rahman, A.F.M.A., Mustafa, M.G., Hossain, M.B., et al. (2012). Nutrient composition of indigenous and exotic fishes of rain-fed waterlogged paddy fields in Lakshmipur Bangladesh. *World Journal of Zoology*, *7*, 135-140.https://doi.org/10.5829/idosi.wjz.2012.7.2.63162.

Alam, A., Chadha, N.K., Chakraborty, S.K., Joshi, K.D., et al. (2019). Studies on the growth and mortality of invasive *Oreochromis niloticus* (Linnaeus, 1758) in sub-tropical river Yamuna, part of Gangetic River system, India. *Aquatic Ecosystem Health & Management*, *22*, 473–480. https:// doi. org/ 10. 1080/ 14634 988.2019. 16909 26.

Alam, A., Vaisakh, G., Dharma, N.J., Kripal, D.J., et al. (2020). Food and Feeding Biology of Commercially Important Freshwater Eel, Mastacembelus Armatus (LACEPÈDE, 1800) from the Ganga River, India. *Oceanography & Fisheries Open Access Journal*, *11*(4): 555819. https://doi.org/10.19080/OFOAJ.2020.11.555819.

Al-Hussaini, A.H. (1949). On the functional morphology of the alimentary tract of some fish in relation to differences in their feeding habits: anatomy and histology. *Journal of cell Science*, *3*(10), 109-139.https://doi.org/10.1242/jcs.s3-90.10.109.

Almeida, A.P.G., Zardo, E.L., Toni, C., Behr, E.R., et al. (2018). Composition of gastrointestinal content, protease and lipase activities in summer and winter of four freshwater siluriforms (Teleostei: Actinopterygii) with two different feeding habits. *Zoologia* (*Curitiba*), *35*, e13286. https://doi.org/10.3897/zoologia.35.e13286.

Al-Zibdah, M., & Odat, N. (2007). Fishery status, growth, reproduction biology and feeding habit of two scombrid fish from the Gulf of Aqaba Red Sea. *Lebanese Science Journal*, *8*(2), 3-20.

Anson, M.L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *Journal of General Physiology*, *22*(1), 79-89. https://doi.org/10.1085/jgp.22.1.79.

Babare, R., Chavan, S., & Teerth, R. (2013). Gut content analysis of *Wallago attu* and *Mystus* (Sperata) *seenghala* the common catfishes from Godavari River System in Maharastra state. *Advances in Bioresearch*, *4*(2), 123-128.

Begum, M., Alam, M., Islam, M., & Pal, H.K. (2009). On the food and feeding habit of an estuarine catfish (*Mystus gulio* Hamilton) in the south-west coast of Bangladesh. *University Journal of Zoology, Rajshahi University*, *27*, 91-94. https://doi.org/10.3329/UJZRU.V27I0.1962.

Bernfeld, P. (1955). Amylase, α and β: Colorimetric assay methods. In: S.P. Colowick, N.O. Kaplan (Ed.). *Methods in Enzymology* (pp. 149-158). Academic Press. New York.

Bhatnagar, G.K., & Karamchandani, S.J. (1970). Food and feeding habits of *Labeo fimbriatus* (Bloch) in river Narbada near Hoshangabad (MP). *Journal of Inland Fisheries Society of India*, *2*, 30-50.

Bhutia, R.N., Prakash N.R., Ahmed I., & Hussain I. (2021).Small indigenous fish based paddy cum fish cultivation in land shaping models in Sundarbans. *Food and Scientific Reports*, *2*(5), 21-23.

Champasri, C., Phetlum S., & Pornchoo C. (2021). Diverse activities and biochemical properties of amylase and proteases from six freshwater fish species. *Scientific Reports*, *11*, 5727. https://doi.org/10.1038/s41598-021-85258-7.

Chan, A.S., Horn, M.H., Dickson, K.A., & Gawlicka, A. (2004). Digestive enzyme activities in carnivores and herbivores:

comparisons among four closely related prickleback fishes (Teleostei: Stichaeidae) from a California rocky intertidal habitat. *Journal of Fish Biology, 65*(3), 848-858. https://doi.org/10.1111/j.0022-1112.2004.00495.x.

Chaudhuri, A., Mukherjee, S., & Homechaudhuri, S. (2012). Diet composition and digestive enzymes activity in carnivorous fishes inhabiting mudflats of Indian Sundarban estuaries. *Turkish Journal of Fisheries and Aquatic Sciences, 12*(2), 265-275. https://doi.org/10.4194/1303-2712-v12_2_11.

Das, S., Nandi, S., Majumdar, S., & Saikia, S.K. (2013). New characterization of feeding habits of *Puntius sophore* (Hamilton, 1822) through morphometry. *Journal of Fisheries Sciences.com, 7*(3), 225-231.

Das, S.K., & Kalita, N. (2006). Seed production technology of ornamental gouramis *Colisa fasciata* and *C. Ialia* under captive conditions-an experience in Assam, India. *Aquaculture Asia, 11*(4), 13-14.

Duarah, P., & Das, K. (2019). Diversity of Small Indigenous Freshwater Fish Species (SIFs) in Assam; Nutritional Contents and Medicinal Importance: A Review. *International Journal on Emerging Technologies, 10*(2), 357-361.

Dutta, M., Pradhan, A., Mandal, B., & Mahapatra, B.K., (2020). Feeding and reproductive biology of blue perch, *Badis badis* (Hamilton, 1822) under captivity. *International Journal of Fisheries and Aquatic Studies, 8*(2): 98-102.

Emmanuel, M., Neethu, G.P., Sreekanth, G.B., & Pramod Kiran, R.B. (2019). Food and feeding habits of *Etroplus suratensis* (Bloch, 1790) in Vellayani Lake, Kerala. *Journal of Aquatic Biology and Fisheries, 7*, 120-126.

Erlanger, B.F., Kokowsky, N., & Cohen, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics, 95*(2), 271-278.https://doi.org/10.1016/0003-9861(61)90145-X.

Froese, R., & Pauly, D. (2021). Fish Base 2021. World Wide Web electronic publication. Retrieved from: http://www.fishbase.org.

Garcia-Carreno, F.L. (1992). The digestive proteases of langostilla (*Pleuroncodes palanipes*, Decapoda): their partial characterization and the effect of food on their composition. *Comparative Biochemistry and Physiology, 103B*, 575-578.https://doi.org/10.1016/0305-0491(92)90373-Y.

Gioda, C., Pretto, A., de Freitas Souza, C., Leitemperger, J., et al. (2017). Different feeding habits influence the activity of digestive enzymes in freshwater fish. *Ciência Rural, 47*(03), e20160113. https://doi.org/10.1590/0103-8478cr20160113.

Gogoi, B., Das, D.N., & Saikia, S.K. (2020). Feeding ecology of Pachypterus atherinoides (Actinopterygii; Siluriformes; Schilbeidae): A small freshwater fish from floodplain wetlands of Northeast India. *Croatian Journal of Fisheries, 78*, 105-120. https://doi.org/10.2478/cjf-2020-0011.

Gupta, M.D. (2004). Relative length of the gut of some freshwater fishes of West Bengal in relation to food and feeding habits. *Indian Journal of Fisheries, 51*(3), 381-384.

Gupta, S.,& Banerjee, S. (2014). Food and feeding habit of a freshwater catfish, *Mystus tengara* (Siluriformes: Bagridae). *Journal of Ichthyology, 54*(9), 742-748. https://doi.org/10.1134/S0032945214060071.

Hidalgo, M.C., Urea, E., & Sanz, A. (1999). Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture, 170*(3),267-283. https://doi.org/10.1016/S0044-8486(98)00413-X.

Hossain, M.I., Nipa, F.R., Tumpa, A.S., Mannan, M.A.,et al. (2012). Food and feeding habit of *Puntius sarana* in the river of Padma, Rajshahi, Bangladesh. *Trends in Fisheries Research, 1*(3), 2319-4758.

Jewel, M.A.S., Ali, S.M.W., Haque, Md.A., Ahmed M.G.U., et al. (2020). Growth and economics of Silver Barb (*Barbonymus gonionotus*) in rice-fish-vegetable integrated culture system at different stocking densities in a Rainfed Arid Zone. *Egyptian Journal of Aquatic Biology and Fisheries, 24*(6), 459-476. https://doi.org/10.21608/ejabf.2020.117948.

Khabade, S.A. (2015). Study of gut contents of major carps for their food habits from Sidddhewadi lake of Tasgaon tahsil of Sangli district Maharashtra. *International Journal of Fisheries and Aquatic Studies, 2*(4),1-4.

Khan, W., Naqvi S.M.H.M., Khan H.U., Rafiq, M., et al. (2020). Feeding habit of Brown trout (*Salmo trutta fario*) in upper parts of river Swat, Pakistan. *Brazilian Journal of Biology, 82*(e239219), 1-7.https://doi.org/10.1590/1519-6984.239219.

Khongngain, O., Das, S., & Bhakta, D. (2017). Study on food and feeding biology of *Trichogaster fasciata* Bloch and Schneider, 1801 from a wetland of Nadia district of West Bengal. *Journal of Inland Fisheries Society of India, 49*(2), 3-9.

Koundal, S., Dhanze, R., Koundal, A., & Sharma, I. (2012). Relative gut length and gastro-somatic index of six hill stream fishes, Himachal Pradesh, India. *Journal of Environment and Biosciences, 27*(1), 11-18.

Kumar, J., Alam, A., & Das, B.K.(2022). The first report on food and feeding habits of yellowtail mullet, *Mini mugil cascasia* (Hamilton, 1822), of the tropical River Ganga, India. *Environmental Biology of Fishes, 105*, 645-652.https://doi.org/10.1007/s10641-022-01263-3.

Kumar, J., Datta, S.N., Tewari, G., Hassan, S.S., et. al (2021). Population dynamics of *Puntius sophore* (Hamilton, 1822) of river Sutlej in Punjab (India). *Journal of Environmental Biology, 42*, 1505-1511.

Langeland, M., Lindberg, J., & Lundh, T. (2013). Digestive enzyme activity in Eurasian Perch (*Perca fluviatilis*) and Arctic Charr (*Salvelinus alpinus*). *Journal of Aquaculture Research and Development, 5*(208), 1-8. https://doi.org/10.4172/2155-9546.1000208.

Lanthaimeilu, K., & Bhattacharjee, D.P. (2018). Relative gut length and gastro-somatic index of *Pethia conchonius* (Hamilton, 1822) and *Trichogaster fasciata* Bloch and Schneider, 1801, Tripura. *Journal of Entomology and Zoology Studies, 6*(2), 2403-2407.

López-Vásquez, K., Castro-Pérez, C.A., & Val, A.L. (2009). Digestive enzymes of eight Amazonian teleosts with different feeding habits. *Journal of Fish Biology, 74*(7), 1620-1628. https://doi.org/10.1111/j.1095-8649.2009.02196.x.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with folin phenol reagent. *Journal of Biological Chemistry, 193*, 265-275. https://doi.org/10.1016/S0021-9258(19)52451-6.

Manorama, M., & Ramanujam, S.N. (2017). Diet of threatened fish *Pethia shalynius* (Yazdani and Talukdar 1975) in the Umiamriver, Northeast India. *Asian Fisheries Science, 30*, 38-49. https://doi.org/10.33997/j.afs.2017.30.1.004.

Meshram, M.M., Rajesh, M., Suyani, N.K., & Rajesh, K.M. (2022). Diet composition, feeding dynamics and proximate composition of obtuse barracuda Sphyraenaobtusata (Cuvier, 1829) in the southeastern Arabian Sea. *Egyptian Journal of Aquatic Research, 48*, 163–168.

Mitu, N.R., Alam, M.M., Hussain, M.A., Hasan, M.R., et al. (2019). Length-weight and length-length relationships, sex ratio and condition factors of the Asian striped dwarf catfish *Mystus tengara* (Hamilton, 1822) (Siluriformes: Bagridae) in the Ganges River, Northwestern Bangladesh. *Iranian Journal of Ichthyology, 6*(1), 21-30. https://doi.org/10.22034/iji.v6i4.334.

Odedeyi, D., & Fagbenro, O. (2010). Feeding habits and digestive enzymes in the gut of *Mormyrus rume* (Valenciennes 1846) (Osteichthyes Mormyridae). *Tropical Zoology, 23*(1), 75-89.

Pirroni, S., Dezen, L.D.P, Santi, F., & Riesch, R. (2021). Comparative gut content analysis of invasive mosquitofish from Italy and Spain. *Ecology and Evolution,11*,4379–4398.

Rahman, M.A., Islam, M.S., Hossain M.Y., Hasan M.R., et al. (2019). Morphometric and Meristic Characteristics of the Banded Gourami, *Trichogaster fasciata* (Bloch & Schneider, 1801) in a Wetland Ecosystem from Northwestern Bangladesh. *Jordan Journal of Biological Sciences, 5*(12), 561-566.

Rao, K.R. (2017). Food and feeding habits of freshwater catfishes (Siluriformes: Bagridae: *Mystus* sp.). *International Journal of Life-Sciences Scientific Research, 3*(1), 786-791. https://doi.org/10.21276/ijlssr.2016.3.1.7.

Risal, A., Shrestha, S., & Mahaseth, V.K. (2019). Food and feeding behaviours of Spotfin Swamp Barb *Puntius sophore* (Hamilton, 1822) of Singhiya river, Biratnagar. *Our Nature, 17*(1), 31-36. https://doi.org/10.3126/on.v17i1.33989.

Sabbir, W., Basonti, M., Mukta, D., Nuruzzaman, K. et al. (2017). Biological aspect of Nona Tengara (*Mystus gulio*) in Khulna Region, South West Bangladesh. *Journal of Biomaterials, 1*(1), 19-24.https://doi.org/10.11648/j.jb.20170102.11.

Solovyev, M.M., Kashinskaya, E.N., Izvekova, G.I., Gisbert, E. et al. (2014). Feeding habits and ontogenic changes in digestive enzyme patterns in five freshwater teleosts. *Journal of Fish Biology, 85*(5), 1395-1412. https://doi.org/10.1111/jfb.12489.

Talwar, P.K., & Jhingran, A.G. (1991). *Inland fishes of India and adjacent countries*. New Delhi, India: Oxford and IBH Publishing Co. Pvt. Ltd.

Velasco-Reyes, L.E., Aguilar-Betancourt, C.M., González-Sansón, G., Flores-Ortega J.R., et al. (2022). Feeding and Diet Overlap of Six Estuarine Fishes (Family Carangidae) from the Mexican Pacific Region. *Estuaries and Coasts, 45*, 302-313.https://doi.org/10.1007/s12237-021-00950-1.

Vishwanath, W., Lakra, W.S., & Sarkar, U.K. (2007). *Fishes of Northeast India* (pp. 1-290). Lucknow, India: National Bureau of Fish Genetic Resources Publications.

Weinrauch, A.M., Schaefer, C.M., & Goss, G.G. (2019). Activity and post-prandial regulation of digestive enzyme activity along the Pacific hagfish (*Eptatretus stoutii*) alimentary canal. *PloS One, 14*(4), e0215027. https://doi.org/10.1371/journal.pone.0215027.

Yadav A.K., Das K.K., Das P., Raman R.K., et. al. (2020). Growth trends and forecasting of fish production in Assam, India using ARIMA model. *Journal of Applied and Natural Science, 12*(3), 415-421. https://doi.org/10.31018/jans.v12i3.2353.