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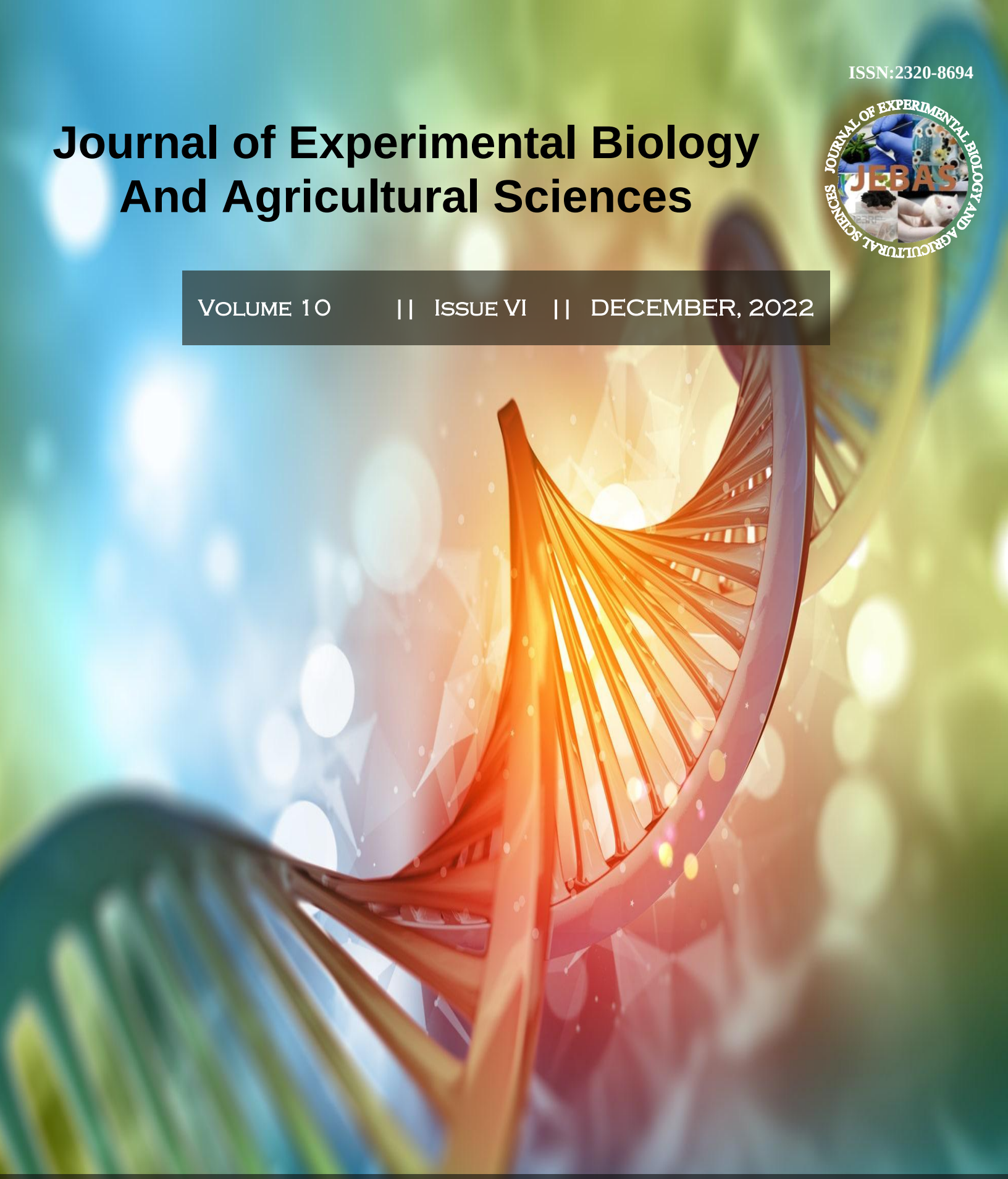

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Table of contents

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SARS-CoV-2 emerging Omicron subvariants with a special focus on BF.7 and XBB.1.5 recently posing fears of rising cases amid ongoing COVID-19 pandemic

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ABSTRACT

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Omicron versions have been the sole one circulating for quite some time. Subvariants BA.1, BA.2, BA.3, BA.4, and BA.5 of the Omicron emerged over time and through mutation, with BA.1 responsible for the most severe global pandemic between December 2021 and January 2022. Other Omicron subvariants such as BQ.1, BQ.1.1, BA.4.6, BF.7, BA.2.75.2, XBB.1 appeared recently and could cause a new wave of increased cases amid the ongoing COVID-19 pandemic. There is evidence that certain Omicron subvariants have increased transmissibility, extra spike mutations, and ability to overcome protective effects of COVID-19

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Pandemic

Rising cases

Mitigation strategies

neutralizing antibodies through immunological evasion. In recent months, the Omicron BF.7 subvariant has been in the news due to its spread in China and a small number of other countries, raising concerns about a possible rebound in COVID-19 cases. More recently, the Omicron XBB.1.5 subvariant has captured international attention due to an increase in cases in the United States. As a highly transmissible sublineage of Omicron BA.5, as well as having a shorter incubation time and the potential to reinfect or infect immune population, BF.7 has stronger infection ability. It appears that the regional immunological landscape is affected by the amount and timing of previous Omicron waves, as well as the COVID-19 vaccination coverage, which in turn determines whether the increased immune escape of BF.7 and XBB.1.5 subvariants is sufficient to drive new infection waves. Expanding our understanding of the transmission and efficacy of vaccines, immunotherapeutics, and antiviral drugs against newly emerging Omicron subvariants and lineages, as well as bolstering genomic facilities for tracking their spread and maintaining a constant vigilance, and shedding more light on their evolution and mutational events, would help in the development of effective mitigation strategies. Importantly, reducing the occurrence of mutations and recombination in the virus can be aided by bolstering One health approach and emphasizing its significance in combating zoonosis and reversal zoonosis linked with COVID-19. This article provides a brief overview on Omicron variant, its recently emerging lineages and subvairants with a special focus on BF.7 and XBB.1.5 as much more infectious and highly transmissible variations that may once again threaten a sharp increase in COVID-19 cases globally amid the currently ongoing pandemic, along with presenting salient mitigation measures.

1 Introduction

Coronavirus disease 2019 (COVID-19) pandemic, caused by Severe Acute Respiratory Syndrome Coronavirus - 2 (SARS-CoV-2), has now entered into its fourth year, with over 655 million confirmed cases and 6.6 million deaths recorded globally as of January 6, 2022, overall leading to devastating adverse health consequences and socio-economic impacts on mankind globally (Dhama et al. 2020; WHO 2023a). This pandemic is not seeming to an end owing to continuous evolution and emergence of several variants, strains, subvariants and lineages of SARS-CoV-2 despite developing vaccines, progressive massive vaccination drives, booster shots and finding out drugs and therapies for treating COVID-19 patients (Kopsidas et al. 2022; Wong 2022; Dhama et al. 2023). This urges for developing better diagnostics and more efficacious and newer vaccines, drugs and therapeutics to combat COVID-19 pandemic (Fernandes et al. 2022; WHO 2022). SARS-CoV-2 variants have been classified into variants of concern (VOCs), variants of interest (VOIs), and variants under monitoring (VUMs) (Reynolds et al. 2022; WHO 2023b). The SARS-CoV-2 Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2) variants (VOCs) have now been designated as previously circulating VOCs, and its Omicron (B.1.1.529) variant that emerged in the late 2021, is now considered as the only circulating VOC along with its subvariants BA.1, BA.2, BA.3, BA.4, BA.5, and descendent lineages (Kopsidas et al. 2022; Dhama et al. 2023; WHO 2023b). Additionally, co-infections with different SARS-CoV-2 variants may pave ways to genetic recombination that may produce newer chimeric, recombinant and hybrid variants (such as XD, XF, and XE) with unpredictable pathogenic properties, which could feasibly pose a new alarm (Dhama et al. 2023; WHO 2023b).

2 Omicron variant, its emerging lineages and subvariants

Omicron variant (Pango lineage B.1.1.529) has produced different sub-lineages BA.2, BA.3, BA.1, BA.1.1, BA.2.12.1, BA.2.75, BA.2.75.2, BA.4, BA.5 (BA.4/5), BA.4.6, BA.5.1, and one after another these became dominant and predominant strains from time to time by displaying additional immunological escape mechanisms posing significant global health hazards (Ai et al. 2022; Aleem et al. 2022; Ferré et al. 2022; Farahat et al. 2022; Graham 2022; Tian et al. 2022; Elliot et al. 2022; Kurhade et al. 2022; Planas et al. 2022; Qu et al. 2022; Mohapatra et al. 2022a; Dhama et al. 2023). Omicron variant and subvariants have been implicated to compromise vaccine and infection-induced immunity and modify virus biology eventually to a higher extent (Evans et al. 2022; Cui et al. 2022; Qu et al. 2022). Additionally, newer Omicron subvariants, such as BA.4.6, BF.7, BQ.1, and BQ.1.1 (descended from BA.4/5), and BA.2.75.2, have emerged as a result of the spread of the BA.4/5 and BA.2.75 subvariants (derived from BA.2.75) (Hachmann et al. 2022). These new variants have been on rise and could replace as most common subtype in the future (Saito et al. 2022; Uraki et al. 2022; Wang et al. 2022).

Multiple sublineages of Omicron variant and its lineages revealed dynamic molecular phylogenetics and mutational landscape analysis, which demands approriate prepredeness planes to be excecuted (Chakraborty et al. 2022; WHO 2022). Some of these

variants gained higher mutations, are more contagious with higher transmissibility than the original Wuhan SARS-CoV-2 strain, can evade protective immunity, decreases antibody neutralization in vaccinated individuals and adversely affects therapeutic potential of monoclonal antibodies (mAbs) , and lead to reinfection, notably Delta variant has additional ability to cause severe COVID-19 disease too (Arora et al. 2022; Du et al. 2022; Chen et al. 2022; Tian et al. 2022; Hanai 2022; Kurhade et al. 2022; Mohapatra et al. 2022a, Mohapatra et al. 2022b; Uraki et al. 2022; Qu et al. 2022; Zhou et al. 2022).

Omicron has a much higher mutation rate than any other previously circulating VOCs and became the worldwide dominant variety after acquiring new mutations and splitting into several subvariants, each with its own unique epidemiological, clinical, and viral signature as it expanded over the world (Aleem et al. 2022; Dhama et al. 2023). Omicron variant shares at least 50 alterations/mutations with the reference strain, and about 30 of these alterations were detected in the viral S protein, that may lead to receptor binding domain (RBD) motif accumulations. Mutations in the Omicron S protein RBD increase its affinity for the human ACE2 receptor, allowing efficient virus entrance into human cells (Chen et al. 2022). Remarkably, Delta variant lead to a rapid surge in COVID-19 cases and higher deaths during 2021 due to being causing more serious COVID-19 disease, and thereafter Omicron variant that emerged in November 2021 from South Africa caused a very huge massive surge in COVIID-19 cases during early 2022, though not causing severe disease but cumulative number of deaths increased highly owing to very high surge in cases globally (Kannan et al. 2021; Balint et al. 2022; Khandia et al. 2022; Kurhade et al. 2022; Mohapatra et al. 2022b).

Of late, emergence of Omicron subvariants and sublineages such as BQ.1, BQ.1.1, BA.4.6, BF.7, BA.2.75.2, XBB.1 (a BA.2 subvariant) and BF.7 may pose an alarming global health situation and might lead to a new wave of surge in cases amid the ongoing COVID-19 as reflected by the most recent start of rise in cases being observed presently in few countries particularly in China and others (Graham 2022; Wong 2022; News 18, 2022; Sagar 2022; Ai et al. 2022; Aleem et al. 2022; Wang et al. 2022). Some of these Omicron subvariants have been implicated to possess additional spike (S) mutations, higher transmissibility, and immune evasion properties to escape protection rendered by neutralization antibodies of COVID-19 vaccines and boosters, and mAbs as therapeutics (Hanai 2022; Fernandes et al. 2022; Kurhade et al. 2022; Uraki et al. 2023; Qu et al. 2022). Notably, the RBD of the spike (S) protein, the primary target of COVID-19 vaccines and therapeutic mAbs, is more heavily modified in BQ.1.1 and XBB than in BA.5 and BA.2. These versions may, therefore, be more difficult for the immune system to combat than BA.5 and BA.2 (Imai et al. 2022). According to a recently amended FDA information sheet, bebtelovimab will not neutralize Omicron subvariant BQ.1 or BQ.1.1, while Paxlovid should “retain activity” against the new subvariants (Thakur and Ratho 2022).

Since Omicron BA.1 was supplanted by BA.2, the latter has diverged into the sublineages BA.2.12.1, BA.2.75, BA.2.75.2, BA.4, and BA5, with the latter now predominating in many countries. Both BA.4 and BA.5 share the same spike sequence (henceforth BA.4/5), and their progeny, BA.4.6, BF.7, and BQ.1.1, are on the rise. Vaccine efficacy may be compromised by the accumulation of spike mutations in the recently revealed SARS-CoV-2 Omicron sublineages, such as BA.2-derived BA.2.75.2 and BA.5-derived BQ.1.1 and XBB.1 (Kurhade et al. 2022; Qu et al. 2022). Omicron subvariant BQ.1 (a subvariant of BA.5), its sublineage BQ.1.1, and XBB (a recombinant of two separate BA.2 subvariants) have shown rise in many countries, including the United States, France, Singapore, and India. The RBD of S protein is the primary target for vaccinations and therapeutic mAbs against COVID-19. BQ.1.1 and XBB have substitutions in this region compared to BA.5 and BA.2, respectively. As both BQ.1.1 and XBB include the substitution R346T, which gives resistance to certain therapeutic antibodies, there is cause for worry that mAbs or vaccinations may be less successful against these strains than they are against other omicron strains (Uraki et al. 2022).

2.1 BF.7

Recently, BF.7 subvariant of omicron has been in the news owing to its spread in China and other few countries, posing worrisome situation of rise in COVID-19 cases again, and more recently, XBB.1.5, a more contagious and highly transmissible Omicron variant to date has attracted global attention due to rise in cases in the USA. BF.7 is a sublineage of Omicron variation BA.5, has stronger infection potential since it is highly transmissible with a shorter incubation time, and can also reinfect or infect the immunized population (Sagar 2022). Of note, Chinese cities have been afflicted by the highly transmissible Omicron strain, especially BF.7 that is spreading in Beijing and leading to a COVID outbreak after a long time since the first deadly disease outbreak started as a pandemic during early 2020 (Graham 2022; Wang et al. 2022). The BF.7’s high transmissibility in China may be due to inadequate immunity from past SARS-CoV infections and less potentially vaccination. It has also been found in few countries including such as the USA, Brazil, UK, Belgium, Germany, France, China, Denmark and India (Sagar 2022; WHO 2022). The basic reproductive number R0 of Omicron was noted to be an average of R0 of 5.08, while BF.7 is implicitated to presumably have an R0 of 10 to 18.6, which might be potentiating its ability of higher transmission and infectivity.

2.2 XBB and XBB.1.5

To be specific, XBB is a hybrid of the BA.2.10.1 and BA.2.75 variants. However, preliminary research suggests that XBB has a greater reinfection risk than other circulating Omicron sublineages. Only those who contracted XBB before the introduction of the Omicron variant were at risk of reinfection. There is currently no evidence suggesting additional Omicron lineages can evade the recent immunological responses they have elicited. The magnitude and timing of prior Omicron waves, as well as the COVID-19 vaccination coverage, appear to impact the regional immunological landscape, which in turn affects if the increased immune escape of XBB is adequate to drive fresh infection waves (Wang et al. 2022; WHO 2023b).

The rapidity of the XBB.1.5 Omicron subvariant's spread in the northeastern United States has raised concerns among health agencies including the World Health Organization (WHO). Until now, this subvariant had the highest transmission rate. While the WHO still lacks data on XBB.1.5's severity, there is currently no evidence to suggest that it is more dangerous than earlier subvariants. This is due to the fact that mutations in this particular omicron subvariant make the virus particularly well-suited to adhering to cells and replicating within them (CNBC 2023a; CNBC 2023b). No statistics on the severity of XBB.1.5 have been collected by the WHO just yet, although there is currently no evidence to suggest that it is more dangerous than prior Omicron strains. Researchers have shown that XBB.1.5 shares similar abilities to its related XBB and XBB.1 in avoiding the immunological responses prompted by vaccinations and infections. However, XBB.1.5 possesses a mutation that increases its ability to connect to cells, which provides a growth advantage (CNN 2023; Forbes 2023). The ability of a virus to infect people who have been exposed to it before, either by infection or immunization, is known as immune evasiveness. XBB.1.5 achieved this by developing an uncommon form of mutation termed F486P, found in its RBD. Whether or if it contributes to more severe disorders is unknown. This is deemed highly implausible by experts (Livemint 2023; WFLA 2023).

Despite a lower risk of severe COVID-19 and death than the previous SARS-CoV-2 variants comparatively, the high transmission levels and more contagious nature of Omicron and it's different subvariants and sublineages could lead to a significant increase in the COVID-19 cases and hospitalization rates, continue to overwhelm healthcare systems in many countries, and may lead to a considerably higher morbidity rate, especially in vulnerable populations (Kandeel et al. 2022; Dhama et al. 2023).

3 Mitigation strategies

Vaccination is the most effective method for conferring anti-COVID-19 protective immunity and avoiding infections of SARS-CoV-2 and its emerging variants, initial immunizations and recommended vaccine boosters need to be administered to cover up larger population. Vaccines and booster shots can reduce Omicron-related hospitalizations, ameliorate disease severity, deaths, and significant complications (Björk et al. 2022; Mohapatra et al. 2022c; Zhou et al. 2022). Vaccination boosters protect against both SARS-CoV-2 and its Omicron variant and subvariants, though to a different protective level, and despite administering three doses of COVID-19 vaccines only partial protection can be conferred against infection with Omicron variant and subvariants, therefore better and newer vaccines and vaccination strategies are the need of the current times (Wong 2022; Björk et al. 2022; Dhama et al. 2023).

New vaccinations and mAbs are urgently needed since the number of Omicron subvariants keeps growing (Fernandes et al. 2022; Hossain et al. 2022), halting resurgences of resistant strains by designing vaccines targeting Omicron subvariant(s). A variety of vaccination platforms, including those based on mRNA and viruses, as well as protein-based adjuvanted vaccines, which widen present immune responses have been explored. Moreover, RBD-dimeric vaccines, mosaic RBD nanoparticle vaccines, conservative S2-targeting vaccines have demonstrated their potential for countering pan-beta-CoVs (SARS-CoV-2, SARS-like sarbecoviruses), and human endemic CoVs protections by inducing broad-spectrum neutralizing antibodies (nAbs). Together, these active as well as passive vaccination strategies advance us further along the road to pan-beta-CoV, or pan-CoV immunity (Akkız 2022; Ke et al. 2022; Xia et al. 2022). Nasal vaccines generating both humoral and respiratory mucosal immunity could be beneficial in limiting transmission and spread of SARS-CoV-2 and its variants and subvariants.

Recent surge in COVID-19 cases in China and USA and other countries should be kept in mind, and optimal infection prevention and control methods should be closely followed until this pandemic comes to an end. Much is to be known about Omicron and its continuously emerging newer subvariants and lineages. Explorative research and deeper investigations are required for studying transmissibility, effectiveness of vaccines, immunotherapeutics and antiviral drugs against recently emerging Omicron subvariants and lineages as well as enhance surveillance and monitoring, strengthening genomic facilities for tracking their spread, tight vigilance, and throwing more light on their evolution and mutational events which would aid in formulating appropriate mitigation strategies (Hanai 2022; Qu et al. 2022; Uraki et al. 2022). These Omicron subvariants could increase the risk of serious illness and hospitalization altogether under threats of cumulative rise in COVID-19 cases again, especially in vulnerable population and under vaccine breakthrough events and reinfection (Farahat et al. 2022; Tuekprakhon et al. 2022). Of note, circulation of SARS-CoV-

2 and its variants among animals including pet and wild animals also need to be checked by strengthening of one health approach and promoting its importance in tackling zoonosis and reverse zoonosis associated with COVID-19, which will also aid in limiting events of mutations and recombination to happen in the virus.

4 Conclusion and Future Directions

We need to now be prepared holistically and act proactively to avoid any new dangerous COVID-19 wave and adopt appropriate and recommended COVID-19 protective and control measures. COVID-19 appropriate behaviours and safety measures including wearing of face mask, regular hand washing and norms of social / physical distancing, hygiene and disinfection practices, avoiding crowded places and mass gathering events must be remembered to be followed strictly. Along with these, enhancing immunization rates / vaccination coverages, checking vaccine hesitancy and throwing up reluctance to promote booster shots, equal global access of vaccines at global level, generate adequate herd immunity, could save us from facing again a massive surge in COVID-19 cases in the form of a new pandemic wave by restricting circulation of the virus in unprotected people and rapidly accumulate mutations to boost viral transmissibility and infectivity and closely watching the emergence of newer strains. This will ultimately help to avoid increase in COVID-19 cases and associated deaths as well as countering incidences of newly emerging subvariants of Omicron.

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Potential effects of essential oils in safeguarding the health and enhancing production performance of livestock animals: The current scientific understanding

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ABSTRACT

The food sector competes in a cutthroat environment, and it constantly struggles to maintain or even grow its market share. For customer confidence and consumption to remain strong, consistent animal products are needed. The qualitative attributes of the derived goods appear to be improved by the addition of bioactive substances to food, such as essential oils (EOs), and consumers are shielded from the impacts of bacterial and oxidative deterioration. Due to the current controversy surrounding synthetic chemicals and their alleged carcinogenic potential, a substantial study has been done to find effective and safe substitutes. Aromatic plants and the corresponding EOs from them are considered natural products and are typically employed in ruminant nutrition. Since dietary supplementation has been demonstrated to be an easy and practical method to successfully suppress oxidative processes or microbial deterioration at their localized sites, the addition of EOs in animal diets is now becoming a

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regular practice. However, there is just a little amount of evidence supporting the notion that these compounds may improve nutrient absorption and gastrointestinal health. Additionally, a variety of factors affect how well EOs works in animal diets. These variables can be, on the one hand, the erratic composition, and the many additions to the diet, and, on the other hand, erratic animal genetic elements. Maximizing the use of EOs and creating high-quality products require a deeper understanding of the composition and activity of the gastrointestinal tract microbiota. Numerous EOs contain bioactive substances with the potential to serve as multifunctional feed supplements for animals, with impacts on growth performance, the digestive system, the growth of pathogenic bacteria, and lipid oxidation, among others. To establish their regular use in animal production and to determine their precise mechanism of action, more research is required. The potential advantages of EOs for livestock health and production are highlighted in the current article.

1 Introduction

Essential oils (EOs), sometimes called volatile oils, are fragrant liquid distillates that come from various plant parts like flowers, buds, seeds, leaves, twigs, bark, wood, and roots (Miguel 2010). These extracts have been utilized traditionally for millennia in a variety of regions of the world, and are either steam-volatile or natural-solvent (ethanol, methanol, toluene, or other natural solvents) based. They are regarded as having high-quality flavor and scent in addition to having preservation properties (Prakash et al. 2021a; Prakash et al. 2021b). Certain EO additions can be derived naturally from plants, while others can be synthesized. Terpenes, alcohols, acetones, phenols, acids, aldehydes, and esters are just some of the many chemicals that are commonly found in EOs (Negi 2012). These substances may operate as a barrier against microbial, fungal, or insect invasions. Therefore, EOs can be categorized as herbal oils, complex oils with several factors, and oils with multiple uses (Brenes and Roura 2010; Abd El-Hack et al. 2016; Andri et al. 2020; Buttar et al. 2022). Plant-based feed additives including herbs and their metabolites, phytochemicals, aromatic plant extracts, and their constituent's essential oils have found promising beneficial applications for use as feed additives, and also possess benefits against the usage of conventional antibiotics in livestock animals and poultry (Dhama et al. 2014; Alagawany et al. 2015; Yadav et al. 2016; Dhama et al. 2018; Tiwari et al. 2018; Kuralkar and Kuralkar 2021; Nehme et al. 2021; Uddin et al. 2021; Chandran 2021a; Zhang et al. 2022).

EOs were utilized for their flavoring capabilities and for their capacity to be used as food preservatives since they lengthen the shelf lives of products or reduce the concentration of Clostridium spp. Over the past decade, the use of EOs in the animal field and medicine has skyrocketed. Their potential as antioxidants and immunomodulators in the ruminant region, however, is still insufficiently investigated (Burt 2004; Corbo et al. 2009). Studies have shown that some EOs can increase animal performance and productivity, particularly in the chicken and pig industries, by enhancing digestive secretions (Lambert et al. 2001); increasing the diversity of probiotic bacteria, including *Lactobacillus* spp. (Gill and Holle 2006; Raybaudi-Massilia et al. 2009); stimulating the immune feature and the gastrointestinal microbiota (Oussalah et al. 2006). However, there are still significant discrepancies about the efficacy of EOs, particularly associated with the nature of the compounds and some intrinsic and extrinsic factors such as infection, dietary reputation, environment, and, most importantly, diet composition (Ultee et al. 2002; Saleena et al. 2021a; Saleena et al. 2021b). It is important to note that the genetics, geographical origins, agricultural practices, and harvesting seasons of individual plant species all influence the unique chemical makeup of their respective EOs (Juliano et al. 2000). EOs mostly consists of terpenoid and phenylpropanoid derivatives. About 80% of the EOs from most plants are terpenoids, however, phenylpropanoid derivatives add significant flavor, piquancy, and odor to the EOs (Faleiro et al. 2003).

EOs have a useful role in the fermentation process in the rumen. EOs, due to their antibacterial qualities, can regulate rumen metabolism. Methane, a type of hydrocarbon, is the fuel source from which natural gas is created. EOs from plants including garlic, clove, eucalyptus, origanum, and others are effective in lowering methane emissions (Deepak et al. 2020a; Kumari et al. 2022). *In vitro* batch culture shows that EOs and their constituents can improve nitrogen and potential utilization in ruminants. It is believed that the effects of EOs on ruminal nitrogen metabolism are mediated by their effect on hyper-ammonia producing bacteria, leading to a decrease in amino acid oxidation and ammonia nitrogen synthesis. The natural antioxidants in EOs benefit animal health by reducing oxidative stress, and they also help the food industry by halting the oxidative processes that lead to spoilage. Natural antioxidant uses of EOs are an exciting new area of research. This is because the safety of some synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), has been called into question, especially when it comes to animal consumption. EOs have beneficial effects on antioxidative enzyme activity and inhibit the generation of reactive oxygen species, two factors in lipid metabolism in animal

tissues. High antibacterial action was indicated, and EOs are effective against both gram-positive and gram-negative bacteria (Panghal et al. 2011). The concentration of EOs is directly related to their antibacterial efficacy (Ultee et al. 2000). The effects of both room temperature and storage heat are detrimental. Among the several secondary metabolites found in many EOs, phenolic compounds have the greatest antibacterial activity. EOs are commonly used as preservatives in some foods. At concentrations of 5-20 µl/g, plant compounds like eugenol and coriander, clove, oregano, and thyme oils repressed *Listeria monocytogenes*, *Aeromonas hydrophila*, and autochonous spoilage organisms in meat products (Skandamis and Nychas 2001), while mustard, cilantro, mint, and sage oils were either ineffective or less effective (Tassou et al. 1995). Because of their perceived safety, EOs and their derivatives are widely used in the feed industry. *In vivo* results suggest that EOs are included in animal diets as a natural means of promoting growth. EOs added to animal feed enhance both meat and milk quality. EOs make the meat softer because they include oxidation proteases. Milk total bacterial and somatic count, yield, and feed efficiency were all found to be enhanced when early lactation Holstein dairy cows were fed modest doses of an EOs mix consisting of clove, juniper, and oregano in the same proportion (Al-Suwaiegh et al. 2020; Deepak et al. 2020b; Patange et al. 2022a: Patange et al. 2022b).

EOs have been shown to have several positive benefits on livestock health and productivity, and those are the focus of this review. It tells us everything we need to know about the role of EOs in animal nutrition, from their effect on rumen fermentation and the digestive system to their antioxidant and antibacterial properties and their ability to modulate the immune system.

2 Essential oils (EOs)

Paracelsus von Hohenheim, a Swiss medical reformer, is credited with coining the word "essential oil" somewhere in the 16th century. He is also credited with coining the name of a beneficial component in the Quinta essential medication. Even though spices have been employed as preservatives, perfumes, and flavors in food since antiquity, turpentine oil is the only EO recorded in Roman and Greek history. A few thousand years ago, civilizations in the East, including Persia, India, and Egypt, began using distillation as a means of EO extraction. Pharmacological effects were not documented in pharmacopeias until the 13th century, and only then did pharmacies begin producing them. Before the development of the London market in the 16th century, European countries had little use for EOs (Dhama et al. 2014; Dhama et al. 2018; Dosoky and Setzer 2018; Kumar et al. 2022a; Kumar et al. 2022b). In the eighteenth century, when Europeans first settled in Australia, records began to appear detailing the medical use of tea tree oil (Carson and Riley 1993; Aziz et al. 2018). EOs are named after the fragrant properties of the plants they were extracted from. Since "essential oil" is a poorly defined term that dates back to the medieval pharmacy, the term "volatile oil" is being proposed as a replacement (Hoffmann 2020).

EOs are potent because they contain more characteristics than dry herbs and perform an essential function as a natural protection for host plants. Eos are fascinating not only for their therapeutic effects, but also for their antimicrobial, antiviral, antifungal, and bacterial characteristics. They are not only aesthetically pleasing but also have excellent scent and flavor preservation qualities (Bakkali et al. 2008; Aziz et al. 2018). EOs are also known as ethereal or volatile oils and are aromatic lipophilic liquids that are obtained from plant parts like flowers, leaves, twigs, bark, seeds, buds, herbs, fruits, roots, and wood (Miguel 2010). EOs are mainly obtained through enfleurage, expression, extraction, or fermentation but for the commercial production of EOs, the most commonly used method is steam distillation. Some components of the EO can be obtained not only from the plant parts but also from synthetic manufacture. As we have seen above, we know about 3000 EOs of which 300 are used in the commercial markets for flavouring and fragrance. The increase in the interest of scientists in these compounds is not only due to their antibacterial properties but also due to insecticidal, antiviral, anti-toxigenic, antimycotic, and antiparasitic properties. These properties may be the functions of the compound in the plants. EOs are natural and complex oils not only that they can be considered also multi-component oils (Brenes and Roura 2010; Hoffmann 2020).

Nowadays, EOs are used for a wide range of purposes. ‘DMC Base Natural’ is a commercially available natural food preservative made from 50% EO (sage, citrus, and rosemary) and 50% glycerol. Protea 1 and Protea 2 by 'Bravia Corp.' are a standardized extracts of a proprietary blend of herbs (Cutter et al. 2000). Perfumes and aftershaves are only two examples of how EOs can be employed in the beauty industry. Pure molecules of EOs are employed in a variety of applications, including animal feed supplements, antiseptics, and oral care products. Due to their enhanced biological activities as antioxidant, antibacterial, and antifungal, EOs are of tremendous interest in the cosmetic, pharmaceutical, and food supplement industries (Leherbauer and Stappen 2020).

3 Chemical composition and bioactive in essential oils

The genetics, geographical origins, farming practices, and harvesting seasons of different plant species all affect the unique chemical makeup of their respective EOs. There will be a wide variety of similar-looking chemical compounds produced by these variants. The addition of fertilizers like phosphorus and potassium nitrogen will alter the EOs' chemical makeup and raise their yield. The enzymes that catalyze the biosynthesis of organic compounds like terpenoids will proliferate in response to fertilizers (Jerkovic et al. 2001). *Rosmarinus officinalis* EOs derived through irrigation

show more variety than EOs obtained from non-irrigated plants such as linalyl-isobutyrate and trans-verbanol, indicating that irrigation will impact EO quality. EOs extracted from herbs at or around the end of blooming will be the most potent in terms of antibacterial action. Elements of EOs, such as enantiomers, have demonstrated impressive antibacterial efficacy (Marino et al. 1999). Major components of EOs are phenylpropanoid and terpenoid derivatives. Most plants' EOs include around 80% terpenoids, however, the presence of phenylpropanoid gives the EOs its notable flavor, piquant, and smell. These are the two principal metabolites that have been most successfully extracted (Brenes and Roura 2010; Hoffmann 2020; Leherbauer and Stappen 2020). The selected EOs' main features are listed in Table 1, and their major bioactive components are depicted in Figure 1.

EOs are predominantly found in plant tissues, specifically in their glands and intercellular spaces. The seeds and blooms of plants usually contain the highest concentrations of EOs. The majority of EO ingredients evaporate easily in water steam. Although "Esters" are often cited as the main culprits behind the aroma and fragrance of fruits and flowers, it is important to note that other components may also contribute to the taste and scent. The remaining parts are a complicated combination of EOs such as alcohols and carbonyl, as well as hydrocarbons. These chemicals are mostly found in two

Table 1 Significant essential oils and their major bioactive components

Plant name	Botanical name	Major bioactive component	Percentage composition	Major plant part
Thyme	*Thymus Vulgaris*	Cymene	8.41	Leaves Flowers
		Thymol	47.59	
		Terpinene	30.90	
Ginger	*Zingiber officinale Rosc*	Neral	4.9	Roots
		β-Eudesmol	5.4	
		Ar-curcumene	14.5	
		Camphene	14.1	
		β-Bisabolene	22.1	
Bergamot	*Citrus bergamia*	Limonene	59.21	Bark
		Linalyl acetate	16.83	
		Linalool	0.51	
		β-Pinene	4.38	
Sandalwood	*Santalum album*	β-santalol	18	Heartwood
		α-santalol	43	
Lavender	*Lavandula officinalis*	β-farnesene	0.9	Leaves Flower spikes
		Linalyl acetate	4.6	
		β-caryophyllene	0.6	
		Lavandulol acetate	0.8	
		Linalool	28	
Eucalyptus	*Eucalyptus citriodora*	Citronellol	14.5	Leaves
		Citronellal	72.8	
Jasmine	*Jasminum grandiflorum*	Benzyl benzoate	20.7	Flowers
		Benzyl acetate	23.7	
		Linalool	8.2	
		Eugenol	2.5	
		Geranyl linalool	3.0	
Peppermint	*Menthae piperitae*	Limonene	2.6	Fresh leaves
		Menthol	40.7	
		Menthyl acetate	4.2	
		Menthone	23.4	
		1,8-Cineole	5.3	
Cinnamon	*Cinnamomum zeylanicum*	Eugenol	7.2	Inner bark
		Cinnamaldehyde	77.1	
Clove	*Syzygium aromaticum*	β-Caryophyllene	11.54	Dried flower buds
		Eugenol	76.23	
		Caryophyllene oxide	4.29	
Rosemary	*Rosemarinus officinalis*	1,8-Cineole	43.6	Whole plant
		β-pinene	5.0	
		α-pinene	7.4	
		Camphor	12.3	

Source: Brenes et al. 2010; Aziz et al. 2018; Plant et al. 2019; Hoffmann 2020; Leherbauer and Stappen 2020

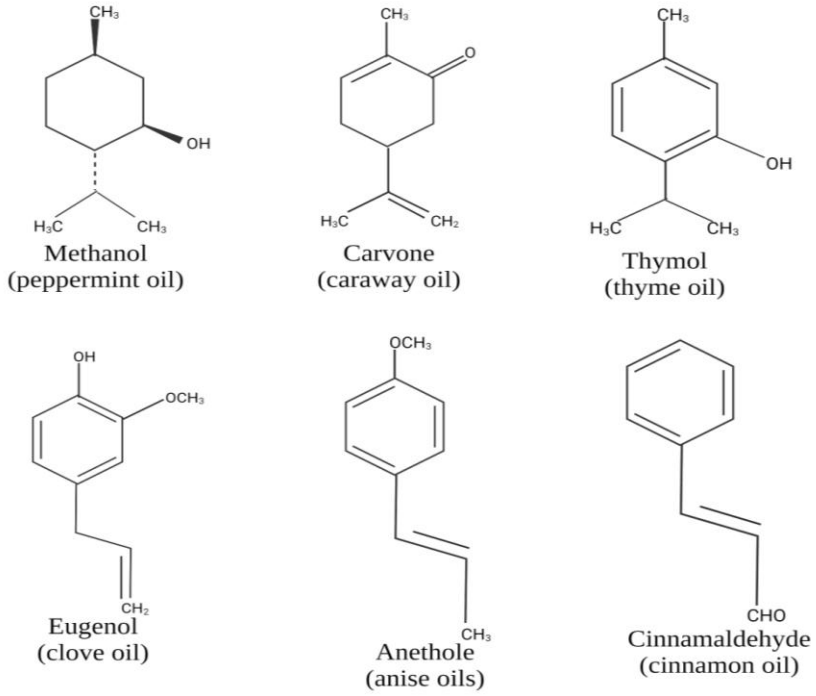

Figure 1 Major bioactive component of essential oils

classes of natural products: terpenes and phenylpropanoids (Plant et al. 2019). Some plants use the shikimic acid pathways to produce phenylpropanoids, or cinnamic acids, from tyrosine and phenylalanine. Phenylpropanoid molecules have a six-carbon based aromatic phenol group coupled primarily to 3-carbon propene tailoring cinnamic acid. Ethanol, myristicin, vanillin, safrole, and cinnamaldehyde are all phenylpropanoids. Both anethole and myristicin have been shown to have anticancer qualities, with the latter also exhibiting anti-inflammatory and anti-proliferative effects, while safrole encourages biological processes such as antibacterial and antifungal ones (Sharma et al. 2019; Eid and Hawash 2021).

Isopentenyl diphosphate is the progenitor of all terpenes, including primary and secondary metabolites numbering over 25000 substances. Named thus because the first few members were first extracted from turpentine, "terpene" refers to any of several related compounds. Due to the thermal decomposition of terpenoid compounds into the alkene gas isoprene, the monomer with five carbon atoms is most commonly referred to as isoprene. Hemiterpenoids, monoterpenoids, sesquiterpenoids, diterpenoids, sesterterpenes, triterpenes, and tetraterpenes are the seven basic categories into which terpenoids fall based on the number of isoprene units they contain. Both antibiotic-resistant and antibiotic-susceptible bacteria are susceptible to the antimicrobial effects of terpenes, which include the promotion of inhibition of protein and DNA synthesis and the prevention of cell rupture (Plant et al. 2019; Hoffmann 2020). Terpenoids, a class of secondary metabolites found in medicinal plants, play a crucial role in the plants' ability to ward off disease. This is because terpenoids, particularly monoterpenoids, have antibacterial properties, disrupt metabolic processes, and in some cases even have pest-repellent effects (Burt 2004; de Matos et al. 2019). Leucine, valine, polyketides, sulphur components, methionine, lipids, isoleucine, and alanine are some of the most common amino acid-derived components found in EOs such as allicin, cis-jasmone, jasmonic acid, and methyl jasmonate (Pandey et al. 2017).

4 Effect of essential oils on rumen fermentation

EOs have a useful purpose in the rumen fermentation process. It may have something to do with ruminants' ability to generate high-quality protein via synthesis. Methane and ammonia, both of which are produced during microbial fermentation, are both significant environmental pollutants and a by-product of the process (Aziz et al. 2018). EOs, due to their antibacterial qualities, regulate rumen metabolism. Methane is the most abundant component of natural gas and a type of hydrocarbon. It has a 21 times greater impact on the potential for global warming than carbon dioxide does, making it another greenhouse gas. Depending on diet and feed, ruminants can save 2% to 12% of their total digested energy by reducing the amount of methane produced in their digestive tracts (Boadi et al. 2004; Cobellis et al. 2016). Thus, the use of EOs to reduce methane emissions is beneficial for both animals and the environment (Benchaar and Greathead 2011). The "hyper-ammonia-producing bacteria" in the rumen are responsible for the

degradation of amino acids to ammonia through the influence of protein metabolism and a decreased rumen ammonia range, resulting in the efficient management of dietary nitrogen (Mclnotosh et al. 2003; Calasmiglia et al. 2007). In one of the experiments commercial blend of essential oil decreased methane production in Holstein-Friesian cows and increased milk yield (Hart et al. 2019). As they improve VFA production and utilization they can improve the production performance of animals and the quality of products (Simitzis 2017; Hart et al. 2019). Besides decreasing methane production, essential oil supplementation has reduced gross energy consumption, apparent total tract digestibility, and rumen valerate concentration in beef heifers (Jiménez-Ocampo et al. 2022).

Many EOs, including garlic, clove, eucalyptus, origanum, etc., can help achieve the goal of decreased methane generation. The importance of EOs in ruminal fermentation has not been exaggerated, but at high concentrations, these substances are toxic to the beneficial bacteria in the rumen as well as the targeted microorganisms (Benchaar and Greathead 2011; Benetel et al. 2022). EOs can be tested in vitro to determine how they influence factors including food, time, and pH to reduce methane production, total volatile fatty acid concentration, and feed digestion. The *in vitro* effects of EOs on ruminal fermentation at high levels could be applied realistically in vivo, having an inhibiting influence on feed palatability, digestion, and animal output. Positive *in-vitro* responses will be achieved despite the rising cost and toxicity associated with EO production (Beauchemin et al. 2009; Poudel et al. 2019). There will be no change in consumption, average daily procures, or concentration of total volatile fatty acids and rates after the main observation of dietary complement of ruminants with EOs in the form of single compounds and mixes. As a result, the benefits of rumen methanogenesis do not convince. Rumen microorganisms' capacity to metabolize and modify volatile oils provides a plausible explanation (Benchaar et al. 2011; Aziz et al. 2018; Ku-Vera et al. 2020). Figure 2 provides the impact of EOs on microbial fermentation in the rumen.

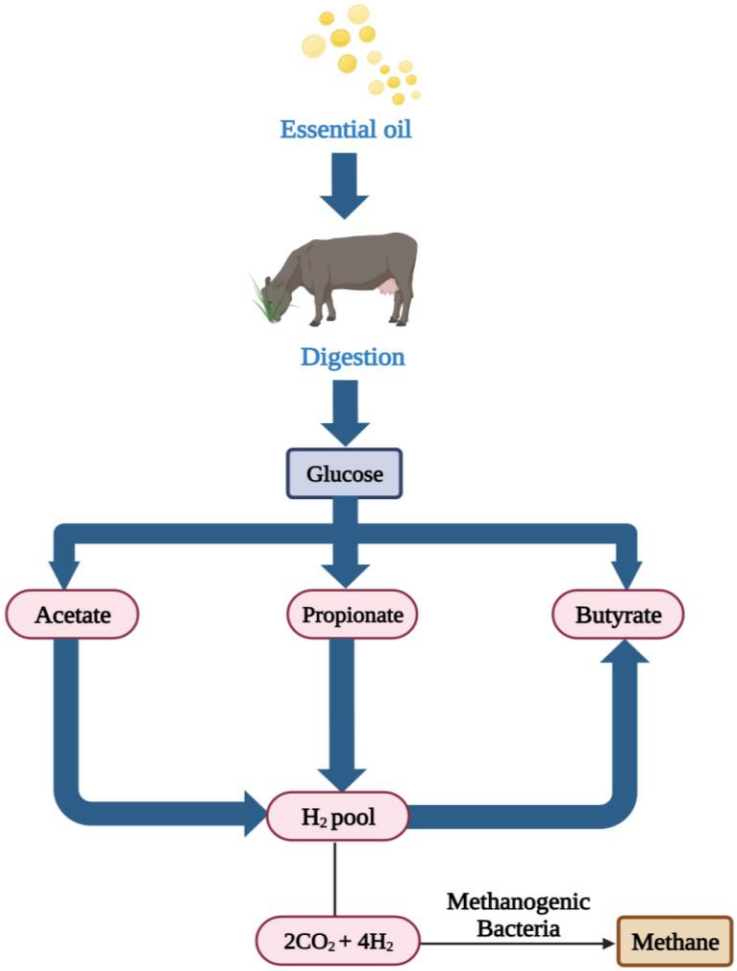

Figure 2 Impact of essential oils in the microbial fermentation pathway in the rumen

Table 2 Effects of essential oils or their components on rumen characteristics

Essential oil or component	Level of incorporation	Animal	Effects on rumen metabolism
Coriander oil	14 ml/day	Domesticated cow	Increased milk yield, nutrient, and digestibility
Mood essential oil	25 g/day	Domesticated cow	Improved carcass oil, cow quality, milk yield, and feed efficiency
Thyme	100 g/day	Domesticated cow	Increased milk yield
Cashew and caster	2 g/day	Domesticated cow	Alter the ruminal pH
Orgenao	100-150 mg/kg	Domesticated cow	Improved feed efficiency and reduced incidence of diarrhoea
Blend of essential oils	1 g/day	Domesticated ruminants	Increase Immunity; improvement and better feed efficiency
Thyme	1.25 g/kg	Domesticated sheep	Increased nutrient; metabolism and rumen fermentation

Source: Greathead 2003; Cobellis et al. 2016; Aziz et al. 2018; Poudel et al. 2019; Ku-Vera et al. 2020; Dorantes-Iturbide et al. 2022

4.1 Dietary ruminal effects of essential oils

In response to the emergence of antibiotic-resistant bacteria and the spread of these pathogens from livestock to humans, the efficacy of pharmacological materials in ruminants has improved. Alterations to rumen metabolism that increase feed productivity and animal health have been lauded in a variety of ways. Processing of microbial activity takes place in the rumen to build an energy source with antibacterial characteristics to substitute antibiotics (Benchaar et al. 2011; Ku-Vera et al. 2020). EOs and their constituents can improve nitrogen and potential ruminant utilization through in vitro batch culture. It is believed that the effects of EOs on ruminal nitrogen metabolism are mediated by their effect on hyper-ammonia producing bacteria, leading to a decrease in amino acid chemical alterations and ammonia nitrogen synthesis. There is conflicting evidence regarding the effects on methane generation, however, current data suggests that it is possible to select EOs or other hand-active components that selectively inhibit ruminal methanogenesis (Benchaar et al. 2008; Dorantes-Iturbide et al. 2022). There is currently no evidence that aromatic plant EOs have antibacterial activity in ruminants *in vivo*. EOs have been utilized to alter rumen metabolism, leading to greater feed efficiency and increased animal productivity (Greathead 2003). Table 2 displays how EOs or their constituents changed rumen characteristics.

5 Effects of essential oils on the digestive system and the gut microbiota

Up to this point, we have looked at how EOs in the diet affects gut flora, enzyme function, and performance. EOs naturally arise to destroy harmful bacteria and stimulate beneficial bacteria like *Lactobacillus* spp., which regulates enzyme function and protects intestinal villi, and all without having any major practical effects on reaching a healthy body weight. Similarly, feed ratio transformation is typically improved upon (Dorantes-Iturbide et al. 2022). By enclosing low-molecular-weight peptides, a bacterial group called Lactobacilli has been shown to boost the enteric immune system, which in turn boosts resistance to disease and the immune system's ability to activate (Muir et al. 2000; Chandran 2021a). The rising numbers of Lactobacilli put up resistance against pathogenic bacteria by enhancing their receptor usage among themselves. Antimicrobial properties of EOs have been shown to prevent the attachment, colonization, and proliferation of *Escherichia coli*, *Clostridium perfringens*, and *Eimeria tenella* in the gut (Jamroz et al. 2006). Reducing the prevalence of pathogenic bacteria in the intestine and maintaining a healthy equilibrium between beneficial and harmful bacteria has been shown to enhance the epithelial cells' ability to revitalize the villus (Chandran 2021a; Benetel et al. 2022).

For livestock, the gut is a crucial organ used in the treatment of nutritional absorption. EOs mitigate the negative effects of oxidative stress on the digestive tract by supporting the health of the microorganisms that live there. Accordingly, improved intestinal health for absorption, which extends villus length and surface area in the gut, and increases growth and performance parameters, is crucial (Windisch et al. 2008; Franz et al. 2010; Zeng et al. 2015). In addition, digestive secretions like saliva, bile, mucus, etc., and enzymes like trypsin, amylase, lipase, etc., will increase incomplete infuriation of the epithelial tissues and decrease the depth of the crypts in the ileum. Increasing the time that food spends in the stomach has a positive effect on nutrient absorption (Platel and Srinivasan 2004; Chandran 2021a). A hypothetical explanation works perfectly to account for the discrepancy. However, approval for a range of EO types and concentrations is available. There is potential for making advantage of the wide range of bioactive component concentrations present in plants across parts, including those present in barks, leaves, flowers, etc. Complementary species and their range will be utilized as well. The content of the food, the amount of feed consumed, the level of hygiene practiced, and the state of the ecosystem are only a few of the environmental factors that influence how effective EOs are in boosting agricultural output (Brenes and Roura 2010; Poudel et al. 2019).

6 Antioxidant effects of essential oils

Many of the EOs also have antioxidant properties. The natural antioxidants found in EOs benefit animal health by reducing the effects of oxidative stress and the food industry by halting the oxidative processes that lead to spoilage. Using EOs as natural antioxidants is a fascinating area of study. This is because the dangers of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) to animals are contested. The ability to donate an electron to free radicals and the removal of the unpaired electron from the aromatic structure are the two main elements that govern the antioxidant processes of EOs (Fernandez-Panchon et al. 2008; Aziz et al. 2018; Hoffmann 2020). Sanchez-Moreno (2002) has found many techniques for assessing the antioxidant capabilities of phenolic components in EOs. Compared to vitamins E, C, and carotenoids, phenolics are described as being more powerful antioxidants. Essential oils alone or in association with other agents like cobalt have proven antioxidant activity and hence improve antioxidant capacity thereby lowering oxidative stress hence improving production performance (Lei et al., 2018; Seyidoglu et al., 2021).

There is a positive correlation between the degree of unsaturation of fatty acids and the oxidative damage caused by the lipids found in EOs (particularly phospholipids). In contrast, the polyunsaturated fatty acids (PUFAs) found in EOs help keep the permeability and fluidity of cell membranes. Hydroperoxides are formed when the proxy radicals in EOs combine with the polyunsaturated fatty acids, and these products then undergo a decomposition process to yield volatile non-radical aromatic chemicals. Animal products lose some of their nutritional content and spoil more quickly because of these chemicals (Pisoschi and Pop 2015; Poudel et al. 2019). There is a rising market for antioxidants that come from natural sources. Although some of them, like BHA, BHT, tert-butylhydroquinone (TBHQ), etc., are employed to postpone or halt the adverse consequences of lipid peroxidation in EOs, they do so by scouring the chain for peroxyl radicals. The diet of an animal plays a crucial role in inhibiting the development of free radicals in living organisms and their byproducts. Based on the nutritional safeguards and inferences of oxidizing stress, the optimal utilization of antioxidant levels in animal feed is proposed. The quality of meat and other animal products is improved when they are fed a diet containing both rosemary and oregano EOs (Amorati et al. 2013; Valdivieso-Ugarte et al. 2019).

Since EOs have more favorable redox characteristics and a more stable chemical structure, they are a rich source of natural antioxidants such as phenolic compounds. EOs have beneficial effects on the activity of antioxidative enzymes and prevent the generation of reactive oxygen species, two mechanisms by which they influence lipid metabolism in animal tissues. Depending on the concentration and nature of the EO, the membrane and organelle integrity of cells can be compromised (Bakkali et al. 2008; Aebisher et al. 2021). Incorporating EOs into one's diet is a straightforward and appropriate method of introducing natural antioxidants into phospholipid membranes, where they decrease oxidative reactions by preventing radical production and boosting their breakdown at localized regions (Govaris et al. 2004; Benetel et al. 2022).

7 Antimicrobial effects of essential oils

The use of synthetic chemicals aids in the prevention of spoiling and the spread of harmful germs in animal products. As a result of concerns over teratogenicity, carcinogenicity, acute toxicity, etc., the use of these substances has been curtailed. To this purpose, it was suggested that antimicrobial-containing animal products benefit from the addition of naturally occurring substances that improve their quality and longevity on store shelves (Faleiro et al. 2003). EOs are widely employed as functional chemicals in the pharmaceutical industry and for food flavoring (Corbo et al. 2009; Negi 2012). While most EOs are GRAS (Generally Recognized as Safe) ingredients, flavor concerns may prevent their widespread usage as food preservatives (Lambert et al. 2001; Leyva-López et al. 2017). EOs have proven antimicrobial effects (Evangelista et al., 2022). The antimicrobial effects of multiple bioactive compounds in EOs are not due to a single mechanism of action, but rather to the additive effect on many different boards in many different areas of the cell (Burt 2004; Ebani and Mancianti 2020). They influence zootechnical indices (Evangelista et al. 2022). It has been hypothesized that their efficacy is contingent on factors such as pH, chemical structure, concentration, or specific bioactive chemicals, in addition to the population and microbial species involved. They modulate gene regulation (Evangelista et al. 2022). Natural antimicrobials in food systems, including EOs, have been the subject of preliminary research into their antimicrobial effects (Brut 2004). The usefulness of EOs is reduced by the presence of lipids, water activity, pH, proteins, and enzymes, all of which are crucial components of food systems (Burt 2004; Sakkas and Papadopoulou 2017; Winska et al. 2019).

Since EOs are hydrophobic, they are useful in cleaving the lipid bilayer of mitochondria and bacteria, which results in changes to the cell's osmotic pressure due to disruptions in membrane integrity and ion transport systems. When the H^+ and K^+ ion gradients suddenly degenerate and the intracellular ATP pool depletes, it is easier to detect because of the fall in ATP production and a large amount of hydrolysis. Both the decrease in trans-membrane electric potential and the increased permeability to protons have an inhibitory effect on bacterial growth. When the bacterial tolerance limit is exceeded, cell death may occur as a result of a dramatic reduction in the concentration of essential chemicals and ions (Burt 2004). The transport of ions across the

cytoplasmic membrane is disrupted due to a hydroxyl group in a phenyl ring and the ring's capacity to release its proton (Ultee et al. 2002; Burt 2004).

Disruption of the cell membrane, suppression of ATPase activity, and the release of intracellular ATP are just some of the antibacterial processes of EOs compounds including thymol, eugenol, and carvacrol (Brut 2004; Lambert et al. 2001). These substances tend to render the cell membrane permeable because they enter the phospholipid bilayer and form associations among the fatty acid chains (Ultee et al. 2000; Lambert et al. 2001). As a result, passive permeability increases as the membrane expands and becomes more brittle, and the fluidity of the membrane rises (Negi 2012). Hydrophobic binding of thymol to proteins in bacterial membranes alters their permeability. Gill and Holle (2006) reported that EOs inhibits ATP and ATPase activity inside *E. coli* O157:H7 and *Listeria monocytogenes* cells without causing any obvious changes to the membrane. Improved membrane permeability and cytoplasm leakage were also reported by Kim et al (1995), while Farag et al (1989) noted that cinnamaldehyde might interact with enzymes on the cell surface. These interactions can also lead to membrane distraction, impeding the diffusion of the proton motive force, or they can inhibit the activity of enzymes necessary for amino acid production.

Among the various secondary metabolites found in EOs, phenolic compounds have the strongest antibacterial activity. Consistent with their hydrophobicity, they primarily target the bacteria's cell membranes. Phenols alter membrane properties by rearranging lipid and protein components and stimulating the outflow of potassium ions. Catechins have been linked to liposome leakage due to the disruption of membrane integrity. Catechins and epigallocatechin gallate affect the outer polar area of lipid bilayers of the liposomes, which likely contributes to membrane rupture. Similar antimicrobial effects of vanillin were also demonstrated at the cell membrane and via cell inhibition in a variety of flora bacteria. Terpenes can also cross the membrane and alter the lipid structures' capacities. Phenolic chemical manipulation has been linked to the cell wall (Valdivieso-Ugarte et al. 2019; Winska et al. 2019).

Research has shown that EOs have antibacterial activity against both gram-positive and gram-negative bacteria, and this antimicrobial activity is quite potent (Panghal et al., 2011). It is well-accepted that EOs are slightly more efficient against gram-negative food spoilage bacteria and food-borne pathogens than they are against gram negative bacteria (Burt. 2004). Given that gram negative bacteria have an outer membrane enclosing their cellular wall, which confines the imposition of hydrophobic substances via lipopolysaccharide-protecting lipid bilayers, it stands to reason that they are more resistant to the action of antibacterials (Vaara 1992). Some studies with EOs have shown, however, that gram-negative bacteria can be more difficult to cultivate (Wilkinson et al. 2003). One of the most delicate bacteria is *Aeromonas hydrophila*. When added to Greek salad appetizers such as taramasalata and tzatziki, it was found that mint (*Mentha piperita*) EOs had a more potent antibacterial impact on *Salmonella enteritidis* than *Listeria monocytogenes* (Tassou et al. 1995). No significant difference in antimicrobial activity was found between gram-positive and gram-negative bacteria after 24 hours, while growth inhibition was typically prolonged to 48 hours with gram negative bacteria compared to gram-positive bacteria (Ouattara et al. 1997). There was no difference in susceptibility between gram-positive and gram-negative microbes in a report that tested 50 commercially available EOs against 25 taxa (Deans and Ritchie 1987). In a later investigation using newly distilled EOs, the same testing procedure and bacterial isolates were used, and it was found that gram-positive bacteria were more susceptible to two of the EOs tested, and equally sensitive to four others, compared to gram-negative ones (Dorman and Deans 2000; Valdivieso-Ugarte et al. 2019).

The antibacterial efficacy of EOs is dose-dependent. The effects of both room temperature and storage heat are detrimental. Also, the shape and arrangement of the tannins' ortho-phenolic hydroxyl groups can have a chilling influence on their organic effect. EOs are widely used as preservatives in a small number of food items. EOs of eugenol, coriander, clove, oregano, and thyme, when used at 5-20 µl/g, inhibited the growth of *Listeria monocytogenes*, *Aeromonas hydrophila*, and autochonous spoilage microbes in meat products, whereas oils of mustard, cilantro, mint, and sage were ineffective. Overly fatty meats may diminish the effectiveness of EOs. EOs of mint and cilantro were not as potent as they usually are in pate (30% fat) and a ham coating made of canola oil (Brochot et al. 2017; Rapper et al. 2021).

EOs can be used in food systems to minimize foodborne infections, hence extending the shelf life of animal products (Calo et al. 2015; Anand et al. 2022). Nutritional additions of EOs in animal feed aid in the establishment of natural microorganisms, which are then kept in the tissues. When combined at a concentration of 5-20 µl/g, mint oil prevented *Salmonella enteritidis* growth in low-fat yoghurt, among other dairy products. A rise in yoghurt starter culture species was observed when mint oil was employed at concentrations of 0.0.5-5 µl/g; however, cinnamon, cardamom, and clove oils were shown to have a more potent effect. EOs have been proven to be highly effective as food preservatives, and their implementation is anticipated on a wide range of food items containing a wide variety of ingredients (Cannas et al. 2016; Valdivieso-Ugarte et al. 2019; Rapper et al. 2021). Figure 3 provides evidence of the effectiveness of EOs against bacteria and other pathogens.

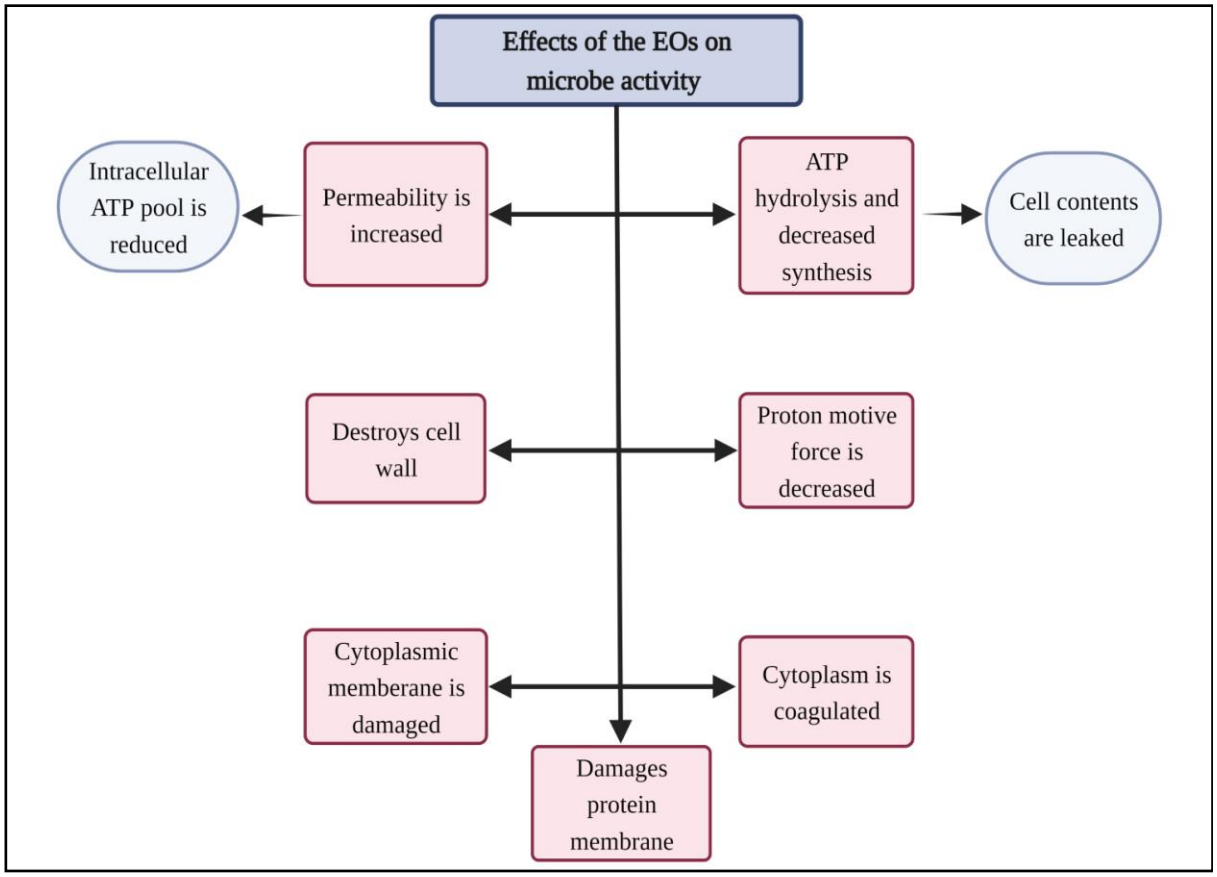

Figure 3 Anti-microbial effects of essential oils in livestock

8 Inflammation reduction by essential oils

When body tissues are harmed or infected, they trigger a physiological response known as inflammation. The production of cell adhesion molecules and the release of pro-inflammatory cytokines are both triggered by inflammation, which in turn increases the permeability of mucosal endothelial cells (Sandner et al. 2020). EOs have shown antiinflammatory nature in animal studies (Schabauer et al. 2017). They can have direct or indirect anti-inflammatory actions via immunomodulatory or counter physiological and inflammatory mechanisms including hyperemia and blocking synthesis and secretion of inflammatory mediators (Nehme et al. 2021). As a result of their antioxidant properties and their ability to signal through regulatory transcription factors called cytokines, EOs can influence the expression of both anti-inflammatory and pro-inflammatory genes. Hyperemia, for example, will increase leukocytes and anti-inflammatory actions while also blocking the secretion and synthesis of inflammation mediators like nitric oxide, histamine, and pro-inflammatory cytokines, acting at multiple levels to reduce inflammation (Asif et al. 2020).

EOs have been used for quite some time for their immunomodulatory effects on human patients. *Lavandula angustifolia* serves as an example due to its anti-inflammatory properties, since it can promote phagocytosis and lower the pro-inflammatory cytokinins (Giovannini et al. 2016). Even though there is less information on the degradation rate of EOs and their constituents in the gastrointestinal tract and how they should be prepared and fed to animals, interest in their use continues to rise. Previously, it had been established that in *Mollugo verticillata* EO can lower the number of bacteria in the glands of test mice infected with Enterococcus faecium, suggesting that EOs will enhance immunity to lessen inflammation. Inflammation of the mammary glands, often known as mastitis, is common and can be very expensive to treat. Most cases of mastitis are brought on by an intramammary infection caused by bacteria or other microbes. Bovine mastitis describes bladder irritation in cattle (Salehi et al. 2018; Chandran and Radhakrishnan 2019; Sandner et al. 2020; Chandran 2021a; Chandran and Athulya 2021). The infection of the udder by viruses, yeast, or bacteria causes this disease (Chandran 2021b; Anand et al. 2022). Mastitis and mammary gland infection occur when a pathogen invades the milk supply and multiplies beyond the control of the teat canal barriers. If the immune system is overworked or compromised during parturition, or if the pathogen can evade it, the resulting mastitis is chronic or severe. However, if the immune system can mount an effective

Table 3 Effectiveness of various EOs in treating bovine mastitis

Plant	Use
Cymbopogon citratus	Shows high antimicrobial activity in every type of bacteria
Minthostachys verticillata	Inhibits all bacterial isolates' growth
Origanum vulgare	Showed active antimicrobial activity; Somatic cells are reduced and *E. coli* was not present in milk
Copaifera spp.	Reduced antimicrobial activity
Origanum floribundu	Exhibit high anticandidal activities
Cymbopogon citratus	Exhibit satisfactory antimicrobial activity
Alpinia zerumbet	Strains showed extreme sensitivity to EOs at 100mg/mL
Cinnamomum zeylanicum	Exhibit high inhibiting action against the bacterial strains
Syzygium aromaticum	High antimicrobial activity and biofilm formation is affected
Eucalyptus globulus	Has shown small inhibitory actions in time
Thymus vulgaris	Effective against algal strains

Source: Reshi et al. (2017); Valdivieso-Ugarte et al. (2019); Sandner et al. (2020); Chandran (2021a)

defense, the resulting mastitis is just temporary and mild (Chandran et al. 2021a; Chandran et al. 2021b; Lejaniya et al. 2021a; Lejaniya et al. 2021b; Sharun et al. 2021).

The fundamental benefit of EOs is their all-natural nature, as long-term use of conventional medications will have resulted in resistance (Chandran et al. 2022). *Terminalia chebula* ethyl acetate extract has been shown to have antibacterial activity against milk bacteria associated with subclinical mastitis, and similar activity to amoxicillin (Chandran and Arabi 2019). *Sanguisorba officinalis* ethanolic extract is used in traditional Chinese medicine and has been shown to prevent the biofilm formation of *Staphylococcus aureus* and methicillin-resistant strains of the bacteria. Vaseline in EOs has potent antibacterial properties, as evidenced by its ability to speed up wound healing by a factor of 100 when combined with thymus EO (Reshi et al. 2017; Sandner et al. 2020). Excitingly, the extracts led to noticeable improvement after treatment. *Adiantum capillus*, also known as *Fumaria indica*, has been shown to produce excellent clinical results. Plants with these properties are employed as adjuvants in antimicrobial treatment, specifically for the treatment of cow mastitis (Valdivieso-Ugarte et al. 2019). Table 3 might help us learn more about the effectiveness of EOs in treating bovine mastitis.

8.1 Limitations in using essential oils for inflammation

When using EOs for medical purposes, there are a few caveats to keep in mind. The mix of secondary metabolites, responsible for the plant's medicinal and biological capabilities, will change according to environmental parameters such as climate management, phenological stages, and soil (Burt 2004; Andrade et al. 2017). The use of EOs as a substitute for mastitis also faces the challenge that their commercial scale manufacturing will require a substantial volume of plant biomass. We should also evaluate the antimicrobial activity of the EOs to compare and utilize it because the different methods of application used will have different impacts, so this is a time-consuming task that needs careful consideration before being undertaken (Amber et al. 2018; Valdivieso-Ugarte et al. 2019). Clinical applications in livestock can be appropriate after proper in vivo evaluation in experimental animals.

9 EOs in animal nutrition

EOs and their bioactive components play an important role in the feed industry because of their perceived safety. *In vivo* results suggest that EOs are included in animal diets as a natural means of promoting growth. The quality of meat and milk can also enhance by the use of EOs in animal feeds. Meat quality, shelf life, and even antioxidant activities like reduced lipid peroxidation can sometimes be improved by feeding animals low dosages of EOs (between 1.33 and 4 g/animal/day). Oxidation proteases in EOs improve meat softness. 3.5 grams of EOs per animal per day is the standard recommendation for feedlot cattle (Rivaroli et al. 2016; Chandran et al. 2019; Chandran 2021a). But a pro-oxidant impact is caused by higher concentrations of EOs, which is harmful to the health of animals. This occurs because mitochondrial permeabilization can be induced by large doses, causing an alteration in electron flow and a subsequent increase in free radical production within mitochondria. Absorption, distribution, metabolism, and excretion (ADME) theory does not evaluate the transfer of metabolites from one component of EOs to another to determine whether or not EOs improve meat quality when fed to animals. As a result, many studies cannot attribute changes in meat quality to the direct or indirect impacts generated by multiple chemicals in EOs at the meat level (Omonijo et al. 2018;

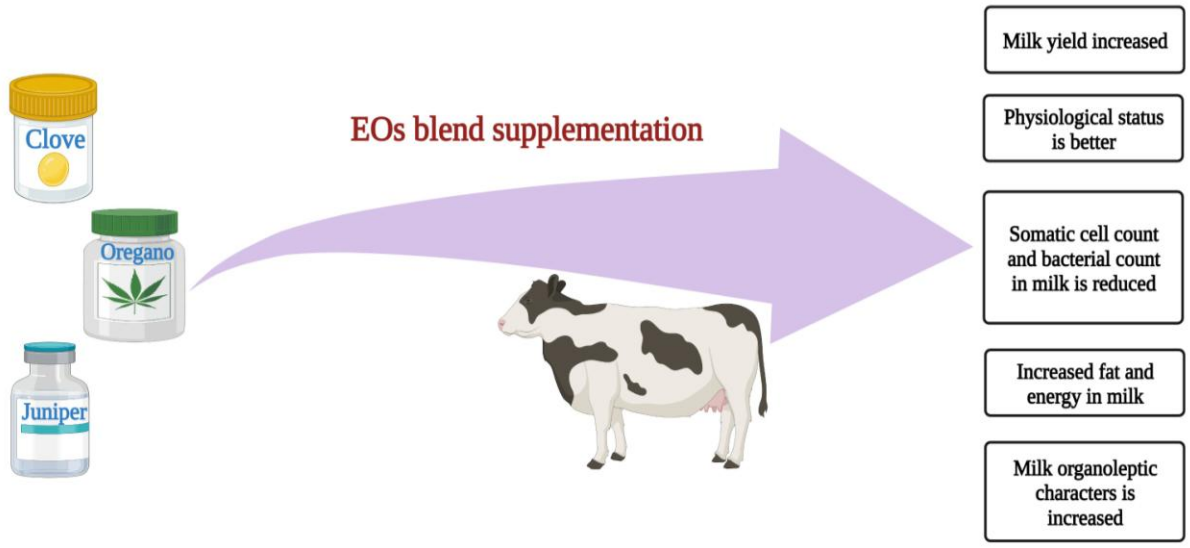

Figure 4 Impact of a feed supplement containing essential oils on dairy cow productivity

Valdivieso-Ugarte et al. 2019; Nehme et al. 2021). Al-Suwaiegh et al. (2020) showed that early lactation Holstein dairy cows fed modest dosages of an EOs blend consisting of clove, juniper, and oregano in the same proportion had an increase in milk total bacterial and somatic count, yield, and feed efficiency. Because EOs in low doses does not affect microbial populations in the rumen, this is the case. Milk production is not affected by giving a blend of EOs to eight lactating sheep, but the resulting fermentation is (El-Essawy et al. 2021). Figure 4 shows how the EOs blend affected the productivity of dairy cows.

The rosemary EOs in the diet has improved the lamb's flavor and aroma. It has been revealed by El-Essawy et al. (2021) that adding Thymus EOs to the diet of dairy cows does not enhance the fermentation process. Soltan et al. (2018) created a blend of EOs that had a suppressing effect on methane generation in Santa Ines sheep. The blend included eugenol, capsicum oleoresin, carvacrol, and cinnamaldehyde. EOs given to animals might affect the monetary worth of their meat and milk by inhibiting the microbes responsible for the bio-hydrogenation of unsaturated fat. Feeding dairy goats with EOs including nails, clove, anise, and juniper increases their levels of omega-3 and conjugated Linoleic Acids (Morsy et al. 2012; Prakash et al. 2021a; Prakash et al. 2021b; Kumari et al. 2022). Kholif et al. (2018) found that supplementing Farafra ewes with a mixture of thymus and capsicum EOs and fibrolytic enzymes increased their milk yield, fat content, and feed efficiency. Fiber digestibility is improved by this EO blend because it promotes the growth of cellulolytic bacteria, which in turn raises the amount of fat stored by the animal. There are positive results for total protozoa when EOs like linalool, diallyl disulfide, and alpha-pinene are used in animal feed for more than 70 days. Serum metabolite data show that EOs improves the antioxidant status of small ruminants' blood.

10 Conclusion and future prospects

Scientists are interested in EOs and its components not just because they are antibacterial, but also because they kill insects, viruses, toxins, fungi, and parasites. The enhanced biological activity of EOs as antioxidants, antibacterials, and antifungals provides them an edge in these industries. During and immediately following flowering is the best time to gather herbs for extracting their EOs, as this is when the EOs will be at their most potent against microbes. Supplementing ruminants' diets with EOs, both singly and in various combinations, had no effect on food intake, daily procurement rates, or total volatile fatty acid concentrations, according to the major observation. Consequently, rumen methanogenesis benefits are not universally convincing. A possible explanation is that bacteria in the rumen have the potential to transform and break down EOs. Some of the factors that will determine how effective EOs are in boosting agricultural output include dietary diversity, feed consumption rates, sanitation practices, and environmental health. Nutritional supplementation with EOs is a practical and efficient way to introduce natural antioxidants into phospholipid membranes, where they decrease oxidative reactions by preventing the production of radicals and boosting their breakdown at localized regions. EOs are very effective as food preservatives, and their incorporation into a wide range of food items and formulations is anticipated. The antibacterial and anti-inflammatory properties of EOs found in plants are utilized as adjuvants to treat bovine mastitis. Feed efficiency, yield, and the number of bacteria and somatic cells in milk all go up. This is because EOs in low doses do not affect microbial populations in the rumen. In addition to their other beneficial properties, EOs and their constituents are potent anti-inflammatories, anti-microbial, and immune system boosters. As feed additives, these chemicals are safe to consume.

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Author's contribution

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Medicalization of sexuality and sexual health: A perspective review

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ABSTRACT

Sexuality has become a medical issue in the context of aging due to a variety of aspects, such as growing life expectancy, an optimistic societal paradigm that indorses sexuality as significant for the superiority of life with age, and the medicalization of sexuality with the emergence of remedial medicines to extravagance sexual dysfunction. At any age, a reduction in the desire for sexual activity or inadequate performance of sexual intercourse is considered atypical and requires a medicinal treatment response. However, despite concerns that this is leading to an unhealthy obsession with sexuality from a medical perspective, this line of thinking is likely to continue. In this context, people can identify and take advantage of sexual problems. Sexual desire and performance are affected by normal physiological changes associated with aging in both genders. Medical experts must understand these changes to optimize sexual functioning in older patients. Sexual health can only be improved by addressing both sexual rights and enjoyment, even in the current politically charged context. Through legislation, programming, and lobbying, we may all work to enhance health, happiness, and quality of life by fostering more positive associations between sexual health, sexual rights, and sexual pleasure. This calls for not just a

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thorough understanding of the real-world consequences of these ideas' interconnectivity, but also conceptual, individual, and systemic approaches that properly acknowledge and alleviate the problems imposed on people's lives due to insufficient consideration of these links. This review describes the factors associated with aging and sexuality, the normalization and medicalization of sexual health, and unusual situations associated with aging, including institutionalized care and the prospects of elder abuse.

1 Introduction

Sexuality continues to be imperative for older people and should be recognized as a vital module of their inclusive care. The ongoing appearance of sexuality throughout the lifespan and into old age is gradually acknowledged in the medical literature and research, which indicates that sexuality remains significant for the well-being of older people (Haesler et al. 2016). The definitions of sexual health and sexuality given by the World Health Organization (WHO) have a utopian cast. WHO defines "sexual health" as more than just the absence of sickness, infirmity, or dysfunction; it encompasses a person's physical, mental, emotional, and social well-being as it relates to their sexuality. Sexual health requires an optimistic and reverent attitude towards sexual relationships and sexuality, as well as the capability to have pleasant and safer sexual practices that are free of violence, coercion, and discrimination. WHO, on the other hand, considers "sexuality" to be an integral part of the human experience that spans a person's entire life and includes such diverse concepts as sex, sexual orientation, eroticism, intimacy, reproduction, pleasure, and gender roles and identities. Sexuality is practiced and articulated in feelings, desires, beliefs, behaviors, fantasies, attitudes, practices, and relationships (Beasley 2008).

Sexual complications are usually experienced by both males and females throughout their lifespans. Even though several sexuality issues have an impact on a person's physical or mental health, people may avoid discussing their sexuality apprehensions or queries with a healthcare expert due to shame, embarrassment, or a lack of time. Considering that sexuality is an offensive subject in many cultures, it is perhaps not astonishing that people commonly access sexual information privately. They may pursue the study of sexuality via magazines, newspapers, and television, all of which may diverge in their accuracy. Moreover, individuals may seek out information related to sexuality from those nearby them, including friends, family members, or partners (Herbenick et al. 2009; Hoch 2022; Stanley and Pope 2022).

The growing medicalization of sexuality has another impact on how later-life sexuality is recognized and practiced. It is now widely recognized that the extent to which a population achieves sexual satisfaction is a major public health problem, and several treatments have expanded to include the realms of sexual pleasure and performance. This trend has negative effects on the elderly since they are less likely to engage in sexual intercourse, the "gold standard" of sexual expression, due to factors such as health and the availability of romantic partnerships. Erectile dysfunction and female sexual dysfunction are two examples of "sexual dysfunctions" that are more common in older adults (Gott 2006; Stegenga 2021).

As political currents and social movements at the national, regional, and global levels influence health, legal, and policy norms, and their effects on people's lived experience of their sexuality, sexual health, sexual rights, and sexual pleasure, it is necessary to pay attention to these dynamics. A perfect triangle of sexual health, sexual rights, and sexual happiness for all people around the world is a goal that must be actively pursued. We want to go beyond simply drawing attention to the negative by highlighting positive examples of how sexual health, sexual rights, and sexual pleasure have been and can be jointly addressed in light of the current political climate, which is characterized by local to global retreats, increased conservatism in every corner of the globe, and a shrinking space for civil society (Gruskin et al. 2019; Mollaioli et al. 2020). It is important to remember that addressing sexual rights and sexual pleasure through the lens of sexual health has been and continues to be a legitimate method of doing business for practically all actors operating at the international and state levels. By focusing on sexual health, we may bring in the health sector and reach out to programmers and legislators who might not be immediately open to the significance of rights and enjoyment. Even in the current politically charged climate, addressing sexual rights and enjoyment is essential if we are to make any progress in improving sexual health (Logie et al. 2021). Creating an environment where laws, media, and activism all support sexual health, sexual rights, and sexual pleasure can have a profound impact on people's physical and mental well-being. This requires not only an in-depth understanding of the real-world implications of the interconnectedness of these ideas, but also conceptual, individual, and systemic approaches that fully acknowledge and address the harms imposed on people's lives when these connections are not adequately considered (Heidari 2015; Miller et al. 2015; Castellanos-Usigli and Braeken-van Schaik 2019). This study examines the medicalization of sexual health and sexuality, spanning both theoretical and applied grounds.

2 Sexual Health

Sexual health has been well-defined as "the amalgamation of emotional, somatic, social, and intellectual features of sexual being in customs that are positively enriching" and is characterized as a growing area of curiosity for practitioners, investigators, and policymakers (Gott et al. 2004). Nowadays, taking care of one's sexual health is seen as fundamental to one's whole psychological and physiological wellbeing. It is a fundamental part of being human, right up there with the freedom to speak one's mind, have a family, and not be treated unfairly. Sexual fulfillment and equitable relationships, as well as access to information and services, are essential components of good sexual health to evade the peril of unintentional pregnancy, disease, or illness (Evans 2006). The term "sexual health" elevates the specialty out of the clinical domain, emphasizing lifestyle and behavior rather than clinical practice, shifting the emphasis to the patient rather than the consultant, and emphasizing prevention rather than treatment (Wellings and Cleland 2001). Various domains and their variables related to sexual health (Hensel and Fortenberry 2013) are described in Figure 1.

2.1 The Stigma of Sexual Health

Messages that promote sexual health are deemed more acceptable when they are unpretentious and don't interfere with social media practices. Due to the stigma surrounding sexual activity, especially sexually transmitted infections (STIs), several studies have found that anything relating to sexual health and practice is unlikely to be shared amongst their peers on social media. Including comedy in messages or online films about sexual health might boost its chances of being shared throughout young people's peer networks, leading to greater understanding and acceptance into mainstream media practices. Therefore, rather than bringing pre-packaged programs into the field of practice, promotion strategists of sexual health should immerse themselves in the culture of social media, where stigma is a communal concern, privacy is significant, and information distribution is moderated via one's performance (Byron et al. 2013).

3 Normalization and Medicalisation of Sexual Health

There is a lot of curiosity about what testing, therapeutic evidence, and social media expertise can do for sexual health (Davis 2015).

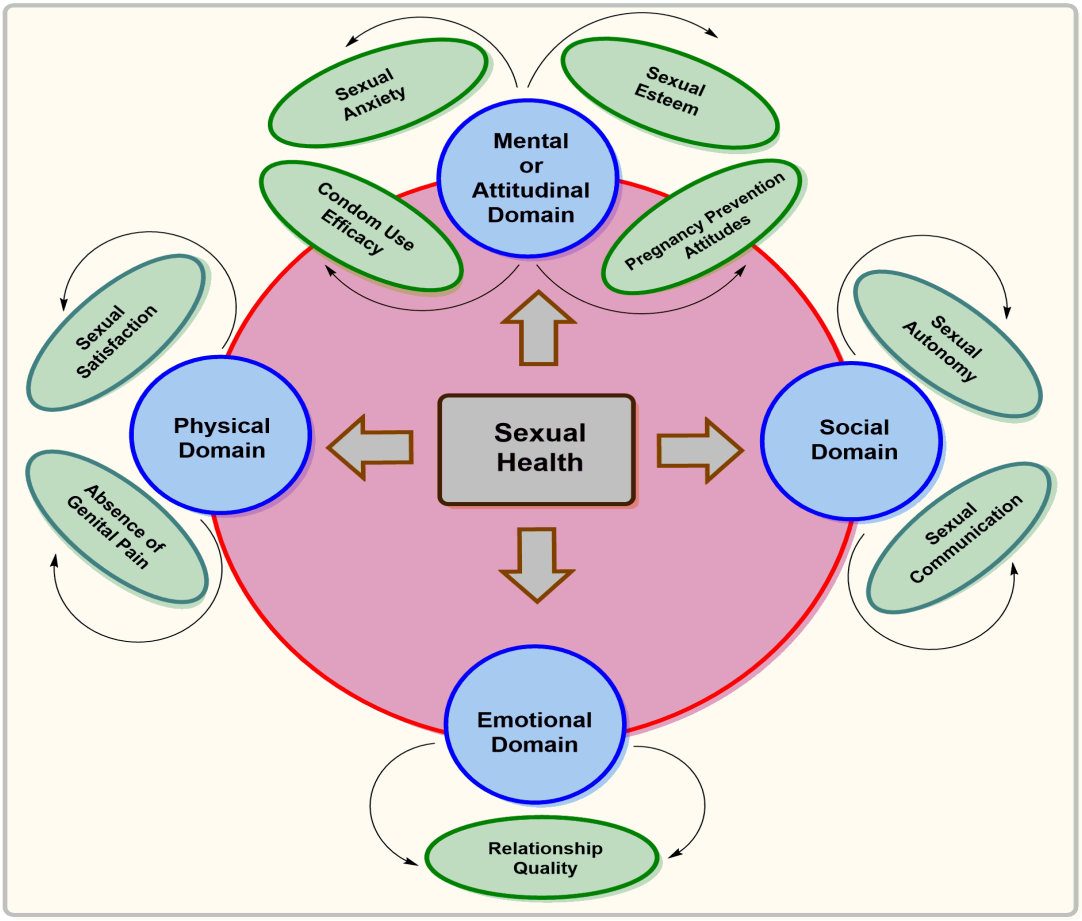

Figure 1 Various Domains and their variables related to sexual health

Medicalized, heteronormative descriptions, and practices have influenced perceptions of "risky" and "safe" sex, as well as those who engage in these behaviors (Grant and Nash 2018). Medicalization is defined as a process by which a growing number of human living circumstances and practices become defined, understood, and accomplished by using medicinal and medically-related proficiency (Bell 2017; Thomas 2021). When we talk about the "medicalization" of sexuality, we are referring to the pharmaceutical industry-funded effort to elevate doctors to the position of final arbiters of one's intimate emotions, thoughts, and bodily sensations. This process is commonly associated with sexual dysfunction pharmacotherapy and the associated epidemiological perceptions of sexual health conflicts (Stulhofer 2015). The term "medicalization" refers to the practice of labelling and solving problems that are not medical in nature as though they were. At its heart, this transformation is based on expanding the illness model to encompass phenomena that were previously considered either wholly or mostly outside the purview of medical science. By looking to Michel Foucault for guidance, this critical sociological view of medicalization contends that it is a type of social control because it not only establishes unique norms (of normality, healthiness, etc.) that come to influence conventional attitudes and activities (Srinivasan et al. 2019). The term "medicalization of sexuality" refers to the pharmaceutical industry-backed trend of medical professionals asserting greater control over individuals' subjective sexual experiences, sensations, and emotions. Typically, this method is associated with the epidemiological view of sexual health problems and the medication for sexual dysfunctions. The critical discussion of the medicalization of sexuality has recently expanded to include concerns about female genital plastic surgery (Marshall 2012; Verrastro et al. 2020).

Public health's acceptance and promotion of sexuality represents a continuation and a ratcheting up of the trend toward medicalizing both sexuality and society at large. By "medicalization," we refer to the following: (1) a social organization of health professions based on professional training and certification, professional practices, and interaction between members of health professions (doctors and psychologists in particular) and their patients; (2) a method of social control and regulation of sexuality based on efficient technologies and the authority of trained professionals; and (3) a body of basic knowledge and scientific concepts (Gruskin et al. 2019). During the medicalization process, clinical care and the curative model within the context of doctor-patient contact became the norm. Since sexuality has become a public health concern, it has taken on a more systemic character, requiring attention on the part of policymakers, educators, and clinicians. Naturally, it is rooted in the development of scientific and medical understandings of sexuality, but it uses social intervention and behavioral regulation that differ from the clinical paradigm (Carter et al. 2022). Clinical medicine is no longer the only conceptual and methodological basis for public health. Now more than ever, fields such as education, epidemiology, statistics, economics, and law are being included in the individual clinical approach. Thus, the traditional model of a doctor-patient interaction within the context of clinical treatment has given way to a wide variety of other forms of intervention (Gruskin and Kismödi 2020; Logie et al. 2021).

Sexual health normalization and medicalization is a critical strategic moves for clinical staff. As revealed in the motif of "engaging with sexuality," the usage of these strategies positioned sexual health as an adequate topic, eventually employed to dispel the stigma associated with the sexual activity of people with infirmities. Nevertheless, there are some hazards associated with the normalization or medicalization of sexual health in the context of disabilities. Precisely, certain prejudices (i.e., heterosexuality) may become normalized at the expense of others, which are labeled as inappropriate or aberrant (i.e., transgendered identities and homosexual relationships). The level of comfort with specific sexual health aspects (such as holding hands vs. sexual intercourse) of healthcare providers and families may predispose them to normalization. Modern biomedicine is obsessed with normalization strategies. In practice, this is embodied by assessments, measurements, and documentation against the norms. When sexual health is normalized for a group, it establishes standards or prospects against which individuals and behaviors can be compared and restrained. Therefore, the medicalization of sexual health for one group may have unintended consequences for those who are not members of that group (McCabe and Holmes 2014).

The discussion about medicalization must consider the fit between the sexuality model and the medical model. Sexuality is a social construction, whereas the new social construction is termed "medicalization" (Tiefer 2002). Although several researchers have labelled medicalization as a "gendered" theory, the literature on medicalization is limited in its incorporation of men and masculinity. This expanding literature places a strong emphasis on sexuality and begins to give attention to medicalized masculinities, in which masculine behaviors are considered a health risk. In addition to expanding knowledge about masculinity and infertility, the study advances the "gendered" medicalization theory (Bell 2016). The medicalization of sexual behavior has expanded recently into the realm of sexual pleasure. Irrespective of desire, both men and women are stimulated to prolong their sexually active lives. Viagra, also known as sildenafil citrate, is the first oral medicine used in the treatment of erectile dysfunction or impotence and ranks as one of the supreme victories in pharmaceutical antiquity (Hart and Wellings 2002). There was a moral and ethical conundrum created by this innovation for both the government and the public. Though there is solid proof of its effectiveness and user happiness, its rather high price tag came at a

time when the National Health Service's (NHS) medicine budget was already severely stretched. As a result, more and more men are seeing erectile dysfunction as a medical problem that the NHS should try to remedy. While the NHS may be able to provide Viagra to some men, this treatment option is restricted to those who fall into narrowly defined categories of sickness. Critics were outraged by the ruling, with one saying that "it is solely unethical to differentiate the patients based on the cause of their erectile dysfunction" This was the position of the Chairman of the British Medical Association's General Practice Committee (Evans 2006).

In the instance of female sexual dysfunction, it is now claimed that sexual disorders not only affect a significant number of women of all ages but also have a reflective influence on mood, self-esteem, relationships, and quality of life. The introduction of Viagra and subsequent advances of other medicinal drugs intended to increase the desire for sexual activity and fulfillment of both genders have refocused the popular media and clinical courtesy on sexology and philosophies of what constitutes normal and healthy sexual activity for them, with the underlying assumption that "good sex is good for you" (Nicolson and Burr 2003). The "Viagra phenomenon" is the most evident and well-studied manifestation of a large global medicalization process of male sexuality. The process of associating male health with self-control and the appearance of sexual potency is forming a new public discourse on masculinity, enchanting the form of a medicalized virility that is scientifically validated and re-establishes the fundamentals of a naturalized notion of men and their sexuality. "Viagra, commonly referred to as a quality-of-life drug, can also be viewed as an identity drug, offering men the opportunity to do masculinity and perform better sexual activity with the assistance of the pill" (Camoletto and Bertone 2017). The role of medicalization (Viagra/sildenafil citrate) in the treatment of sexual dysfunction (Cruz-Burgos et al. 2021) is illustrated in Figure 2.

Medicalization can be hazardous to your health. There are reliable concerns about the perils of infections while in the hospital, over-prescribing, the dangers of pressure sores, and the inappropriate

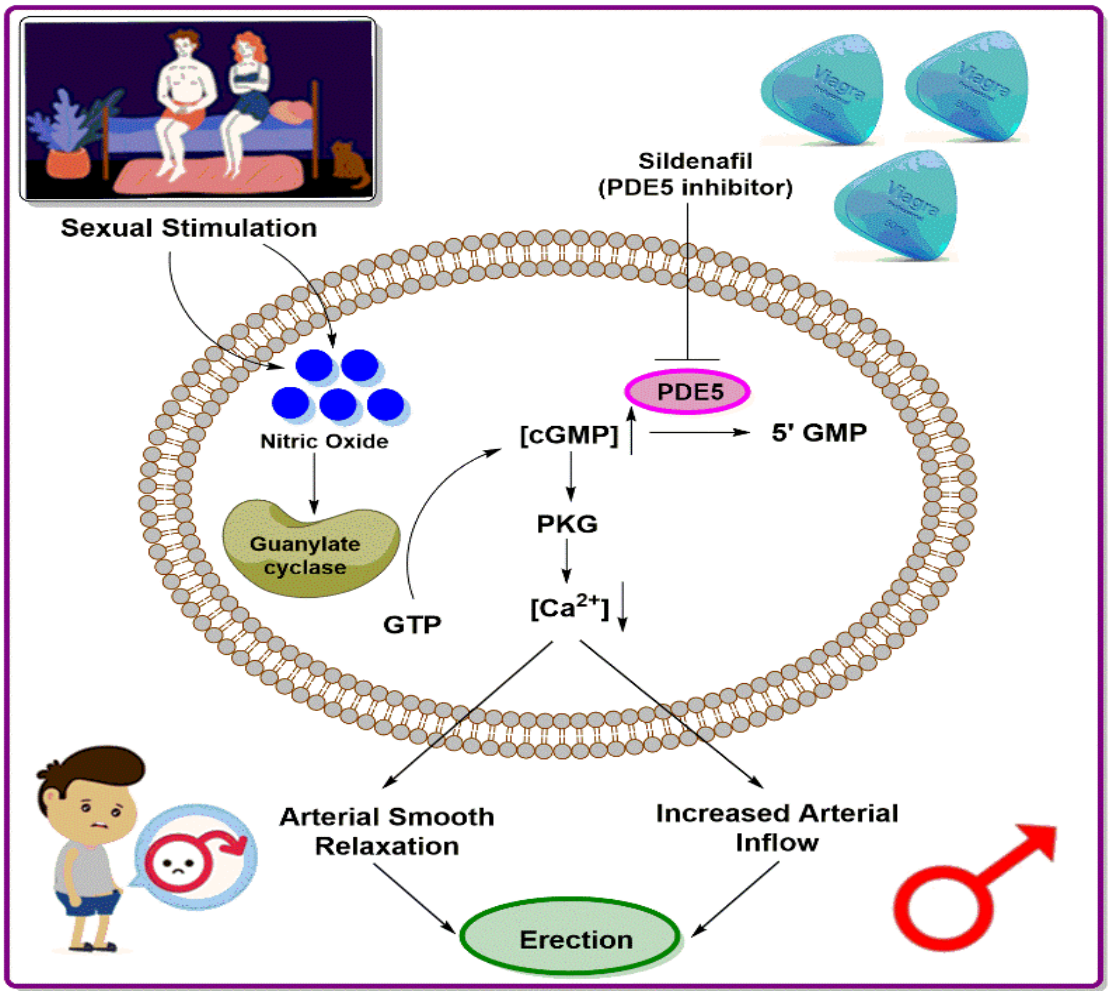

Figure 2 Role of medicalization in sexual dysfunction

use of tranquilizers for restraint. However, many of these issues arise in social care and are indicative of poor practice standards. Medical risks occur at any age, and they are not valid reasons for preceding the therapeutic benefits of treatment. Additionally, many of the risks associated with medicinal care are avoidable. The medicalization of old age should not be condemned but rather encouraged. A large admission to medical care for the elderly will reduce disability and mortality. Anti-aging treatments should be subject to the same regulatory framework as any newer medicinal technology (Ebrahim 2002).

3.1 Benefits and Potential Risks

Particularly with regard to women's sexuality, many have been quite loud in their disapproval of the medicalization trend. Recent evidence has shown that many men are reluctant to utilize selective serotonin reuptake inhibitors (SSRI) antidepressants for quick ejaculation, and several studies have highlighted less gender-specific worries about the low adherence to treatment for erectile dysfunction. Despite the negative feedback, the medicalization of sexuality has been helpful for sexology in many ways. Research into sexual physiology has been revitalized by renewed interest and funding from pharmaceutical corporations, and it has also prompted the growth of psychological examinations of sexual health issues (Mollaioli et al. 2020). Evidence-based interventions and solutions became more of a priority in sex therapy as a result of this process. Last but not least, the revival of interest in sexology, especially among medical specialists, led to higher status and institutional acknowledgment of the area, as seen in the creation of new sexual medicine departments in academic institutions. Similarly, there are valid worries about how sexuality is being medicalized. Some scientists and medical professionals have expressed concern that similarities between the sexes are often overlooked in favor of differences (Logie et al. 2021). A major criticism leveled at the medicalization of sex is that it ignores the social and cultural significance of sexuality and does not take into account relational variables in male and female libido. Finally, there is evidence to suggest that people are becoming warier in sex therapy as sexual medicine becomes more mainstream (Hoch 2022; Stanley and Pope 2022). Even while more people are likely to seek help for sexual issues because of increasing media coverage, the expenses (health insurance policies often do not cover sex therapy) and time commitments are often insurmountable for many people who have had sexual problems. Further, "criticism levelled at psychosexual therapists for not providing ‘evidence-based' treatments" has diminished sex therapists' authority and credibility. Medical and nonmedical sexologists have had a harder time working together because of this. This has slowed the development of an interdisciplinary clinical approach to sexual health issues. Although the effects of medicalizing sex may be different from one culture to the next, it appears that there is a global paucity of government support for studies of the psychosocial components of sex (Carter et al. 2022).

The promotion of discourse on sexual health carries with it the risk that sexual health itself may be elevated to the status of ultimate good or benchmark for what constitutes acceptable sexual behavior. But people participate in sexual activity for reasons other than health. People's sexuality can be attributed to a wide range of unique factors. Participation in sexual activity largely determines one's health, either positively as a result of a sense of well-being or poorly in the form of a sexually transmitted disease (Bell 2016; Bell 2017). Consequences for the research of sexuality and for the practice of sexual health promotion stem from the fact that people give health only a marginal role in their considerations of being sexual, at least if we remove procreation as a purpose for sexual activity (Thomas 2021). An overly narrow focus on health in sexuality research would restrict us from learning more about people’s sexual habits and the significance of sexuality in their own lives and the lives of others. It is critical for successful health promotion to recognize that people's sexual behaviors are not solely determined by their concerns for their health (Castellanos-Usigli and Braeken-van Schaik 2019; Verrastro et al. 2020). Adopting the idea of sexual health carries with it the risk of medicalizing sexuality and promoting an understanding of sexuality in terms of normal and abnormal, given that health is first and foremost regarded as a scientific category. Sexuality is a social activity that occurs in distinct sociohistorical contexts, and this may be lost if sexual problems and their solutions are framed solely in scientific terms as a result of medicalization. However, one need not look just to the biomedical sciences for an explanation of what constitutes mental and physical health and how these conditions might be improved. The fields of medical sociology, anthropology, history, and health psychology have greatly widened our understanding of health. These fields, which extend far beyond medicine, have made important contributions to our knowledge of sexual health (Gruskin et al. 2019; Gruskin and Kismödi 2020; Stegenga 2021).

3.2 Medicinal Plants on Sexual Health: Implications and Mechanisms

Environmental, psychological, and biological variables all have a role in the development of sex problems. Therefore, the use of medicinal plants and chemicals found in nature to treat various forms of these illnesses is controversial (Chandran 2021; Sharun et al. 2021; Alajil et al. 2022; Buttar et al. 2022; Chandran et al. 2022). Plants can assist to improve hypoactive sexual disorder (HSDD) in both men and women by influencing some of the variables that contribute to sexual desire, such as endocrine (androgen), hereditary, neurological (such as brain neurotransmitters), and psychological. Antioxidant-rich medicinal

plants protect the brain and genital tract cells from damage caused by oxidants. Therefore, maintaining general health by fostering healthy cells and organs is directly beneficial to sexual health (Prakash et al. 2021a; Prakash et al. 2021b; Khan et al. 2022; Kumar et al. 2022a; Kumar et al. 2022b; Kumar et al. 2022c; Kumar et al. 2022d; Kumari et al. 2022a). Also, the nitric oxide and opioid systems in the corpus carvenosum's smooth muscle cells are affected by the substances found in medicinal plants, which finally results in the creation of cGMP to treat erectile dysfunction in males. cGMP then expands the penis and increases blood flow there. However, there are still many unsolved questions about the effects of medications, especially herbal drugs, on premature ejaculation. Meanwhile, research has shown that sedative SSRIs, which work by blocking serotonin reuptake, can be effective in treating this condition. In general, medicinal plants are beneficial in treating sexual dysfunction in multiple ways (Castellanos-Usigli and Braeken-van Schaik 2019).

Biochemical studies show that the active chemicals in plants and other compounds found in nature can moderate the levels of androgens, gonadotropins, and prolactin to some degree. It is also widely believed, incorrectly, that most medicinal plants may be used to increase sexual desire or treat sex issues without causing any negative side effects. These misunderstandings raise the risk of adverse reactions and overdosing on certain plant-based substances. These negative consequences can extend to the mind and even threaten life (Najaf Najafi and Ghazanfarpour 2018). Some additional herbs, such as *Pistacia* species and *Nigella sativa*, are used to treat sex abnormalities in Iranian traditional medicine as well. However, *Crocin sativus* and its derivatives are getting a lot of attention, not only for their usefulness in treating sex issues but also because of their refreshing characteristic. The effects of medicinal herbs on sex diseases have been the subject of much research, with mixed results (Prakash et al. 2021a; Prakash et al. 2021b; Kumari et al. 2022b). It might be argued that sex abnormalities are not always treatable by medicinal plants because they are often caused by underlying physical or mental health issues. Therefore, in such circumstances, specific therapies such pharmacological pharmacotherapy, mental treatments, and surgery can be beneficial. Antioxidant and anti-inflammatory actions present in medicinal herbs and their derivatives have the potential to aid in the treatment of a wide range of illnesses (Logie et al. 2021; Thomas 2021; Carter et al. 2022; Hoch 2022; Stanley and Pope 2022).

4 Gender Differences in Sexuality

There is undeniable evidence that sexuality and its manifestations continue to be imperative to both males and females as they age. Physiological fluctuations in their sexual responses can inhibit or improve sexual activity and performance (Lindau et al. 2007), which are influenced by the interaction of the sexual abilities of each partner, their motivation, behavior, and attitudes, as well as the quality of the dyadic relationship itself. Nevertheless, there are substantial gender alterations in the likelihood of being sexually active at an older age. Sexual interest, sexual activity, and quality of sexual life tend to be continuously higher among men than among women, however, both sexes experience a reduction with age (Waite et al. 2009). This could be due to a variety of psychosocial aspects (Howard et al., 2006). These comprise the health status of the partner, their relationship, and their level of life satisfaction (Woloski-Wruble et al. 2010). When a partner loses interest in sexual activity, both genders experience a decrease in sexual frequency, but women experience a greater decrease than men (DeLamater et al. 2008). Life stressors, previous sexuality, contextual factors, and mental health complications are more substantial predictors of sexual interest in older women than physiological status alone (Hartmann et al. 2004). Women do not experience sexual pleasure because they are unable to climax or because their performance anxiety reduces their sex frequency (Laumann and Waite 2008).

5 Physiological Changes associated with Aging

Aging causes variations in the endocrine, neurological, and vascular systems, which all have direct and indirect effects on sexual arousal and performance (Yee 2010).

5.1 Men

Erectile dysfunction is the most common sexual dysfunction in aging men. This can be owing to hormonal fluctuations as a part of normal aging or to fundamental circumstances like late-onset hypogonadism or vascular and neurological disorder progressions (Wylie and Kenney 2010). Erectile dysfunction is treated with an assessment of cardiovascular risk factors, lifestyle advice, and a trial of PDE-5 inhibitors without contraindication (Smith et al. 2010). The link between erectile dysfunction and features of metabolic syndrome is well recognized and should be investigated further when dealing with erectile dysfunction in older men. Hormonal monitoring in older men has revealed a 1-2 percent annual decline in free testosterone from the age of 45–50 years, as well as a decrease in dehydroepiandrosterone (DHEA) and an increase in follicle-stimulating hormone (FSH) or luteinizing hormone (LH) and sex hormone-binding globulin (SHBG) (O'Donnell et al. 2004). Symptoms of marked falls include mood alterations, decreased strength, lower energy, increased sweating, erectile dysfunction, and decreased sexual drive. These older men with androgen deficiency symptoms may benefit from hormone therapy, though there is debate about how much of this decline is due to normal aging and when replacement is necessary (Bhasin et al. 2007).

5.2 Women

The two most common sexual dysfunctions in aging women are the deficiency of sexual desire and sexual arousal. Local urogenital

manifestations of hormonal decline, which commences with menopause and endures as women age, can cause vaginal and vulval membrane atrophy, urogenital prolapse, urinary incontinence, and frequency. A thorough physical examination of the urogenital system will assist in determining whether these variations are present. This diminution of hormones may also result in decreased muscle mass, a loss of sense of well-being, a loss of bone mass, and decreased energy. Sexually, this may cause vaginal dryness and dyspareunia, as well as a decrease in libido and the capability to attain orgasm. If relevant and safe, medical treatment for these circumstances may include local or systemic hormonal therapies (Yee and Sundquist 2003).

6 Future Development

Many disciplines and communities of practice have divergent predictions for the future of medical and nonmedical approaches to sexual health and how they will be merged. As researchers continue to look for effective treatments, many in the medical community are optimistic about what the future holds for sexual medicine. Furthermore, the concept of comprehensive sexological healthcare, which has been replacing the (over) medicalized viewpoint, seems to be increasingly acknowledged as requiring a blend of medical and nonmedical techniques (Cruz-Burgos et al. 2021; Thomas 2021). There may be hope for improved knowledge and therapy of sexual dysfunction if researchers can find ways to better integrate the available data. Several nonmedical sexologists are enthusiastic about interdisciplinary approaches, but there is also a worry that the ongoing medicalization of sexuality would further marginalize sex therapy and basic sexological research. The medicalization of sexuality has led to the development of cutting-edge diagnostic and therapeutic modalities and, at least in the developed world, more widespread and less-stigmatized access to sexological healthcare (Castellanos-Usigli and Braeken-van Schaik 2019; Carter et al. 2022). Medication for erectile dysfunction has been so effective that not only is the public more aware of sexual health problems, but the stigma associated with getting help is also less of a deterrent. However, the recreational use of erectile dysfunction medication has contributed to normative pressures and social expectations regarding sexual performance, and this has led to the current difficulties in accepting the normal process of age-related changes in sexual functioning, which has been attributed in part to the medicalization process (Hoch 2022; Stanley and Pope 2022).

Conclusion

Respect for the continued significance of sexuality in the lives of the elderly is a crucial aspect of providing for their needs. Sex, sexual orientation, gender identities and roles, eroticism, intimacy, pleasure, and reproduction are all viewed as essential aspects of human sexuality. It is common for both men and women to experience sexual dysfunction. Physical and emotional well-being are intertwined, making sexual health a crucial factor. Like the rights to happy family life, privacy, and to be treated equally, it is an essential aspect of what it means to be human. A decrease in sexual desire or poor performance during sexual interplay is pathological at any age and should prompt a visit to the doctor. The term "medicalization of sexuality" is commonly used to describe the pharmaceutical industry-backed trend of doctors and other medical professionals gaining more sway over individuals' private sexual thoughts, emotions, and sensations. In this review, we discussed the factors surrounding aging and sexuality, as well as gender differences in sexuality, the normalization, and medicalization of sexual health, and also considered special situations with age, such as institutionalized care and the possibility of elder abuse.

Despite widespread agreement on the importance of protecting and promoting sexual health and rights, the existing policy, programming, activism, and discourse on sexuality have failed to fully address the interconnected nature of sexual rights, sexual health, and sexual pleasure. It is more important than ever, given the current political climate, to apply the "triangle approach" to sexual health and rights, which will benefit all people but notably the most marginalized. We can learn from and move beyond conversations about the pleasure that target only specific populations like young people, women, and/or other marginalized populations, or that target specifically sexual orientation, gender, gender expression, or sex characteristics, due to the importance of sexual health, sexual rights, and pleasure as a universal demand.

An intersectional, interdisciplinary, and multi-sectoral approach is necessary to ensure that initiatives are accepted, implemented, funded, and sustained on a local and global scale. To get started, we can make a map of how sexual health, rights, and enjoyment have been conceptually and practically brought together, as well as how gaps have been uncovered with the needs and rights of certain communities. If we had such a map, we might see at a glance where it would be most beneficial to collaborate with others to guarantee uniformity and where it could be most prudent to start from scratch in terms of concepts. Where these ideas have been put into practice, there should be records kept of the programming that went into it and, more significantly, the impact it had on people’s lives. The rigorous evaluation of what has been put in place can be used to create arguments that are more likely to be accepted by states and institutions of power internationally and within countries, opening the door to resources that can then be utilized to support direct impacts on people's lives. It is important to reflect, though, that even the rare pleasure we feel has roots in public health. More "ecosystem" work within institutions, communities, families, etc. is required to create the enabling environment necessary for everyone to enjoy sexual rights and pleasure, as is

consideration of the varied and contextual ways in which individuals exercise choice, receive information, and engage in sexual pleasure. Recognizing that the identities and circumstances of the vulnerable or disadvantaged will vary across and within nations, we must remain vigilant to ensure that our current and future efforts yield better laws, policies, and programs to promote sexual health, sexual rights, and sexual pleasure for all people.

Conflict of Interest

The authors declare no conflict of interest in this article.

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Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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A Review on Bacterial Degradation of Benzo[a]pyrene and Its Impact on Environmental Health

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ABSTRACT

Benzo[a]pyrene is a polycyclic aromatic hydrocarbon (PAH) having a high molecular weight. Benzo[a]pyrene and other PAHs are induces severe acute or chronic human health hazards and are extremely carcinogenic, mutagenic, immunotoxic, and teratogenic. Microorganisms play a crucial part in the degradation of benzo[a]pyrene from polluted environments. Such micro-organisms synthesize monooxygenase and di-oxygenase enzymes that proceed with the aerobic or anaerobic catabolic degradations of benzo[a]pyrene. Bioaugmentation, biomineralization, and biostimulation methods can be used for the decontamination of benzo[a]pyrene from hydrocarbon contaminated sites. In this review paper, we thoroughly explained the impacts of benzo[a]pyrene pollution on human health and the environment. Further, this study also described various pathways regarding the bio-degradation of benzo[a]pyrene and also an updated overview of future prospects of benzo[a]pyrene biodegradation.

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1 Introduction

Benzo[a]pyrene and hydrocarbons are persistent organic pollutants (POPs), and polycyclic aromatic hydrocarbons (PAHs), and stand for a group of pollutants that are present in the surrounding of the environment (Nzila and Musa 2021). These PAHs pollutants harm our lives and, some have less beneficial effects on the environment and human health (Jong-Su et al. 2009). PAHs are used as raw materials for the manufacture of all hydrocarbons which generate toxic environmental pollutants due to natural and anthropogenic activities (Elyamine et al. 2021). Millions of tonnes of hydrocarbon products spill into soil and water bodies due to oil spills and vehicle emissions (Stogiannidis and Laane 2015) and the major amount of oil spill sites have hazardous conditions for human health and the environment. PAHs are treated to photo-degradation and react with SO_2, NO_2, and O_3 producing stacks of gas and, mutagenic compounds in the atmosphere. The environment contains benzo[a]pyrene in the air, water, and soil, and they can persist there for months or years, the concentration of PAHs in urban air may be ten times higher than in rural environments (Keyte 2015).

Benzo[a]pyrene and its relevant other PAHs are abundant in nature and cause a wide range of life-threatening issues and are commonly found in oil and fuel-contaminated soil; soil particles absorb the aromatic compound, then cause soil contamination and release it into the environment (Kuppusamy et al. 2017). The Groundwater is also contaminated by Benzo[a]pyrene and PAH compounds, which are continuously released into the environment by incomplete combustion of fuel, during anthropogenic and natural atmospheric activities. In large quantities, water is contaminated by oil spillage sites and causes the death of aquatic species (Montuori et al. 2022) and so, it is very important to control and low emission of benzo[a]pyrene in the environment because it causes mutagenic, carcinogenic, immunogenic and teratogenic (Nzila and Musa 2021). Biological, chemical, and physical remediation are used for decontamination of benzo [a]pyrene, and one of the best methods for microbial degradation, is bioremediation, the most cost-effective and eco-friendly method (Jong-Su et al. 2009; Wang et al. 2018). Breakdown of benzo[a]pyrene, is a mineralization and conversion process and various aerobic and anaerobic bacteria species contribute to this process (Elyamine et al. 2021). Multiple microorganisms and their species have been discovered, which have degraded highly stable and waste polycyclic hydrocarbons. Its degradation rate and other hydrocarbon compounds also depend on various terms such as environmental conditions, availability of nutrients, temperature, pH, microbial load, source of the growth medium, chemical properties, and structure of aromatic compounds (Mortazavi et al. 2022). Oil-eating bacteria produce enzymes and secrete for the utilization of aromatic hydrocarbons; they produce different types of metabolic pathways depending on the optimal condition of the environment (Mishra et al. 2021). Furthermore, biodegradation of benzo[a]pyrene is a more advantageous biological, physical and chemical method, which is used for cleaning up the benzo[a]pyrene-polluted environment (Houshani et al. 2021). PAHs, such as benzo [a]pyrene, have more potential for microbial degradation, the bacterial degrading process by which benzo[a]pyrene is removed from the environment using micro-organisms is known as bioaugmentation. Microorganisms are a major role play in the de-contamination of benzo[a]pyrene in soil and water bodies. Potable water and soil are important elemental requirements for sustainable development, so uncontaminated soil and water can be used for industrial contributions and agricultural production to improve health and economic development (Wang et al. 2018).

Information about microbes in oil spills degrading Benzo[a]pyrene is available in this review, the mechanism of its impact is also fully described and more than 100 species of bacteria from the group's Actinobacteria, Proteobacteria, Firmicutes, Bacteroids, and Cytophaga have been found in the PAH-contaminated areas. It has been discovered that Flavobacterium-Bacteroides assimilates pyrene and has some of the primary abilities of natural self-recovery (Zada et al. 2021). Some normal methods of biodegradation have been used to degrade the PAH compound, but most of these methods have the limitation of being costly and implication is hard under the environmental conditions. For effective PAH-reducing emissions, integrated PAH remediation approaches have also previously been reported. The most prevalent, potentially diversified, and plentiful group of bacteria for benzo[a]pyrene breakdown from the PAHs-contaminated site are reported to include fungi, protozoa, and actinobacteria. Alpha proteobacteria have been observed to be more widespread than beta and gamma proteobacteria among the proteobacteria (Zada et al. 2021; Kumari et al. 2022). We should look for introducing new techniques or novel strains break down of PHAs such as benzo [a]pyrene so the primary goal of this review is to describe the physicochemical properties and microbial biodegradation mechanism by aerobic and anaerobic and as well as to illustrate the health impact on mankind of benzo[a]pyrene.

2 Benzo[a]pyrene

Benzo[a]pyrene and its relevant compound are high molecular weight (HMW) PAHs, and it has great stability due to the presence of an aromatic ring (Nzila et al. 2021). One or more rings fused, yellow, solids, whitish in a specific solvent, angular, low and un-soluble in water, low vaporizing pressure, high melting, and boiling point (Patel et al. 2020), it has high potential toxicity and resistance to biodegradation also and might cause recalcitrant of mutagenic and carcinogenicity (Hsu et al. 2005; Cao et al. 2020). There are a priority list of 16 PAHs given by USEPA (the United States Environmental Protection Agency) which harm biological

Table 1 Properties of Benzo[a]pyrene

Molecular structure	
Molecular Formula	$C_{20}H_{12}$
Molecular Weight	252.31
Solubility in water	0.2 to 6.2 µg/L
Melting point	179[2] °C (354 °F; 452 K)
Boiling point	495 °C (923 °F; 768 K)
No. of benzene rings	5

(humans, animals, plant) and environmental health (Dudhagara and Dave 2018; Dhar et al. 2019).

2.1 Benzo[a]pyrene-related physiochemical properties

Benzo[a]pyrene have five fused benzene ring structure and comes in high molecular weight PAHs. It is a pale yellow color, crystal, slightly soluble in water (hydrophobic), thermodynamically isomer, and stable form in nature (Subashchandrabose et al. 2014). IUPAC's name is 3,4- benzo[a]pyrene and benzo[pqr]tetraphene, and the formula is $C_{20}H_{12}$ (Table 1). This benzo[a]pyrene molecules dissolve in a special solvent like acetone, acetonitrile, dimethyl sulfoxide(DMSO), dichloromethane, hexane, etc., and different PAHs are less water-soluble as their molecular mass increases. (Logeshwaran et al. 2022). Because of the recalcitrance and hydrophobic nature of benzo[a]pyrene, a major concern is ultimately deposited in natural soil and water (Sun et al. 2018; Li et al. 2022).

2.2 Source of Benzo[a]pyrene

Benzo[a]pyrene is a major organic pollutant, one of which is the high molecular weight organic compound PAH and is found not only on earth whereas, but in all spheres, the universes, and planets (Tielens 2005; Dhar et al. 2019; Adeniji et al. 2019). Sources of benzo[a]pyrene are divided into two categories and these are given below:

2.2.1 Natural Source

Benzo[a]pyrene, is emitted from natural forest fires, volcanic eruptions, rangeland fires, and bush fires**.** At natural and some artificial crude oil reservoirs, erosion of sedimentary rock is released by PAHs. It is a pyrogenic source of PAHs, which automatically raises the temperature (Patel et al. 2020).

2.2.2 Anthropogenic sources

Anthropogenic sources are divided into four major categories based on PAH emission routes. They are subcategorized below:

2.2.2.1 Agricultural source

In agricultural sources, PAHs are emitted by the burning of peat cow-dung cake straw (Ravindra et al. 2008; Tsibart et al. 2014) and stubble, rice husk briquettes, bushland and forest, and moorland health (Abdel-Shafy and Mansour 2016).

2.2.2.2 Industrial sources

The industrial sector contributes significantly to PAHs emissions in the environment, particularly in the air through cement production, bitumen and asphalt production, rubber and tire production, metallurgical processes, coal distillation, coal tar and coke production, petroleum residue, gas plant manufacturing, dye, paint, plastic production, and food preservatives (Hesham et al. 2014).

2.2.2.3 Domestic sources account for the majority of PAHs emissions

Cooking (Gupte et al. 2016; Patel et al. 2020), grilled meat foods (Rose et al. 2015), heating, burning activities of coal, oil, gas, garbage, and wood at high temperatures (Johnsen and Karlson 2007); cigarette smoking and fireplaces also come under PAH emission sources from domestic sites. PAH emission is high in daily use, we should bring it to use in less amount little.

2.2.2.4 Automotive sources

Automotive sources of benzo[a]pyrene in environmental contamination include jet engines, trains, exhaust gases, ships, aircraft, and motor vehicles, which secrete a high amount of benzo[a]pyrene (Stogiannidis and Laane 2015).

3 Microbial degradation of Benzo[a]pyrene

Mainly PAHs compounds formed with one and more than three or four benzene rings, namely benzo[a]pyrene (BaP), and its relevant compound polycyclic aromatic hydrocarbons (PAHs). In this

study, we will refer to benzo[a]pyrene and other relevant benzene ring compounds as HMW-PAHs because of their long persistent in nature and high toxicity which cause mutagenic and carcinogenic properties (Jong-Su et al. 2009). Since the 1970s, benzo[a]pyrene has been biodegraded and the first reports of its degrading bacteria and bacterial strains cultured in succinic acid and biphenyl during benzo[a]pyrene exposure were published in the 1970s, with *Beijerinckia B-836* being one of the reported bacteria strains (Gibson 1975; Nzila and Musa 2021). *Pseudomonas* strains have degraded benzo[a]pyrene and its substrate salicylate completely removes it (Logeshwaran et al. 2022). Microbes can utilize hydrocarbons through different methods like the bioaugmentation process the addition of mico-organism in hydrocarbon contaminated areas; the biomineralization method is metals removal by microbes (Lawniczak et al. 2020). Metabolism of benzo[a]pyrene by microbial species is an example of biomineralization, bioaugmentation, bioremediation, and pollutant transformation (Kour et al. 2022). With the help of biological, chemical, and physical (Peng et al. 2018) approaches, by way of several bacterial species and other microbes, PAHs compounds are being eliminated from the environment (Tyagi et al. 2011; Adams et al. 2014). Biodegradation demonstrates microbial mechanisms that aid in the ecological recovery of the contaminated sites of benzo[a]pyrene. Specifically, two main types of enzymes identified, which are most abundantly that are monooxygenase and di-oxygenase; which have been characterized by various methods and these enzymes are play important roles in forming cis-dihydrodiols from PAH rings secreted by bacteria and other microbes (Ghosal et al. 2016). In biodegradation mechanisms, enzyme attacks on benzo[a]pyrene rings in the presence of oxygen molecules are called aerobic metabolism. As a first step, the dioxygenase-enzyme helps catabolize oxidation of arenes (organic molecules made from C, H, atoms) which occur in an aerobic environment bacterial metabolic activity to concern for cis-dihydrodiols, catechol (Elyamine et al. 2021), that is an early product (cis-dihydrodiols, catechol) formed by the aerobic bacterial system. This pathway indicates major middle intermediates such as sodium succinate, salicylate (Nzila and Musa 2021), and catechols, which go through intermediates of the tricarboxylic acid (TCA) cycle, in the meta-cleavage pathway, dioxygenase enzyme secretes by bacteria and enzyme attacks on the ortho position of benzo[a]pyrene and intradiol and extradiol product produced during ring cleavage of aromatic compounds (Pimviriyakul et al. 2020). In this system, they have multiple enzymatic systems that involve different types of proteins, iron molecules of nonheme, and NADH (Peng et al. 2008). The dioxygenase (DO) enzyme system, which belongs to a broad family of oxygenases, determines the specific substrate of DO and contains subunits with Rieske [2Fe-2S] centre and mono-central nonheme iron molecules (Gibson and Parales 2000). One of the main contributors to aquatic ecosystem production is algae, which also significantly contributes to the degradation of benzo[a]pyrene in the environment's aromatic pollution (Dell''Anno et al. 2021). However, well-described strains of algae have been shown in previous studies to mineralize or metabolize benzo[a]pyrene and PAHs compounds such as naphthalene, pyrene, phenanthrene, anthracene (Safonova et

Table 2 Benzo[a]pyrene Degrading Microbes and Their Source

S. N.	Microbes	Degraded PAHs	Benzo[a]pyrene source	Reference
1.	*Sphingomonas sp.GY2B;* *S. koreensis ASU-06,*	Benzo[a] pyrene	PAH contaminated soil, Oil-contaminated soil	Tao et al. (2007), Hesham et al. (2014)
2.	*Pseudomonas putida, P. citronellolis,* *P. stutzeri* *P. aeruginosa*	Benzo[a] pyrene	Hydrocarbon contaminated soil and landform used for petrochemical effluent treatment.	Kumar et al. (2006), Zhao et al. (2009), Jacques et al. (2005)
3.	*Bacillus subtilis* *B. megaterium* *B. simplex*	Benzo[a] pyrene	PAH contaminated soil Plankenburg river, oil spill sites.	Lily et al. (2009), Alegbeleye et al. (2017)
4.	*Cellulosimicrobium cellulans CWS 2*	Benzo[a] pyrene	PAH –contaminated soil	Qin et al. (2018)
5.	*Orchrobacterium, Enterobacter cloacae,* *Rhodococcus sp., Staphylococcus*	Benzo[a] pyrene	Contaminated soil, swab samples from human skin	Arulazhagan and Vasudevan (2009), Sowada et al. (2014)
6.	*Penicillium sp.06, Penicillium sp. CHY-2,*	Benzo[a] pyrene	Petroleum contaminated sites, Antarctic soil	Zheng and Obbard (2003), Govarthanan et al. (2017)
7.	*Fusarium, Fusarium sp. E033*	Benzo[a] pyrene	Crude oil-contaminated Soil, soil site at gas station, and Leaves of *Pterocarpus macrocarpus*	Li et al. (2005), Mineki et al. (2015), Chulalaksananukul et al. (2006)
8.	*Trichoderma sp.*	Benzo[a] pyrene	Petroleum contaminated soil	Mineki et al. (2015)
9.	*Scopulariopsis brevicaulis PZ-4*	Benzo[a] pyrene	PAH-contaminated soil	Mao and Guan (2016)

al. 2005; Chan et al. 2006) (Table 2), but this review focus on most of the bacterial degradation rather than algae.

Benzo[a]pyrene and its organophosphorus pesticides degraded by *Microbacterium* sp. MM1 has a 57.8% of the degraded ability of benzo[a]pyrene in 15 days incubation period at 30° C using a carbon source and energy (Logeshwaran et al. 2022), through a chromosomally encoded path, *B. subtilis BMT4i* (MTCC 9447) degrades benzo[a]pyrene effectively, up to 84.66% in 28 days (Bhatt et al. 2018), *P. aeruginosa PSA5* and *Rhodococcus sp.NJ2* degrades benzo[a]pyrene at 88% and 47% respectively (Mishra and Singh 2014). The aerobic biodegradation process of benzo[a]pyrene by different types of bacterial species usually secretes dioxygenase enzymes: and releases oxygen atoms and the substrate of the compound (di-hydrodiol and enoic). Bacterial species of benzo[a]pyrene degrading and their source are given below (Table 2).

3.1 Benzo[a]pyrene degrades aerobically

Aerobic degradation is the process of the breakdown of a complex compound into simple compounds by microorganisms in the presence of oxygen (Jong-Su et al. 2009). Various micro-organisms grow in aerobic conditions; in these conditions, bioventing techniques can use for in-situ bioremediation of benzo[a]pyrene bioventing helps in the cleanup of pollutant and aromatic compounds (Kour et al. 2022). Benzo[a]pyrene has five-aromatic rings and high molecular weight PAH which is one of the most carcinogenic compounds. The low water solubility of benzo[a]pyrene is related to its high recalcitrance and resistance to bacterial and other microbial degradation (Patel et al. 2020). Benzo[a]pyrene is found in relatively high concentrations in environmental samples, and its degradation is limited to catabolic activity by bacterial cultural mass (Nzila et al. 2021). The concentrations of benzo[a]pyrene and relevant hydrocarbons, in soils and water bodies at industrial sites, can significantly depend on the industries, oil-spilling sites, and land activities associated with the contaminated sites (Peng et al. 2008). There is a lack of information about the bacterial degradation of benzo[a]pyrene that has five rings and some bacterial species have previously reported, they have the degrading capability of benzo[a]pyrene including *Mycobacterium sp., Sphingomonas paucimobilis,* and *Stenotrophomonas maltophilia*, as well as *S. maltophilia VUN 10,003*, that degraded benzo[a]pyrene by 22% after 14 days of incubation period and 37°C temperature (Juhasz et al. 2002) when grown on fluoranthene, *S. paucimobilis EPA 505* degraded 33% of benzo[a]pyrene (Story et al. 2001).

During the dehydration of benzo[a]pyrene, *Beijerinckia sp. strain B1* degrades Benzo [a]pyrene into cis-9,10-BaP-dihydrodiol and 7,8-BaPdihydrodiol are produces using their enzymes secreted by *Beijerinckia sp. strain B1*(Gibson 1999). *Mycobacterium sp. strain RJGII-135* aids in the forms of ring cleavage metabolites and forms 7,8-BaP-dihydrodiol (Schneider et al. 1996) the transformation of oxidation of the Benzo[a]pyrene ring. Benzo[a]pyrene rings metabolites degrade using dioxygenase enzyme, one ring oxidized in one ring and comes forms into cis-4-(8-hydroxypyrene-7-yl)-2-oxobut-3-enoic acid and 7,8-dihydrobenzo[a]pyrene. In this metabolism, enzymes attack the 4,5,7,8, and 9,10 positions of benzo[a]pyrene by using *Sphingomonas yanoikuyae JAR02 metabolites* (Nzila et al. 2022). Pyrene-8-hydroxy-7-carboxylic acid was synthesized by Rentz et al. (2008) and cis-4-(8-hydroxypyrene-7-yl)-2-oxobut-3-enoic acid (Mishra and Singh 2014). In this metabolism, the enzyme attacks the 9,10- and 7,8-positions of benzo[a]pyrene. The alpha subunit of the PAH ring is broken up by referring to the different dioxygenases that are produced from gene encoding and it is concerned with PAH metabolism and especially benzo[a]pyrene metabolic activity by bacteria (Ghosal et al. 2016) and was defined as benzo[a]pyrene cis 7,8 dihydrodiol catabolized into 7,8 dihydro-pyrene-8-carboxylic acid by Cebron et al. (2008); Lozada et al. (2008) (Figure 1).

On PAHs contaminated sites, *Mycobacterium sp. PYR-1* (Bhatt et al. 2018) degraded benzo[a]pyrene into benzo[a]pyrene-11,12-epoxide and trans-11,12-benzo[a]pyrene-dihdrodiol (Kim et al. 2007). Other pathways of benzo[a]pyrene degradation *Beijerinckia sp. strain B-836* aids in the metabolism of this compound, and after its metabolism degrades cis-9,10-BaP-dihydrodiol, *Mycobacterium RJMII-135* uses this substrate and converts it to cis- 4-(8-hydroxypyrene-7-yl)-2-oxobut-3-enoic acid. The above bacterial strains initiate the oxidation of cis-4-(8-hydroxypyrene-7-yl)-2-oxobut-3-enoic acid and the formation of pyrene-8-hydroxy-7-carboxylic acid by *Sphingomonas yanoikuyae., Mycobacterium RJMII-135* reduces hydroxyl groups and produces 7,8-dihydro-pyrene-7-carboxylic acid (Rentz et al. 2008). *Mycobacterium sp. PYR-1* initiates an oxidation reaction and attaches an oxygen group to the 11,12 position of benzene rings, releasing hydroxyl groups that form two molecules of trans-11,12-BaP-dihydrodiol (Figure 1) (Nzila and Musa 2021).

Benzo[a[pyrene degradation pathway, was initiated by monooxygenase and dioxygenase enzymes attacks on the 11,12 position of benzo[a]pyrene rings, which concluded in the formation of cis and trans-11,12-BaP-dihydrodiol. Moody et al. (2004) have revealed new production methods for benzo[a]pyrene degradation in *M. vanbaalenii PYR-1* and its Cytochrome P-450 enzymes help in the catabolic activity of benzo[a]pyrene and further catabolized into dimethoxy benzo[a]pyrene *via* hydroxyl methoxy benzo[a]pyrene (Abdel-Shafy and Mansour 2016) and the hydrolysis reaction produces benzo[a]pyrene epoxide. This cytochrome P-450 enzyme is a superfamily of heme enzymes and is found in all biological domains (Bak et al. 2011; Bhandari et al. 2021).

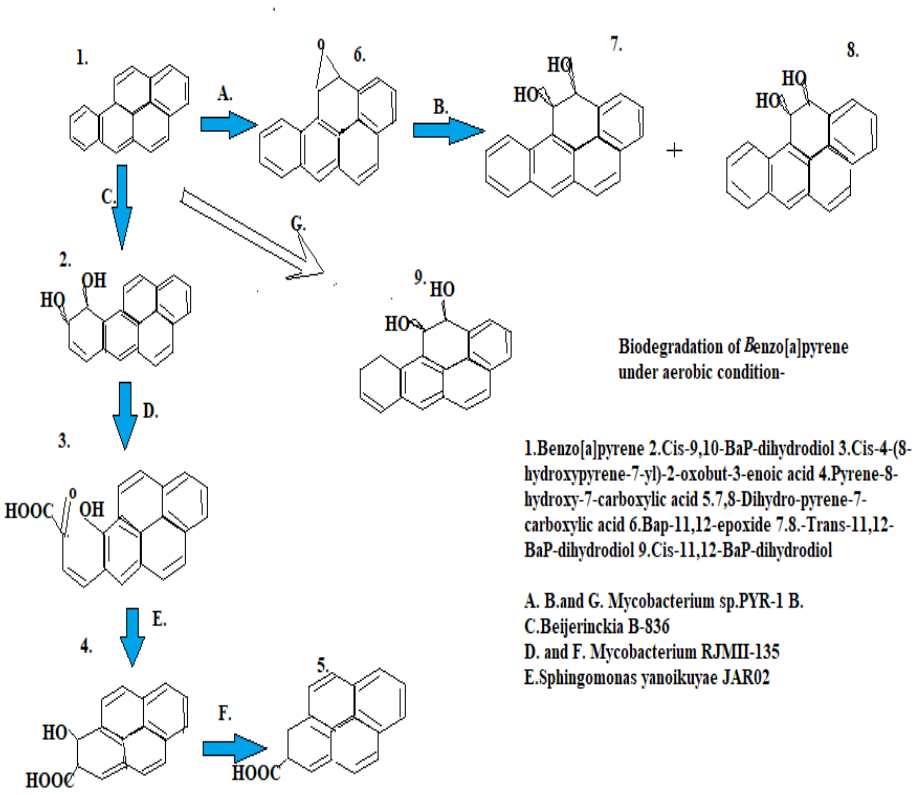

Figure 1 Benzo[a]pyrene Degradation under Aerobic Condition

3.2 Anaerobic Benzo[a]pyrene degradation

Anaerobic degradation is the activity of breaking down a complex compound into simple compounds by microorganisms in the absence of oxygen conditions in the environment. In this pathway, the breakdown of aromatic rings (Chen et al. 2016) by bacterial reduction occurs because of an enzymatic attack on benzene rings secreted by microorganisms. According to benzo[a]pyrene degradation, it was completely oxidized by a mixture of bacteria during the denitrification process (Nieman et al. 2001) and the final product is carbon dioxide and methane (Aydin et al. 2017). Various studies have been conducted on the biodegradation of benzo[a]pyrene by single strains of bacteria under anaerobic conditions. We are aware that the PAH compound, benzo[a]pyrene has a high molecular weight, and *Pseudomonas* sp. JP1 was the first sp. that has specific characteristics and degrades (Liang et al. 2014). *Cellulosimicro biumcellulns CWS2* degraded 78.8% benzo[a]pyrene in 13 days and the occurrence of this species produces pyrene and 1-aminopyrine NH2 groups attached to 1 position of benzo[a]pyrene on contaminated sites (Qin et al. 2018). Opening benzo[a]pyrene rings produce 1-methyl phenanthrene, and attaching hydroxyl groups to two positions produces 1-(2-hydroxypropyl)-naphthalene and 1-methyl-naphthalene. In this reaction, oxidation of this substrate changes it into diethyl phthalate and 2-acetyl-3-methoxybenzoic acid (Figure 2).

Hydrogenophaga sp. PYR-1 degrades benzo[a]pyrene. Furthermore, this species attacks the 5-position of Benzo[a]pyrene rings, producing 5-ethylchrysene and degrading pyrene contents. Therefore, in these mechanism studies, only pyrene, phenanthrene, 1-aminopyrene, 1-methylphenanthrene, 1-(2-hydroxypropyl)-naphthalene, 1-methyl-naphthalene, diethyl phthalate, and 2-acetyl-3-methoxybenzoic acid from the substrate produced by micro-organism enzymatic secretion (Nzila et al. 2021).

Magnetospirillum magneticum strain AMB-1, *Azoarcus sp. EbN1*, *Rhodopseudomonas palustris strain CGA009*, Thauera aromatica, *Geobacillus metallireducens GS-15*, and *Syntrophus aciditrophicus strain SB*; these bacterial strains are famous for anaerobic degradation of aromatic compounds. Cleanup of heavy metals from PAHs using *Pseudomonas putida KBM-1* and *P. putida-CZ1* helps in the binding of Cu-and Zn for chemical transforming and biomass for the cleanup of PAHs contaminated sites in anaerobic conditions (Qin et al. 2017). Carboxylic groups play the main role in the metal-binding in anaerobic conditions, create intermediate substrates and decrease its degradation rates (Kong et al. 2022). Cu (II) (copper) is absorbed by anaerobic bacteria species and other microorganisms in benzo[a]pyrene metabolism under anaerobic conditions, reducing benzo[a]pyrene transport (Chen et al. 2007).

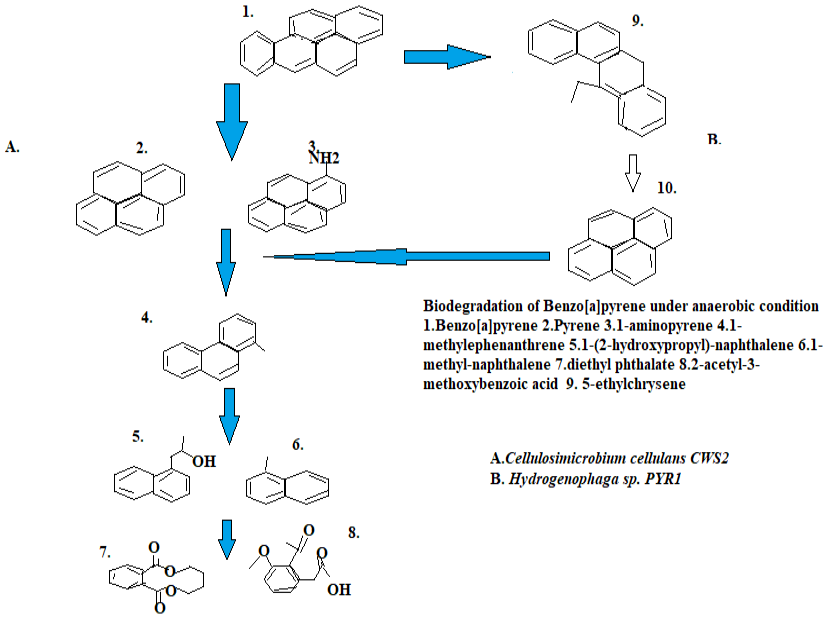

Figure 2 Benzo[a]pyrene degradation under Anaerobic Condition

4 Impact of Benzo[a]pyrene on Human Health

Benzo[a]pyrene is obtained from the environment through human and other animal contact, which is hazardous and unhealthy (Srogi 2007). Despite the case that benzo[a]pyrene is ubiquitous in environmental and food-borne; humans are continuously exposed to it on a daily need through engine exhaust, air, and water. It is widely used in smoking, tobacco, grilling meat, and preparing foods (Bukowska et al. 2022). Benzo[a]pyrene only attacks cells once before being activated by the cytochrome P450-dependent monooxygenase enzyme and converted into other substrates (Peng et al. 2008). Toxically, the substrate binds to DNA cells covalently, and oxygen is produced by micro-organisms during benzo[a]pyrene metabolism, which damages cellular molecules (Rubin 2001; Umannová et al. 2011). When benzo[a]pyrene biotransforms into dihydroxy-epoxy-tetra hydro-benzo[a]pyrene, it attaches to DNA and forms DNA adducts, which causes mistakes in DNA replication and finally unchecked cell division or cancer. Banzo[a]pyrene is a known carcinogen and it also disrupts the immune system's growth and operation, in addition to affecting the fertility of the progeny. After benzo[a]pyrene harmed human health by causing teratogenicity, immunotoxicity, carcinogenicity (Borji et al. 2020; Darajeh et al. 2020), mutagenicity, and neurotoxicity (Burchiel 2005), it was discovered that it also harmed animals (Figure 3). Tumours are induced in different organs during the use of laboratory animals for experimental tests. According to the International Agency for Research on Cancer, it is classified as a group I carcinogen (IARC 2010; Lindeman et al. 2011). According to the WHO (2003), a daily intake of 257µg/day orally causes tumour and carcinogenic effects when in direct contact with the subcutaneous route (WHO 2003).

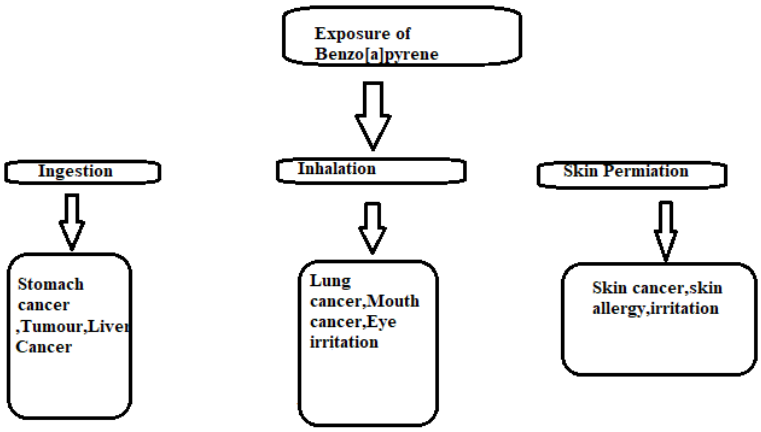

Figure 3 Benzo[a]pyrene Exposure to Human health

4.1 Other Benzo[a]pyrene Side Effects

According to Kungwani et al. 2022, the major side effects of benzo[a]pyrene cause environmental pollution and are responsible for global warming. The major effects of benzo [a]pyrene pollution, according to this study, are contamination of oceans, rivers, ponds, land, and air, loss of animal species, and loss of agricultural productivity due to soil contact with the oil spill (Bukowska et al. 2022). Waterbody species have died in large numbers due to contamination with benzo[a]pyrene and its relevant compounds (Patel et al. 2020).

5 Factors Influencing the Degradation of Benzo[a]pyrene

Bacterial and other microbial degradation of benzo[a]pyrene and its relevant rings fused compounds are affected by environmental physicochemical factors such as various temperatures, pH level, oxygen level, nutrient (energy source) availability, light, and salinity (Rajpara et al. 2017; Zheng et al. 2018).

6 Conclusion and Future perspectives

Benzo[a]pyrene is one of the known toxicants among aromatic compounds. Benzo[a]pyrene has high stability in the environment and severe hazard to human health and causes mutagenicity, carcinogenicity, immunogenicity, and taratogenicity. The main source of benzo[a]pyrene contamination is a petroleum processing plant, petrol pump station, automobile, and garage. Due to their recalcitrant in benzo[a]pyrene, they enter to the biological system through the food chain. Benzo[a]pyrene binds to DNA (DNA adducts forming) genetic material covalently and causes mistakes in DNA replication that ultimately result in unregulated cell division or carcinoma and causes in humans and animals. Bacterial degradation has been reported in previous, aerobic bacteria are *Mycobacterium sp. PYR-1*, *Mycobacterium RJMII-135 sp.*, and anaerobic bacteria are *Pseudomonas* sp. JP1, *Cellulosimicrobium* sp., *Magnetospirillum magneticum strain AMB-1*, *Azoarcus sp. EbN1*, *Rhodopseudomonas palustris strain CGA009*, Thauera aromatica, *Geobacillus metallireducens GS-15*. In co-metabolism anaerobic degradation, *Cellulosimicro biumcellulns CWS2* have generated diethyl phthalate, 2-acetyl 3-methoxybenzoic acid and *Hydrogenophaga sp. PYR1* generated pyrene. In the aerobic degradation pathway, *Mycobacterium sp. PYR-1* converted benzo[a]pyrene into benzo[a]pyrene-11,12-epoxide, trans-11,12-benzo[a]pyrene-dihydrodiol and cis-11,12-benzo[a]pyrene-dihydrodiol and *Mycobacterium RJMII-135 sp.* converted pyrene-8-hydroxy-7-carboxylic acid into 7,8-dihydro-pyrene-7-carboxylic acid in the form of by-product. Degradation and mineralization of benzo[a]pyrene are depends on microorganism and their environmental conditions. Most of the studies were consistent with the degradation of low molecules PAHs but, limited work has been published on benzo[a]pyrene with high molecular weight due to their polycyclic structure. Future work should be revealed in their detailed degrading pathway and enzyme involved during its detoxification. Further, the future process of benzo[a]pyrene degradation are biostimulation, bioventing, and biosparging; the process may be more effective and environmentally sustainable for the benzo[a]pyrene de- contamination from the polluted sites.

Conflict of Interest

All authors declare that they do not have any conflict of interest.

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Comprehensive Review of Aquaponic, Hydroponic, and Recirculating Aquaculture Systems

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ABSTRACT

Hydroponics and aquaponics are emergent agricultural techniques that offer several environmental solutions. It is anticipated that the hydroponic systems will result in a more significant profit from selling vegetables and other plants. The use of new technologies, such as hydroponics and aquaponics, has been demonstrated to increase the number of plants that can be grown. The recirculatory aquaculture system makes it possible to multiply fish production while consuming fewer resources. Essential factors of this technology include higher yield, safety, and water management. In addition, the scope of potential future research in hydroponics and aquaponics has been discussed. Furthermore, the paper identifies and discusses the various applications of hydroponics and aquaponics in agriculture.

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1 Introduction

The need to continuously develop alternative farming methods has always been important, especially in response to the multiple problems faced by conventional agriculture (Manos and Xydis 2019). Various organizations such as European Union, the Environmental Protection Agency, and the WHO often recommend the use of recycled water for irrigation, as a suitable solution for formulating water management strategies. According to studies, Hydroponic systems can be utilized to both produce food and treat wastewater (Sundar et al. 2021). They are a simple technology that enables the soil-free growth of plants in water (Jayachandran et al. 2022). Plants get the nutrients they need for growth from the aqueous solution (Shubham and Shrimanth 2020). Because plants can absorb nutrients, toxic metals, and emerging pollutants, the hydroponic system may also be utilized as a management technique before partially treated effluent is released eventually into the environment (Cifuentes-Torres et al. 2020).

Aquaponics means the co-cultivation of fish and plants together and it is widely used as a solution to the growing food demand in urban landscapes. The combination of aquaculture and hydroponics provides an environment where both can flourish. Filtered aquaculture effluent is supplied into the hydroponic technique for giving food to bacteria along with the roots of plants. This water is reused back into the fish farming tanks once the accumulated nutrients have been removed. To increase the nitrogen utilization rate in aquaponics systems, this analysis investigates the significant consequences of nitrate nitrogen from influent to output, including NH_3, nitrogen generation, nitrogen removal, nitrate absorption, and nitrogen transport (Schmautz et al. 2021).

Most people believe aquaponic farms to be a superior opportunity to hydroponic farms as a soilless growing method. Controlled-farming techniques such as aquaponics and hydroponics have become a great method for feeding the world's population, which is rapidly expanding while using minimum land and water. The hydroponic system, however, might be more environmentally friendly than the aquaponics system if the energy source were switched to renewable sources of energy. This life cycle assessment research can give control to environmental agriculturists with the foundation work to decrease the price of manufacturing (Chen et al. 2020).

The manufacturing system is depending on a stable equilibrium among the bacterial, plant, and fish groups (Figure 1). Compared with aquaculture systems, the core microbial communities of the decoupled and connected systems share other widespread operating systematic groups (Eck et al. 2019).

Aquaponics is a system in which fish waste feeds plants, which in turn cleans the water; when fish produce waste (ammonia) which is converted to nitrate by bacteria so that the plants can take it up (Figure 2) (Puteri Edaroyati et al. 2017).

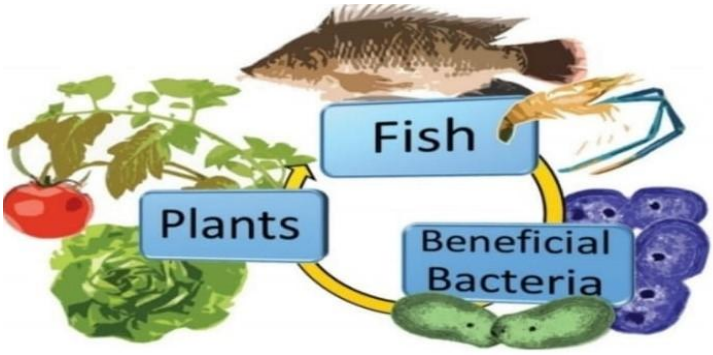


Figure 1 Components of an aquaponic system (Pattillo 2017a).

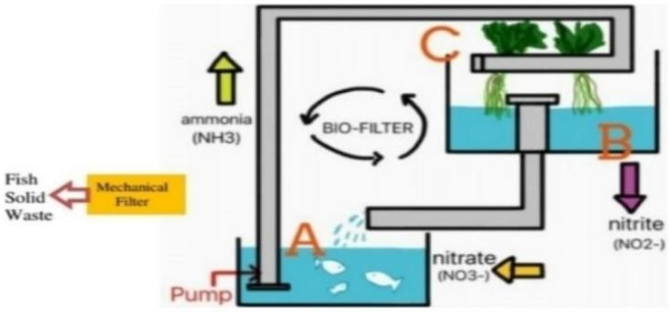


Figure 2 Schematic diagram of the Aquaponic cycle (El-Essawy et al. 2019).

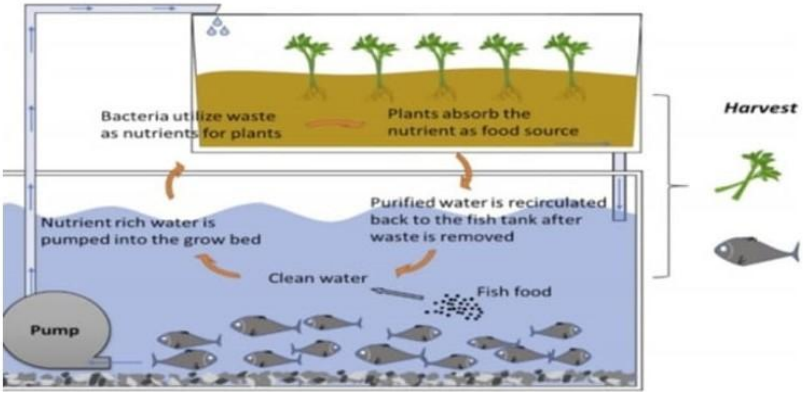


Figure 3 Schematic diagram of Aquaponics (Wei et al. 2019)

This technology makes it possible to use coastal areas, nutrient reuse, reduced pollution, great production, and more effectively. Future aquaponics systems will grow smarter, more efficient, and more effective as a result of technological advancements (Wei et al. 2019) (Figure 3). The futuristic farms have developed the right technology for automated hydroponics, making them pioneers in the hydroponics scene. Monitoring of automated aquaponics technology, the internet of things (IoT), and intelligent systems claimed to have high water efficiency, and consume 90% less water than traditional farming (Bawiec et al. 2018). The technology is environmentally friendly since it uses fewer fertilizers and nil pesticides (Yanes et al. 2020).

The principal objective of aquaponic systems is to stimulate plant growth by employing aquatic animal waste. As a result, aquaponics uses fish waste as plant fertilizer whereas the plants clean the fish's water.

2 Plants used in aquaponics and hydroponics

Herbs like sweet wormwood (*Artemisia annua*), gotu kola (*Centella asiatica*), and especially greens such as spinach (*Spinacia oleracea*), lettuce (*Lactuca sativa*), spearmint (*Mentha spicata*), basil (*Ocimum basilicum*), chives (*Allium schoenoprasum*), and watercress (*Nasturtium officinale*) are perfectly suitable for aquaponics systems. The edible plants like carrot, pumpkin, amaranth, swiss chard, bell peppers, sweet peppers, pak choi, tomatoes, mustard green, kai lan, and cucumbers have an advanced nutritious demand and its efficiency improved in an extensively provided safe aquaponic system.

2.1 Tomato production

Compared to conventional tomato cultivation, evaluating the economic effectiveness of multi-tiered narrow-rack hydroponics showed inherent advantages (Balashova et al. 2019). Askari-Khorasgani and Pessarakli (2020a) observed that in the vermin hydroponic system, the most important thing is in the vermiponic units, the most effective tomato (*S. lycopersicum*) cultural customs provide the best economical and environmentally friendly method of horticultural crops.

2.2 Tomato, basil, and lettuce production

Yang and Kim (2020a) evaluated the phosphorus and nitrogen accumulation stability for tomatoes, lettuce, and basil established in hydroponic and aquaponic technology. The research revealed that the distribution of N and P is mostly affected by these plant species. However, due to its increased biomass output, the tomato was more successful than lettuce and basil at extracting nitrogen from sewage and minimizing denitrification (Yang and Kim 2020a). Their investigation further demonstrated that the profitable give of tomatoes was alike between hydroponics and aquaponics, as lettuce and basil had relatively higher yields (Yang and Kim 2020c).

2.3 Kale production

Kale (*Brassica oleracea l.)* is one of the vegetables produced quickly in hydroponics systems and can be supplied to the market. Kale was cultivated with various stages of natural nutrients in the total hydroponic system to determine its yield, pigment composition, and chemical characteristics. The study showed that Acacia and moringa ferments can be used as nutrient solutions to support kale production in a total hydroponic system and can significantly impact promoting climate-smart organic agriculture (Salas et al. 2020).

2.4 Carrot production

A healthy and manageable root environment is offered by hydroponics. Sakamoto et al. (2020) reported that hydroponic carrots (*Daucus carota*) could be shaped differently based on partially removed early taproots. They observed how partial removal of early hydroponic carrots' early taproots influences their growth mechanisms.

2.5 Swiss chard production

The effects of microelement addition to aquaculture effluent on the swiss chard development (*Beta vulgaris L. spp. cicla*) were found in an aquaponic system using saline groundwater. When microelements were supplied, Swiss chard grown with aquaculture effluent produced sufficient production without chlorosis (Kaburagi et al. 2020).

2.6 Cherry and Tomatoes production

Organic hydroponic fertilizer should be used if certain plants require mineral nutrients supplementation. Hydroponically grown cherry (*Prunus avium*) and fresh tomatoes (*Solanum lycopersicum*) respond to limited fertilization and reduced nutrient concentration when grown in shade nets. Studies on cherries and tomatoes, may minimize nutrient expenditure and result in cost savings of 25% and 50% on fertilizer input costs, as well as decrease the risk of water contamination (Maboko and Du Plooy 2017).

2.7 Mini-Cucumber production

Researchers investigated the nutrients of hydroponically grown mini-cucumbers (*Cucumis sativus* L.) Maboko et al. (2017) studied that the nutritive value was heightened by the condensed nutrient concentration and foliar fertilization. An analysis of the study showed that a reduced nutrient concentration of 75% is enough to maintain cucumber yields and quality, whereas foliar fertilizer has little effect.

2.8 Mint and mushroom herb production

Mint (*Mentha piperitta* L) and mushroom (*Agaricus bisporus*) have excellent nutritional value, making them suitable for aquaponic farming due to the small amount of complement addition required. Hence the modest management effort was causing costs to be raised (Nozzi et al. 2018).

2.9 Pak Choy production

A household-scale aquaponics system was used to optimize the growth of Pak Choy (*Brassica rapa L. var. Chinensis*). Priadi et al. (2019) reported that in the vertical design, Pak Choy's growth parameters were advanced compared with the horizontal one, though the differences were not statistically significant.

2.10 *Nigella sativa* production

Hydroponically grown *Nigella sativa* was studied to determine if additives affected its growth. Using standard plant growth stimulants is a viable alternative to chemical fertilizers. It has been shown that plants treated with *Tricoderma harzianum* spores (fungus) have improved in essential oil and NPK. In hydroponic plant growth, all treatments resulted in an increased yield of seeds, oils, and plants (Nosir et al. 2017).

2.11 Lettuce production

Lettuce is one of the most popular vegetables grown in aquaponic systems. The study showed the potential for year-round lettuce making in the flow-through aquaponic system. Standard yield in the Spring period was the maximum, while productivity in the summer season is elevated than that in spring (Johnson et al. 2016).

Utilizing various growing media, lettuce was produced in hydroponic and aquaponic methods. In both aquaponic and hydroponic systems, the cocopeat-based substrate produced higher yields, making it a better choice for growing lettuce. Lower average harvests were seen in both of the examined production systems when the phenolic foam was used as a medium for agriculture (Jordan et al. 2018). The application of a technique for butter crunch lettuce (*L. sativa*) vertical flowing hydroponic systems coupled with zeolite restoration to create a reusable nitrogen protection system is suggested. The research proved the possibility of fertilizer purification units to concurrently decrease anaerobic membrane bioreactor ammonia-nitrogen concentration while supplying a long-term source of nitrogen for use in hydroponic systems (Calabria et al. 2019).

The study found that fertilizer injection allows a more significant addition of nutrients than water supply treated with chemical fertilizers. Conversely, water supply without supplemental nutrients leads to diminished biomass and nutrient deficits in the vegetation (Mahlangu et al. 2016; da Silva Cuba Carvalho et al. 2018). At the same time, conventional cultivation was done with direct irrigation by using hydroponic solutions, natural irrigation with aquarium waste and wastewater, and managed examination using fresh water with fertilizers. Particularly since the farming region has insufficient water resources, systematic farming is also approximately 80% more water-efficient than topsoil farming (Sayara et al. 2016). The organic hydroponic method was used to study the effects of magnetic field treatments on nutrition solutions on lettuce plant growth (Youssef and Abou kamer 2019). Similarly, Moraes et al. (2020) worked on three lettuce cultivars in a hydroponic system with plants grown below the greenhouse and reported that the cultivar is preferred for the development of leaf length compared with supplementary cultivars.

2.12 Lettuce and water spinach with koi fish and Rainbow trout

Lettuce (*L. sativa L.*) along with rainbow trout (*Oncorhynchus mykiss w.*) crops were grown within a recirculating aquaponic method. According to the study, the lightweight expanded clay aggregate section of a new aquaponics system removed more

ammonium, nitrate, and orthophosphate than the raft portion of the plan (Velichkova et al. 2019). Based on the study, the combination of water spinach plants with koi fish formed the maximum yield and determined the maximum complete development of fish and a survival rate of hundred percent (Andriani et al. 2019).

2.13 Lettuces and lambari fishes

Bianchini et al. (2020) demonstrated that sanitation procedures before consuming vegetables must be achieved, regardless of the agriculture technique. The study showed by slurry that total and thermotolerant coliforms were present in all phases of aquaponics (system combining lettuces and lambari fishes).

Further, to estimate the water quality of an aquaponic system, lettuces and lambari fishes were collected and this study showed that total coliforms and thermotolerant coliforms were present in all phases of aquaponics, demonstrating that sanitation procedures before the consumption of vegetables must be achieved. It suggested that the shrimp concentration was unsuitable, which made the system incapable of raising the concentration of nutrients inside vegetables with reduced yields. It was initially believed that domestic lettuce hydroponic cultivation produced organic wastes, but commercial lettuce production was enhanced to make use of components in the water, including magnesium, potassium, and calcium (Lima et al. 2019).

2.14 Gotukola with koi carp

In aquaponics, phytoremediation wastewater was used to grow gotukola (*Centella asiatica*) and koi carp (*Cyprinus carpio var. koi*). By evaluating the proportions of nutrients removed and biofilter performance, aquaponics was evaluated for its effectiveness. It was determined that aquaculture wastewater treated with phytoremediation could be recycled for fish and plant production in aquaponics (Nuwansi et al. 2019).

2.15 Mint with common carp

In hydroponics with an aquaculture system, healthy typical koi (Cyprinus carpio) were grown using various hydroponic media for mint (*Menthaar vensis*). According to this study, compressed sandstone and balanced raft were significantly more competent than river stone in nutrient removal and maintaining satisfactory water quality for fish culture (Shete et al. 2017). Similarly, in a combined system, the production activities of various plants must be measured on the best levels. This best water application flow value inside hydroponics with aquaculture method by healthy typical koi (*C. carpio*) along with *Menthaar vensis* bio-integration was determined (Mint). The aquaponic recirculation system proved a successful method for carp and mint production and an advantageous method for recycling fish farming effluent to preserve its water wealth (Shete et al. 2016).

2.16 Pumpkin with catfish

Similarly, an aquaponic Catfish-pumpkin system illustrates the impact of various growing mediums both water excellence along with vegetation productivity. Water excellence and the proportion of reduction of mineral nutrients (Ammonia, Nitrite, as well as Nitrate) throughout the structure including in dissimilar growth stages are significant (Oladimeji et al. 2020b). Analyzed the growth of fingerling striped catfish (*Pangasianodon hypophthalmus*) in an aquaponics system with fine bubbles. A study found that aquaponics with fine bubbles led to increased fish growth, fish survival, and the highest plant growth (Naomi et al. 2020).

2.17 Goldfish with microgreen

Kizak and Kapaligoz (2019) monitored changes in the growth of goldfish (*Carassius auratus*) and aquatic habitats in microgreen recirculating aquaponic systems. Their study found that when the pH was high, Arugula micro-greens primarily used ammonia instead of nitrate nitrogen.

2.18 Spearmint with Tilapia production

Aquaponics can also be used for plants high in nutrients like Spearmint. The growth of herbal plants is suitable for a good environment, and they could be employed as biofilters within hydroponic aquaculture production technologies. Spearmint plant has a maximum production rate, which indicates its ability to effectively absorb nutrients within that method (Espinosa-Moya et al. 2018).

3 Hydroponic systems in domestic wastewater treatment

Integrating hydroponic systems with household wastewater treatment can lower expenses by removing fewer contaminants and using less energy and maintenance than traditional wastewater treatment. The decentralized system is employed for vegetable production, home wastewater treatment, and municipal agriculture. A hydroponic system is acceptable for wastewater treatment if it can reduce the health concerns that come with contact with wastewater for farmers, harvested crops, and customers (Magwaza et al. 2020).

It is suggested to treat wastewaters with a high concentration of organic matter and nutrients and poor biocompatible to produce an effluent that may be utilized as a nutrient solution for lettuce hydroponic production (*L. sativa var. crisp*). The generated effluent gives a high pH and nutritional level (nitrogen & phosphorus) for lettuce growing in the hydroponic system. The procedure had a 100% success rate in removing all coliforms (Da Silva Correia et al. 2018). This study's objective was to assess how biologically active component levels in greywater were treated using the hydroponic technique as a treatment system. According

to the findings, whether or not the third stage of purification is used, the efficacy of purifying will be comparable (Bawiec 2019).

Eregno et al. (2017) measured the health risk of using grey water recycled for hydroponically growing lettuce in an eco-friendly wall. It was shown that greywater treatment systems could be made better by adopting suitable research techniques and growing plant varieties capable of capturing less heavy metals and removing them from the greywater.

Hydroponic root mats be a green technology used for various wastewater decontamination. The Vetiver grasses (*Chrysopogon zizanioides*) were suitable for nutrient and organics removal in the bio-remediation of brewery wastewater utilizing hydroponics. The results showed that the phytoremedial ability of the vetiver grass is used in improving wastewater quality (Worku et al. 2018). This study showed that making hydroponic root mats are sustainable. It was found that harvesting before the plants fade is more successful in removing nitrogen and retaining a sustainable structure (Sun et al. 2019).

4 Modern commercial aquaponic systems

4.1 Coupled aquaponics systems

There are two main types of aquaponic systems - coupled and decoupled. Typically, coupled aquaponics systems have an unidirectional flow of water that begins in the fish culture unit, passes through solids and biological filters, and then through the hydroponic unit and sump tank before returning to the fish to complete the cycle. Plant nutrition, solids, biological filtration, and feed consistency are vital to the product's continued and predictable production (Figure 4).

4.2 Decoupled aquaponic systems

A decoupled aquaponics system provides a separation between the fish and plants in the farming process, treating diseases in one part of the system without completely disrupting the entire system (Pattillo 2017a). These methods can develop to be the most successful, long-lasting workflows for the growth of plant and animal resources. The study shows that it decreases carbon dioxide released in a vegetable growing place by 72% by reducing the power of a waste reduction and reprocessing system (Abbey et al. 2019). A statistical study was carried out to improve the system using a multi-loop decoupled aquaponics system (Figure 5). The results showed that nutrient concentration ranges significantly change the presentation of several aquaponic methods within conditions of longevity (Dijkgraaf et al. 2019).

Maucieri et al. (2017) showed that the intercropping technique is a possible solution to improve vegetable quality in an aquaponic system. The multiple cropping of vegetables did not control the oxidation–reduction potential, electric conductivity, pH, temperature, water, nor O_2 content.

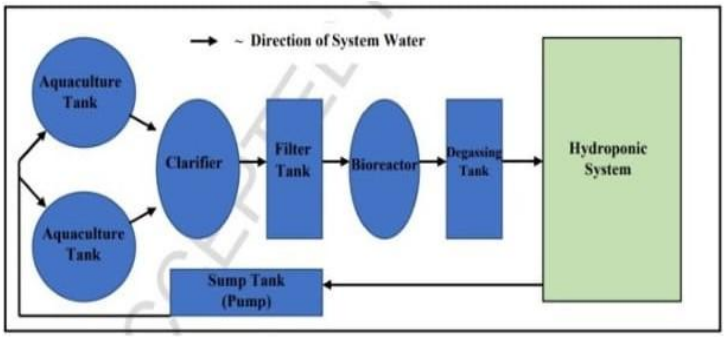


Figure 4 Example of coupled Aquaponics system (Rakocy 2012; Palm et al. 2018)

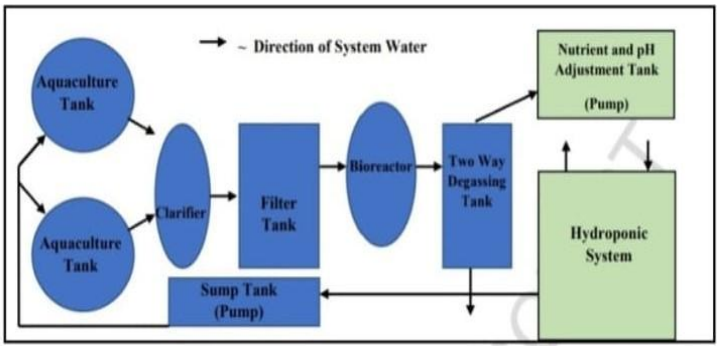


Figure 5 Example of decoupled Aquaponics system (Kloas et al. 2015; Rakocy 2012)

Figure 6 Dutch Bucket hydroponic system for cultivating tomatoes (Osei et al. 2019).

Figure 7 Drip irrigation system (Sahubawa et al. 2020).

4.3 Double recirculating aquaponic system

To cultivate tomato crops that are similar to those in a hydroponic system, dual recirculation aquaponics containing 2 self-regulating phases is necessary. This system lowers the reasonable costs of plant production and recycling of aquaculture effluent along with a related decreased amount of nitrogen production (Suhl et al. 2016).

Studies on low-tech aquaponics systems with different fish-supplying densities by Maucieri et al. (2020) showed that aquaponics systems on modest providing mass-improved crop production, related to hydroponics, but not reducing the quality of the vegetables. Compared to hydroponics, aquaponics gives a higher supply volume and is better economically valuable.

4.4 Aquaponics with subsystems

Macrophytes in the media bed system and NFT are used to remove dissolved phosphorus, total ammoniacal nitrogen, nitrite-nitrogen, nitrate nitrogen, and total nitrogen (Li et al. 2019). Aquaponic systems with two different hydroponic subsystems were used to analyze the development with a yield of fish organisms. This study demonstrates that, throughout the entire process of testing the statistical significance, the aqueous spinach cultivars' proliferation and productivity were not enhanced by hydroponic components (Quí et al. 2020). Hydroponic and immobilized biofilm technology were produced for controlling water quality and increasing feeding effectiveness in pilot-scale aquaponics systems, including mass biosystems.

4.5 Dutch bucket hydroponic system

This system utilized buckets (of varying sizes, filled with inert medium) to grow plants. Plants with extensive roots, such as tomatoes, cucumbers, etc., can be grown in this system. The Dutch bucket works based on the principles of ebb and flow. It is just a variation of that method. The nutrient is forced into a bucket, where it is automatically drained back into the reservoir. The Dutch Bucket is the easiest and most effective way to grow hydroponically indoors or outdoors (Buckets 2018) (Figure 6).

4.6 Dripping method for hydroponics

Irrigation water employs a motor to regularly supply plants with fertilizer and water, making it a dynamic hydroponic design. It is also known as a trickle irrigation system or micro irrigation system. Small emitters are used to drip-wise feed the fertilizer solution onto the plants. Tangune et al. 2016 assessed *Brassica oleracea's* reaction to groundwater concentration below dripping irrigated agriculture during confined surroundings (Figure 7). This study revealed that the soil water tension could not extensively affect water use effectiveness or height of marketable heads.

4.7 Automated hydroponic system

Automated methods will be investigated to provide consistent nutrients in hydroponic solutions by automatically adding nutrients and nutrient concentrations. The automated hydroponic modular system (Figure 8) showed the methodology based on components

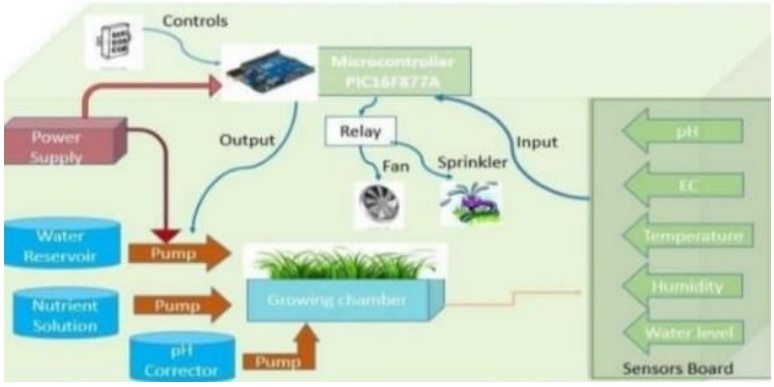


Figure 8 Fully Automated hydroponics system (Shetty et al. 2021).

and provides the pH value of the liquid fertilizer; if the level is insufficient, it will immediately activate the absorption of the solution into the container storage to afterward run the automated irrigation system for hydroponics (González-Linch et al. 2019). Similarly, a computerization system is planned using raspberry microcontrollers and Arduino as a comparative management technique, and it could manage the water temperature and pH (Supriadi et al. 2019). The dissolved oxygen content in an aquaponics system is increased by modifying the aquaculture water and the greenhouse environment (Ren et al. 2018).

The hydroponics by nutrient film technique is a technology for the agriculture of green fodder. This research confirms it is important for agriculture for its extremely used vegetation growth space level and closed-loop water management to plants, which enables it to be readily automated depending on solution conditions. It is beneficial to develop NFT equipment and process management systems in maximizing hydroponic food production methods (Grigas et al. 2020). A hydroponic system integrating pH sensors is suitable for small space areas such as high-rise buildings. The research demonstrates that the computerized method is appropriate for the internet of things and that the user may integrate this information into a created computer to get the best output (Esa et al. 2019).

5 Hydroponic growing methods used in aquaponics

Recirculating nutrient film technique, vertical felt, floating raft, deep floating technique, flood-and-drain system, and aeroponics system are the hydroponic growing techniques utilized in aquaponics systems.

6 Important parameters for Aquaponics and Hydroponics

The environment for the aquaponic system should be patently chosen to avoid pollution factors. The water physicochemical analysis of pH, temperature, dissolved oxygen, nitrate, nitrite, ammonia, calcium, magnesium, and phosphorus should be performed for the maintenance of the aquaponic system (Gavrilă et al. 2019).

6.1 pH and Temperature

Water quality is a very significant parameter for aquaponics and hydroponics cultivation systems. The major factors include total nitrogen, pH, water temperature, dissolved oxygen content, and water hardness. The pH range for hydroponics is 5.5 to 6.5 (Trejo-Téllez and Gómez-Merino 2012) and the optimal pH range for aquaponically grown crops is between 6.5 and 8.0 (Goddek et al. 2015). In hydroponics, it is advised to keep the temperature below 70°F, while in aquaponics, it should be around 82 and 86°F. According to Yang and Kim (2020b), stream speed plays a significant role in the construction of aquaponic crops because it influences the temporal and spatial water quality parameters, which in turn determines the development and productivity of the crops. Aquaponic production requires good water management. A freshwater flow-through fish culture system was used to evaluate the aquaponic crops. The researchers found nutrient recruitment from accumulated solids in the aquaponic channels was responsible for the slightest improvement in water quality (Buzby et al. 2016).

Numerous necessary components for crops are lacking in aquaponics, and the pH of the water can either make these nutrients more or less. To study more about the nutritional kinetics of this mechanism, the investigation into other nutrients offered by the system (such as solid particles conveyed with irrigation water) is recommended (Blanchard et al. 2020). Hydroponic food production is also used to improve crop quality, including taste, sensory attributes, and post-yield durability (Walters et al. 2020).

It was determined through water quality analysis that the aquaponic system's warmth, alkalinity, dissolved oxygen concentration, the total suspended particles have all been within the allowable limit and adequate for the development of Tilapia fish, everlasting greens, green mustard vegetables, and nitrification bacteria. Several necessary economic and fruit cultivation trades can be developed using aquaculture wastewater as part of a large-scale development program (Sahubawa et al. 2020; Alam et al. 2020).

Table 1 Types of Aquaponics and subsystems

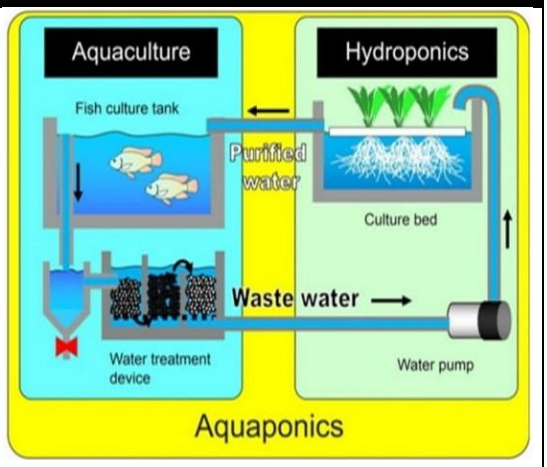
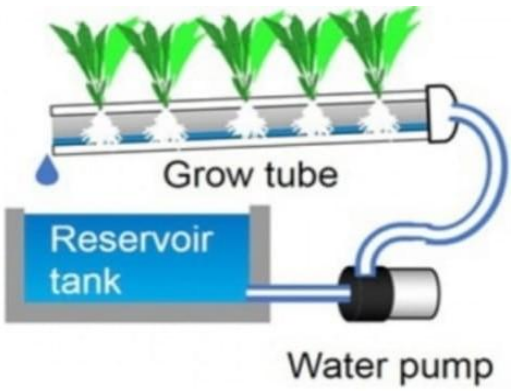
S. No.	Types	Aquaponics and subsystems	Figures
1	Aquaponic system.	In the municipal areas, there are some possibilities for producing supportable seafood and plant production using aquaponics. In this case, some research gaps exist related to the following aspects such as 1. Variety of aquatic and plant species studied. 2. Miscellaneous distribution of the environmental and economic impacts to the co-products. 3. Transportation of formed food 4. Presence of heavy metals, pests, and pathogens with human health consequences (Figure 9) (Wu et al. 2019).	 Figure 9 Aquaponics system (Endo 2018)
2	Aquaponics with subsystems	The comparative studies carried out between deep water culture and integrated aqua vega culture systems at the pilot scale showed that profitability and water conservation were higher in aquaponic systems. This is due to the higher fish production in deep water culture. It is possible to practice by making additional investments in aquaponic system industrial design for reducing expenses and consequently, it is further appropriate for interior agriculture production. (Figure 10) (El-Essawy et al. 2019). This allows an understanding of gaps in product design.	 Figure 10 Aquaponics with sub-systems (El-Essawy et al. 2019).

Table 2 Methods of aquaponic hydroponic gardening

S. No.	Types	Aquaponics uses hydroponic growing techniques.	Figures
1	Nutrient film method	An innovative method of farming known as the "nutrient film method " distributes nutrients by bare roots to provide the nutrients needed by plants for proper growth and development (Qadeer 2020). The studies related to Peppermint (*M. piperita*) and Coriander (*Coriandrum sativum*) performed well as secondary biofilters within a recirculating aquaculture system based on the nutrient film system (Ogah et al. 2020). Suhl et al. 2019 demonstrated that fish wastewater is utilized to nourish NFT hydroponic systems developed vegetation to manage the oxygen assimilation is extremely suggested, particularly at high levels of conductivity (Figure 11). This provides a chance to understand variations in nutrient concentration for various plants.	 Figure 11 Recirculating Nutrient film techniques (Endo 2018)

2	NFT hydroponics system and NFT aquaponics system.	Jordan et al. 2018 designed Lettuce cultivated on different substrates in hydro and aquaculture systems (Figure 12). The cocopeat medium produced higher yields in both hydroponic and aquaculture systems, making it a preferable option for producing lettuce.	 Figure 12 (a) Nutrient film techniques hydroponic systems (Pattillo 2017b)  Figure 12 (b) Nutrient film techniques aquaponic systems (Pattillo 2017b).
3	Different hydroponic subsystems NFT vertical felt and floating raft.	Different hydroponic subsystems were made (nutrient film technology, vertical felt, and floating raft) (Figure 13). Those are evaluated using the United States alimentary and agricultural agencies that serve as models to produce goldfish (*C. auratus*) and lettuce (*L. sativa*). The study showed similar results in fish production (Pérez-Urrestarazu et al. 2019).	 Figure 13(a) Nutrient film techniques: Vertical felt (Pattillo 2017b)  Figure 13(b) Nutrient film techniques: Floating raft (Pattillo 2017b).

Table 3 Other Types of Hydroponics

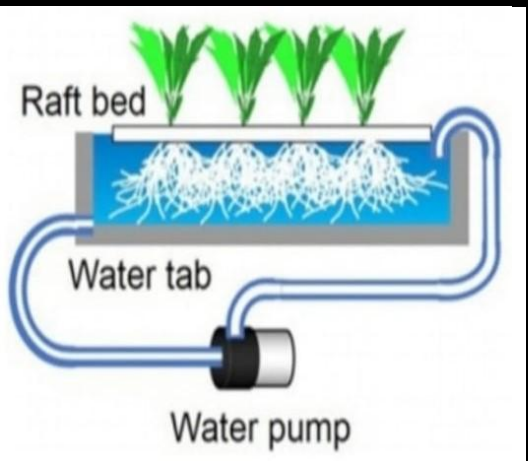
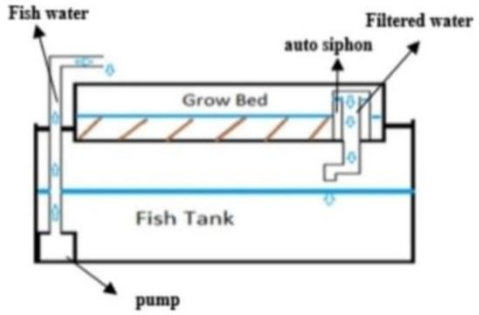
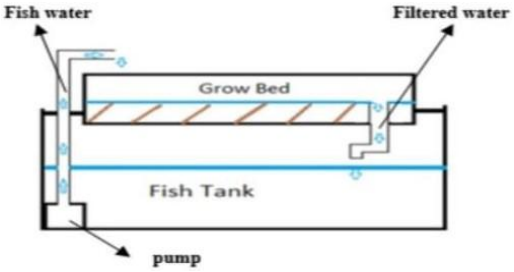
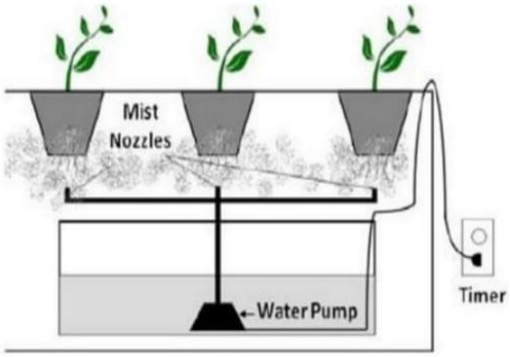
S. No	Types	Other Types of Hydroponics	Figures
1	Deep water culture / Deep floating technique. • It is a hydroponic technique in which plant roots are submerged in nutrient-rich, highly oxidized water (Series and Science 2021).	Triyono et al. (2019) showed how 3 distinct hydroponic containers like a hydraulic cooler, a Polystyrene box, and a bucket affected the growth of three hydroponically grown vegetables such as pak choi, mustard greens, and kai lan. (Figure 14). This study shows that using polystyrene boxes for fertilizer storage did not significantly boost production for tropical locations and that plants growing with nutrients stored in the cooler performed the best for various testing conditions. (Triyono et al. 2019). In bigger commercial-scale systems, it is frequently used (Janni and Jadhav 2022).	 Figure 14 Deep floating technique (Endo 2018)
2	Systems for flooding and drainage and continuous flow. • Drains nutrient solution back into the reservoir after rapidly flooding grow tray by using a submersible pump.	For carps (*C. carpio*) and basil, the flooding and water steady flow methods were examined for variations in water condition, carp production, and plant growth measures (*Ocimum basilicum*). This study showed that both the flooding and drainage and steady flowing methods are suitable for the growing of carp and basil (Homoki et al. 2020) (Figure 15).	 Figure 15 (a) Flood- and- drain system (Homoki et al. 2020)  Figure 15 (b) Constant flow system (Homoki et al. 2020)
3	Aeroponic system	Plant roots are continuously or intermittently in a deeper air or development tank elevated while nutrient solution in droplets is periodically supplied for plant growth. The process provides high aeration (around 100%) and consumes very little water (Biswas et al. 2022). Reena Kumari and Kumar (2019) studied that organic agriculture and hydroponics are both used in aeroponic agriculture. These methods have successfully produced crop potatoes, leafy vegetables, culinary herbs, and reproduction for economic uses. Aeroponics is a very practical way of growing root systems and aerial portions (Figure 16).	 Figure 16 Aeroponics system (Saxena 2021)

Table 4 Merits and Demerits of aquaponic, hydroponic, and related systems

S. No	Aquaponic, hydroponic and related systems	Merits	Demerits
1	Aquaponic system	1. Organic manufacturing method of aquatic organisms and vegetables. 2. Reduce damage from pests and diseases. 3. Significant reduction in the use of water.	1. It is not used for commercial production. 2. There is insufficient lengthy research (Tyson et al. 2011). 3. Reduced efficiency, particularly on the plant side.
2	Aquaponics with subsystems	1. Water quality control and improved nourishment effectiveness. 2. Produced high quality safe organic food. 3. Provide an artificial filtration system for the fish culture environment.	1. High demand for monitoring and control. 2. Lack of skillful technicians and laborers.
3	Wick system	1. Regulates the container's base humidity at a steady level. 2. Simplicity and space efficiency. 3. According to the permeable mat, uses less water.	1. For the manufacture of major commercial crops, the technique is not yet established. 2. It works well for tiny, non-fruiting vegetation, like herbs. 3. It is impossible to maintain slow procedures and crops which require additional water. e.g., tomato
4	a) Recirculating nutrient film technique	1. Perfect for large-scale commercial endeavors. 2. Don't require growing media. 3. Plants grow faster.	1. Purify the culture medium. 2. It is expensive and complicated.
	b) NFT hydroponics system and NFT aquaponics system	1. The potential exists for the general aquaponics technology to provide crop growth at least equal to that of conventional hydroponics. 2. Easy to build & maintain. 3. Low water and nutrient consumption.	1. NFT technology might not be the best technological advancement for an aquatic system. 2. Pump failure can cause the depth of crops to in a few hours.
	c) NFT vertical felt and floating raft are two different hydroponic subsystems.	1. Similar results in fish production. 2. Successful production of leafy vegetables. 3. Very easily adaptable to different spaces & plant requirements.	1. Constant monitoring is required. 2. The roots in the channels can become blocked by blocks of vigorously growing plants (Gaikwad 2020)
5	Deep water culture / Deep floating technique	1. Conservation of water and Fish growth. 2. Used to cultivate a single crop such as lettuce, basil, or other green leafy plants. 3. Does not require farmland with fertile soil.	1. Lack of oxygen in water can happen easily. 2. Nutrient solution needs to be checked frequently. 3. It may be challenging to control the temperature.
6	Drip irrigation system	1. Maximum crop yield. 2. It reduces weed growth. 3. No soil erosion problem.	1. Responsiveness to clogs. 2. Difficulty with humidity dispersion. 3. Risks associated with saltiness (Manda et al. 2021).
7	Flood and drain system and constant flow system	1. Equally suitable for carp and basil cultivation. 2. Efficient with space, relatively low cost. 3. It is easy to build up the structure.	1. Unstable pH levels. 2. Creation of toxicities that are harmful to the crop; this brings losses to the farmer. 3. It is inappropriate for plants that require additional irrigation.
8	Aeroponics system	1. It produces healthier root systems 2. It provides us with a valuable research tool. 3. It is easy to replace old plants with new ones. 4. Due to the abundant oxygen available to plant roots, crops grow quickly.	1. It requires constant monitoring to be successful. 2. It is an expensive growing method to set up initially. 3. It is highly susceptible to power outages. 4. We must have a certain level of technical knowledge.

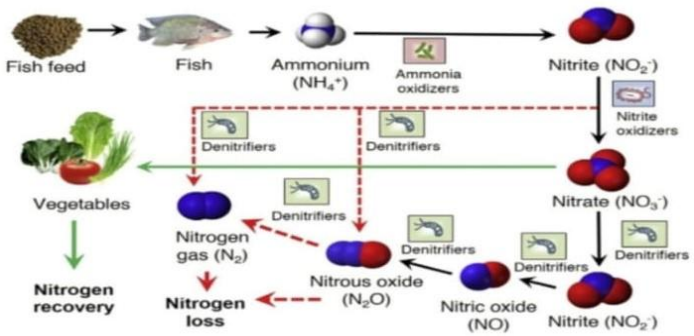


Figure 17 Nitrogen transformations in aquaponics systems (Wongkiew et al. 2020).

6.2 Nitrogen and phosphorus removal by Aquaponics

Nitrogen and phosphorus compound removal efficiency is essential in the hydroponic and aquaponic systems. Aquaponics can be an alternative to reduce inorganic nitrogen and phosphorus accumulation, which can interfere with fish growth. The static tests were conducted to investigate nitrogen removal performance by hydroponics and immobilized biofilm units in an aquaponic system. It was reported that the concentration of total ammoniacal nitrogen and nitrate nitrogen in the aquaponic method satisfied the requirements for fish-friendly water. Equally fish and vegetables are produced well (Zhang et al. 2018). The present study introduced a form of an aquaponic method for minimizing the need for water, electricity, and nitrogen. Nutrients like nitrogen, which can and should be recycled to meet future regulatory discharge requirements, are present in wastewater from industry activities (Lastiri et al. 2016).

6.3 Nitrogen utilization efficiency in Aquaponics

Various studies assessed the nitrogen utilization of the efficiency of aquaponics and suggested ways to boost their effectiveness (Fang et al. 2017; Wongkiew et al. 2017). The nitrogen use efficiency can be used to understand the relationship between the total nitrogen input and the nitrogen output. Aquaponic uniform feeding increased N use efficiency (nitrate nitrogen and nitrite nitrogen) by 30% to 600% compared to those in aquaponic increasing feeding and hydroponics, because it improves the production and yield of herbs and vegetables grown in aquaponic systems by enhancing the quality of the water and nitrogen availability for greater plant production (Yang and Kim 2019).

For increased nitrogen consumption efficiency in aquaponics, it was advised to keep the pH around 6.0, and more research on nitrous oxide reduction strategies is required. In a media-based aquaponic system, the hydroponic bed, where the majority of microorganisms proliferated, was where nitrous oxide release mostly transpired. Advanced nitrous oxide production from aquaponics on lower pH be identified for the prevention of microbial action (Zou et al. 2016b). The lesser nitrifier concentration in the root system and biofilter caused nitrogen removal, which reduced the aquaponic system's ability to use nitrogen effectively. These discoveries about microorganisms and nitrate alterations offered alternative methods to enhance the aquaponic effects in terms of the degree of nitrogen removal from aquaculture wastewater and the quality of the water (Wongkiew et al. 2018a).

To increase the effectiveness of nitrogen utilization in aquaponics methods with a decrease in the discharge of ecosystem-damaging pollutants as well as wastewater, it is important to have a basic knowledge of nitrogen converted in aquaponics. The research demonstrates the rising of dissolved oxygen concentrations by air mixture does not recover nitrogen utilization efficiency and reduce N_2O emission simultaneously (Figure 17) (Wongkiew et al. 2018b).

7 Recirculating aquaculture systems

The recirculating aquaculture system provides a controlled and consistent environment for the optimal production of freshwater aquariums by reusing water in the manufacture. Aquaculture is an industry where various species are grown, such as fish, shrimp, clams, etc., using mechanical and biological filters as part of the irrigation process. Water from the fish tanks is filtered throughout the recirculating process and used again inside the tanks. There is also a need for some other facilities such as pH regulators, heat exchangers, and denitrification units. Aquaponic systems are natural and automatic filters used in aquaculture as techniques for improving water quality. Results showed that the system is more efficient in plant growth than the captive fish supply, so it is not harmful to aquatic life. In this study, biofiltration demonstrated its effectiveness in removing ammonia from water and reducing nitrate formation (Estim et al. 2019).

Nutrient recruitment procedures were investigated by measuring dissolved elements using the spectrophotometric method in the management tanks (nitrate nitrogen, nitrite nitrogen, ammoniacal nitrogen, phosphorus, potassium, magnesium, & iron). Thus, there is a possibility for enhancing crop production with the oxidation

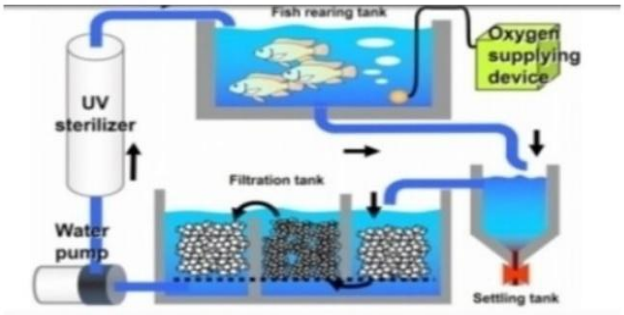


Figure 18 Schematic diagram of recirculating aquaculture system (Endo 2018).

process of the aquacultural waste mixture (Monsees et al. 2017). Recirculating farming practices are more beneficial than conventional agricultural systems in terms of nutrient usage since the fish in these systems give fertilizer to the plants, while the plants remove excess nutrients from the water (Figure 18).

Hydroponic tomato production has been compared to the impact of effluent as a pikeperch recirculating farming practices. Recirculating aquaculture water consists of microorganisms and dissolved organic matter; these act as plant biostimulants and mitigate the salinity pressure and physiological disorders like blossom-end rot symptoms (Delaide et al. 2019). Butterhead lettuces are employed because of their large root systems and aquaponic growing methods. According to the study, analytical calculations were created to relate root development to shoot or root fresh weight and design the nitrification process for an integrated aquaponic system that includes recirculating fish farms (Schwartz et al. 2019).

The goal of the hydroponic filter media project was to design an aquaponic system on a pond scale while treating the nutritional wastewater from the concentrated fishpond. It combined artificial wetlands and suspended grain hydroponic systems. This study demonstrated the feasibility of using rice hydroponic biofilters as an effluent treatment system and producing food plants in an aquaponic method that employs pools with recirculating water (Li et al. 2018). In a laboratory investigation, the impacts of membrane technology remediation on Tilapia and spinach growth (*Ipomoea Aquatica*) and water circulation were examined. The research showed that the sludge tank's sediments and microorganisms were successfully maintained by the membrane treatment, speeding up the nitrification process (Wang et al. 2016). The farming system's hydroponic component proved sufficient for regulating the nitrogen levels during aquaponics within the necessary values for fish farming (Supajaruwong et al. 2020). Fish waste is treated and recycled in an intensive aquaculture system to lower waste concentration in the water and lessen environmental contamination (Calone et al. 2019). There are studies on the effectiveness of removing nutrients from a small-scale recirculating aquaponic system to grow amaranth (*Amaranthus dubius*), sweet wormwood (*Artemisia annua*), and pumpkin (*Cucurbita pepo*). Plants in aquaponic systems take up dissolved nutrients from wastewater from aquaculture. Nutrient removal enables water reuse and lowers wastewater discharge to the residential environment (Gichana et al. 2019). Sustainable food is the source of future water conservation and resources. According to Endut et al. (2016), the aquaponic recirculating system produces crops with a good source of protein and is attaining a sustainable environment.

Aquaculture systems that are concentrated highly controlled, and recirculate waste from fish are treated and recycled to increase fish harvests while saving water. As a separation of recirculating aquaculture systems, aquaponics systems improve water consumption effectiveness along with decrease pollution through a hydroponic technique to facilitate produces good fertilize crops (Medina et al. 2016).

8 Challenges in aquaponics and hydroponics

Even when they are properly designed and successful aquaponic systems, there are major problems in employing the method for crop cultivation. Aquaponics' major agriculture challenge is providing balanced nutrients for substantial nourishing vegetation. According to much research, condensed molasses soluble could be employed as a novel natural fertilizer for crop production to circumvent this nutritional limitation (Li et al. 2020). The countries like India, Nigeria, and Bangladesh are facing a massive loss of nitrogen in the field of aquaculture due to struggling with nutritional and food production security. Thus, aquaponics can be used to evaluate nitrogen transfer by analyzing isotopic signatures or stable isotopes (Adhikari et al. 2020).

In a hydroponic system with artificial lighting, the nitrogen removal efficiency was observed to be elevated in wastewater with exposure to air. Distillation is more efficient in wastewater supplemented with carbon dioxide at the same time, artificial lighting had no significant effect on the reduction of nitrogen forms (Bawiec et al. 2020). Here, the plant is nourished by nutrient-rich fish waste, and the crops help purification by removing toxins harmful to fish (Kumar Sharma et al. 2018).

Few studies have reported that the microorganisms that promote plant growth (sweet peppers, tomatoes, and cucumbers) are likely to be why aquaponics plants can obtain yields similar to those of hydroponics; However, the nutrient level is significantly reduced, and future research in this field is essential for the beneficial use of microorganisms in all plants (Yep and Zheng 2019). With just a basic EC control of the nutrient solution content, vegetables could be grown using recycled hydroponics sustainably (Chowdhury et al. 2021). In protected agriculture, soilless cultivation is significantly employed to maintain the quality of the product while enhancing control over the growing environment and avoiding uncertainties in the soil's water and nutrient condition. In addition to allowing for soilless cultivation and water conservation, soilless methods help for the proximity of food production to urban spaces like residential rooftops (Fussy and Papenbrock 2022).

9 Applications

Aquaponics advanced technologies help to create more sustainable food systems (Krastanova et al. 2022). Aquaponics is beneficial for sustainability in food production as an indirect factor that controls the motivation of environmental awareness and green consumption. Variables impacting the motivation to spend higher ecological production in industrial and theoretical model approaches were studied (Eichhorn and Meixner 2020). Vertical farming utilizes less space for growing extra food. Unlike traditional farming, several novel agricultural techniques such as hydroponics and aeroponics have elevated food production in less space and additional yields in less time (Kumar et al. 2020). Grey water from the bioretention method will be used for the hydroponics of spinach, water spinach, and lettuce, which the community can consume. Thus bioretention plots can decrease the capacity of greywater to have a pollution grade without a pollution index (Widiyanti et al. 2020).

The growing vegetation in hydroponics, like the irrigation of lettuce and beets (rice crops), is measured with phenological conditions like the amount of mixed wastewater collected from domestic effluents, pharmaceuticals, fabric, and fuel discharges. Experiments on seed germination and plant development demonstrate that combined wastewater can be recycled for farming (Egbuikwem et al. 2020). This study aims to examine the efficacy of morning glory in the phytoremediation of hydroponic sewage effluent for use as an alternative to wastewater treatment. The study indicates that the morning glory (*Ipomeaasari folia*) plant can help manage aquaculture effluent to allowable limits, and the treated effluent is safe for reuse (Kiridi and Ogunlela 2020).

Since irrigation water is so limited in desert regions, it is crucial to use alternate water sources, such as tertiary processed wastewater, to cultivate crops. Effluents from hydroponic systems are a key source of fertilizer. It has been suggested that using treated wastewater to irrigate wheat crops using hydroponic systems is an alternate wastewater disposal strategy that poses no risk of heavy metal accumulation in the soil (Al Hamedi et al. 2021). Different aquaponic production systems' degrees of melon (*Cucumis melo L.)* fruit quality were investigated. The study showed that the bioactive compounds in melon should be measured to maintain well-being with age (Piñero et al. 2020).

Strategies for the sustainable and economic development of co-culture and hydroponic systems were studied. This study aimed to discover the possible benefits associated with adopting recirculating aquaculture techniques to avoid over-accumulation of toxic elements while improving nutrient recovery; water exchange and filtration periods were discussed to analyze how these technologies might be used for recirculating aquaculture (Askari-Khorasgani and Pessarakli 2020b). Tomato was produced in aquaponics and evaluation of three hydroponic methods. This study found that the production and fruit quality were comparable among three systems (nutrient film technology, drip irrigation method, and floating raft culture). However, the drip irrigation system gave better results (Schmautz et al. 2016). Healthy amounts of spinach were produced by both the aquaponic and hydroponic spinach treatments, however, the aquaponic system outperformed the hydroponic system in terms of fish development and feed conversion ratio, most likely due to the system's better water quality (Atique et al. 2022). Therefore, aquaponics effectively mobilizes nutrients that are both economically appropriate and ecologically sustainable, not just for a balanced ecosystem but also for humans (Shreejana et al. 2022).

10 Automation

Automation is the hydroponics industry's last challenge. This will allow a single person to employment multiple jobs and cultivate multiple farms simultaneously (Modu et al. 2020). Likewise, the production of catfish and pumpkin using aquaponics and traditional methods was compared. The aquaponics system proved more efficient in producing catfish and pumpkin than other production methods in this study (Oladimeji et al. 2020a).

Aquatic food production from aquaponics systems showed high efficiency and was suitable for production scale. This study confirmed that aquaponics could primarily be used in regions facing water scarcity (Estim et al. 2020). Hydroponic systems for crop production are currently essential to maximize yields. The study reported correct and modernized information about the different nutrients and compositions used hydroponically compared to the traditional production model. They are rationale, nutrient solution technique, and work on fruit crops (Kumar and Saini 2020). Nutrient elimination and growth of arugula were evaluated in the recycled aquaponic method for freshwater fish. The study discovered that biweekly gathering compared with fewer

Table 5 Applications of Aquaponic plants

S. No	Aquaponic plants	Applications
1	Lettuce and beets (*Lactuca sativa* and *Beta vulgaris*)	Seed germination and plant growth experiments showed that mixed wastewater could be reused for farming
2	Morning glory (*Ipomoea purpurea*)	It can be useful in managing aquaculture effluent to allowable limits and the treated effluent is safe for re-use
3	Broccoli microgreens (*Brassica oleracea*)	It is used to enhance the success of farmers and retailers selling microgreens
4	Melon fruit (*Cucumis melo*)	The bioactive compounds in melon are used to maintain well-being with age
5	Tomato (*Solanum lycopersicum*)	It is used to avoid over-accumulation of toxic elements while improving nutrient recovery and water exchange in recirculating aquaculture
6	Watercress (*Nasturtium officinale)*	It can be used to reduce the nitrogen removal effectiveness of aquaponic systems by reducing the ammonia and nitrite removal

than 25 percent of the mass of the developing arugula is advised for nitrification and the economic progress of both the arugula and the carp in aquaponics (Irhayyim et al. 2020).

The cultivation of fish can increase the productivity of fishing technologies. The purpose of the study was to evaluate the entrepreneur's sustainability. Zainal et al. (2021) showed that catfish and mustard greens cultivation in aquaponics yield a high level of profitability and economy. Aquaponics is a system of producing fish using the deep flow technique (DFT) with the result that the partners get an additional income while the environmental improvement makes the area more pleasant and attractive for tourists (Gunawan et al. 2021). An aquaponic system's economic and social benefits in support of the combined trout and water crops were examined. It is used in business models to generate benefit data, which contributes to achieving the multiple potentials of the technology and enabling the development of sustainable food systems from production to consumption (Rizal et al. 2018).

11 Research challenges

Building and sustaining an aquaponic system can sometimes be challenging, as many essential variables need to be measured. The possibility of fish farming developing into a significant sector in the development of environmentally friendly nutrition is restricted by technical constraints and economic challenges. The study highlighted the significance of particular instance analysis taking place in customer choices, financial concerns, and previous marketable advantages in aquaponics (Greenfeld et al. 2020).

The technical challenges of an automated hydroponic system are pH maintenance, nutrient equilibrium, and pest and disease management. Natural cultivating, being a need of great significance, is usually the selected system to overcome the central problem (Devvrat and Ratan 2019). Similarly, the socio-ecological challenges of aquaponics are mineral recycling and overfishing. A review of the fish farming improvement in aquaponics systems was conducted. The potential for fish farming to be a viable technique for the treatment of nitrogen-rich pollutants with year-round principal cultivation of high-quality vegetables and fish while preserving the water is quite high (Oniga et al. 2018).

The optimized aquaponic systems were produced to improve sustainability using Project OASIS. The project's goals were to reduce energy use and construction expenses while using universally available equipment (Nigam and Balcom 2016). The objective of artificial climate-controlled hydroponics farming is to cultivate an electrically controlled environment that encourages plants' growth. Hydroponically grown plants with automatic processing can be used smartly to decrease crops' growth period and save resources such as workforce, water, and fertilizers, thus increasing their productivity (Sanjay and Balasaheb 2021). The buildup of inhibitory allelochemicals that reduce yield and quality due to the autotoxicity phenomenon is a significant concern for recycled hydroponics (Asaduzzaman et al. 2022).

Conclusion

Aquaponics has the potential to be a huge, healthy food production sector, but is constrained by technical issues as well as financial and logistical problems. There is a high demand for green vegetables and small amounts of fish at a relatively high prices. Hydroponics is a profitable technology anywhere there is a shortage of water and poor soil quality. This review article has provided an overview of the various types of aquaponic systems, including those that incorporate standard hydroponically production technology like nutrient film technique, deep water culture, flood and drain systems, aeroponics, and the use of vertical processes. Aquaponics determines the higher removal of nitrogen regaining from aquaculture wastewater through nitrate reduction and nitrogen absorption into organic vegetables and plants. Green leafy vegetables like different varieties of spinach, peppermint, and herb appear to be the best successful species of plants in the aquaponic system because of their low nutritional needs, widespread demand, and usefulness in aquaponics. Aquaponic produce exists to be healthy food that is sustainable and eco-friendly, but it is equally important for the general public to recognize that this product requires a particular level of skill and procedure to function well.

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Conflicts of Interest

The authors declare no conflict of interest.

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Essential oils as valuable feed additive: A narrative review of the state of knowledge about their beneficial health applications and enhancement of production performances in poultry

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ABSTRACT

New research has begun to develop safe and effective alternatives to feed-antibiotics as growth enhancers in response to mounting pressure on the poultry sector to do so. There is a significant demand for poultry products all across the world right now. To achieve this goal, key performance indicators are optimized, such as the rate of chicken growth, the amount of feed used, and the health of the flock as a whole. As a result of this growing need, various alternatives to antibiotics have entered the market. New approaches are desperately needed to keep poultry productivity and efficiency at a high level in the face of mounting pressure to limit the use of antibiotics. Recent years have seen an uptick in interest in the potential of aromatic plant extracts as growth and health boosters in poultry. The great majority of plants' positive effects are accounted for by essential oils (EOs) and other secondary metabolites. EOs have been proven to promote digestive secretion production, improve blood circulation, exert antioxidant qualities, reduce levels of dangerous microbes, and maybe improve the immune status of poultry. EOs are often believed to be safe, non-toxic alternatives because they are all-natural, chemical-free,

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and devoid of potentially harmful deposits. EOs are extracted from plants, and while there are thousands of them, only approximately 300 have been deemed to have significant commercial value. Many different types of bacteria, viruses, fungi, and parasites are negatively affected by EOs in multiple studies conducted both *in vitro* and *in vivo*. The review covers the fundamentals of EOs, their anti-oxidant and immunomodulatory capabilities, their growth-promoting benefits, and their effectiveness against numerous diseases in poultry.

1 Introduction

Preventative antibiotic usage in chicken nutrition is commonly accepted as an effective strategy for maximizing growth, feed consumption, feed utilization, and decreasing mortality from clinical diseases. Fears that antibiotic-resistant microbes will spread across the food supply prompted the European Union (EU) to ban their use in cattle and poultry in 2006 (Chandran and Arabi 2019; Chandran et al. 2019; Aebisher et al. 2021). Therefore, novel plant-based commercial additives, such as aromatic plant extracts and their refined constituents, have been studied for their possible application in future feed alternatives. Due to their lack of residue and widespread acceptance as safe for use in the food business, such products provide several benefits over conventional antibiotics (Chandran 2021a; Chandran 2021b; Chandran and Athulya 2021; Chandran et al. 2021a; Saleena et al. 2021a; Saleena et al. 2021b; Sharun et al. 2021; Uddin et al. 2021; Alajil et al. 2022). In the past decade, these herbs have come under scrutiny for their potential as animal development performance boosters. Since the 1990s, the demand for natural supplements that boost the growth and production performance of poultry has grown (Amorati et al. 2013; Alagawany et al. 2015; Yadav et al. 2016; Dhama et al. 2018; Tiwari et al. 2018; Chandran 2021a; Chandran et al. 2021b; Chandran et al. 2022; Kumar et al. 2022a; Kumari et al. 2022a; Kumari et al. 2022b).

Aromatic plant liquids called essential oils (EOs) are extracted from certain parts of plants. Ethereal oils and volatile oils are two other names for these substances (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots). Secondary plant metabolites called terpenes and low-boiling phenylpropenes are the building blocks of EOs. Quinta essentia, or "herbs and spices," refers to five specific plant essences that have become synonymous with them (Hoffmann 2020; Leherbauer and Stappen 2020; Nehme et al. 2021). Normal extraction procedures involve distillation, most frequently steam distillation. Around 300 EOs are considered to be commercially significant, most of which are employed in the flavoring and fragrance industry. Scientific experiments conducted over the past three decades have confirmed a plethora of positive benefits in addition to those traditionally stated. Their anti-inflammatory capabilities, antibacterial characteristics, antioxidant strength, and capacity to induce digestion are just a few of their many useful attributes in poultry nutrition (Abd El-Hack et al. 2016; Andrade et al. 2017; Omanijo et al. 2018; Alajil et al. 2022; Buttar et al. 2022).

EOs can be extracted from a wide variety of aromatic herbs and spices, including mugwort (*Artemisia vulgaris*), peppermint (*Mentha piperita*), oregano (*Origanum vulgare*), lemon balm (*Melissa officinalis*), onion (*Allium cepa*), fennel (*Foeniculum vulgare*), sage (*Salvia officinalis*), rosemary (*Rosmarinus officinalis*), turmeric (*Curcuma longa*), and many others (Calo et al. 2015; Zeng et al. 2015; Dosoky and Setzer 2018). Even though steam distillation is not the only method used to acquire Eos for commercial reasons, it is by far the most common. Other methods include fermentation, extraction, and expression (Sakkas and Papadopoulou 2017). In addition to dissolving in organic solvents, the EOs also have a distinct aroma. Most EOs are lighter than water because their specific gravity is between 0.80 to 1.17. These oils are sensitive to heat and light, so keep them in dark, cool bottles (Brochot et al. 2017; Pandey et al. 2017). EOs are volatile chemical compounds that are synthesized as secondary metabolites in aromatic plants. Their pungent aromas serve as a distinguishing feature because they are accountable for the aromatic plant's traits. EOs are often lipid- and organic-solvent-soluble, lubricious, and volatile. We can find them in different parts of plants, such as buds, blossoms, leaves, seeds, stems, flowers, fruits, roots, wood, and bark (Brenes and Roura 2010; Oussalah et al. 2006; Leherbauer and Stappen 2020). EOs can be extracted by a wide variety of processes, like solvent extraction, steam distillation, and supercritical fluid extraction (Valdivieso-Ugarte et al. 2019). It is possible to gain insight into the qualities and mechanism of action of EOs by studying their biochemical composition. EOs can have a wide range of chemical compositions, with their major components potentially being an aromatic, aliphatic, or terpenic sequence (Pandey et al. 2017). Some examples of these components are esters, phenols, terpenes, aromatics, volatile acids, ketones, and aldehydes. After harvest, it is not necessarily necessary to process or treat the plant part that underwent hydrokinetics. Except for dry ingredients like lavender, cinnamon, and lime blooms, fresh plants added to solutions have a greater medicinal impact and a more pleasant aroma (Rapper et al. 2021; Raza et al. 2022).

The increasing demand for EOs and other plant extracts can be attributed to the fact that they are following modern thoughts about the future of agriculture in the European Union and with consumer preference for natural products. Moreover, their beneficial

characteristics may be used as a treatment for a variety of illnesses (Plant et al. 2019). There are reports that some EOs can kill microorganisms. Because of their antimicrobial properties, EOs have also been studied as suitable feed additives. In addition to their antibacterial actions, EOs or their components have been shown to have hypolipidemic, antioxidant, digestive stimulant, antiviral, antimycotic, antioxidant, antiparasitic, and insecticidal activities. Perhaps the function of these compounds in plants is related to these characteristics (Giovannini et al. 2016; Asif et al. 2020). However, many herbs and spices are used as condiments in many different dishes. Garlic and chili pepper EOs and oleoresins, as well as cinnamic aldehyde, carvacrol, and pipperine (from black pepper), have all been used for centuries to enhance the flavor of food. While some studies have indicated that adding herbs and botanicals to chicken feed increases feed intake and thus the animals' growth rate, others have found no such conclusive results (Cannas et al. 2016; Aziz et al. 2018; Asif et al. 2020; Sasi et al. 2021).

The purpose of this paper is to provide a synopsis of the literature on the use of EOs and their constituents in poultry nutrition and to discuss the possible mechanisms of action for these compounds. The current state of knowledge on possible antagonistic and synergistic effects is presented, and future directions for research are recommended.

2 Chemical composition and bioactive compounds in EOs

It is believed that the activity of extracted volatile oils is connected to the specific chemical make-up of each oil, as well as its functional groups, potential synergistic interactions between components, concentrations, and other factors. The chemical complexity of EOs varies according to their source, climate, and plant type (Martinez et al. 2006; Cannas et al. 2016). According to this hypothesis, different behaviors emerge as a result of different changes to an EO's underlying structure. Sterols, triterpenes, alkaloids, tannins, flavonoids, and a variety of other naturally occurring medicinal compounds have all been described (Eslahi et al. 2017). Terpenes, a class of secondary metabolites, were synthesized via the head-to-tail paradigm by joining together isoprene units with a 5-carbon base (C5) or 2-methyl-1,3-butadiene. Terpenes, which come in a wide variety of groups, are various combinations of isoprenes that differ in structure and function (Rubio et al. 2013). Terpenes can be found all over the natural world. They can be found in plentiful supplies and have low-priced raw materials. Herbivores are discouraged from grazing on plants by monoterpenes in the air, which also acts as an antifungal defense (Langenheim 1994; Sakkas and Papadopoulou 2017). Specifics on the various plant types that contribute to essential oils and their chemical compositions are presented in Table 1.

Table 1 Specifics on the various plant types that contribute to essential oils and their chemical compositions

Plant source	Scientific name	Main components in EOs	References
Bergamot	*Citrus bergamia*	Limonene Linanine Linalool	Nabiha et al. (2010); Zhai et al. (2018)
Black pepper	*Piper nigrum*	Piperine Piperoleine Piper amide	Singh et al. (2005); Zhai et al. (2018)
Carrot	*Dacus carrot*	Carotol Sabinene Beta caryophyllene Alpha pinenne	Ma et al. (2015)
Cinnamom	*Cinnamomum verum*	Trans cinnamaldehyde Limonene Eugenol	Baratta et al. (1998); Abd El-Hack et al. (2022)
Clove	*Syzgium aromaticum*	Eugenol Eucalyphol	Naveed et al. (2013); Zhai et al. (2018)
Cumin	*Nigella sativa*	Thymoquinone p-cymene Alpha-thugene	Singh et al. (2014)
Eucalyptus	*Eucalyptus* spp	1,8-cineole Cryptone Alpha-pinene	Elaissi et al. (2012); Abd El-Hack et al. (2022)
Fennel	*Foeniculam vulgare*	Trans-anethol Frenchone Methyl cavicol	Hoffmann (2020)
Lime	*Citrus aurantifolia*	Limonene Beta-pinene Alpha-terinine	Costa et al. (2014); Zhai et al. (2018)

Since monoterpenes can include a broad variety of components—hydrocarbons such as p-cimene, camphene, and myrcene, as well as alcohols such as menthol, borneol, and linalool—they are the most versatile structural kinds in EOs. Geraniol and citronellol are aldehydes; camphor, pulegone, and carvone are ketones; linalyl acetate, citronellol acetate, and menthyl are esters; menthofurane and 1,8-cineole are ethers; menthofurane and ascaridole are peroxides; carvacrol and thymol are phenols; and so on (Pavela 2015). Aromatic compounds like terpenes are more common than their aromatic counterparts. These compounds are found in many different plant species, including sassafras, tarragon, parsley, fennel, star anise, nutmeg, and many more. As with phenylpropane, these compounds are often present in lower concentrations than terpenes (Asif et al. 2020; Sharifi-Rad et al. 2021). Plants normally use different biosynthetic pathways to produce terpenes and phenylpropanic derivatives, while there are exceptions. Applications for aromatic compounds are comparable to those for sesquiterpenoids and monoterpenoids. Alcohols, aldehydes, phenols, methoxy compounds, and methylene derivatives are all in this category (Eslahi et al. 2017; Khan et al. 2022). The most common type of terpenoids in EOs is monoterpenes, which can make up as much as 90% of the total and have a very soothing, subtle aroma. A monoterpene consists of two isoprene units, or 10 carbon atoms (Puvača et al. 2022). Numerous terpene synthases monitor the synthesis of monoterpenes. Like monoterpenes, sesquiterpenes can be synthesized by the enzyme sesquiterpene synthase from farnesyl diphosphate, but the structural variety of sesquiterpenes is far larger than that of monoterpenes. Plants have been found to contain these enzymes, which are essential in the creation of sesquiterpenes (Fattahi et al. 2016; Aziz et al. 2018). The chemical composition and structure of major bioactive compounds present in EOs are responsible for their beneficial health effects (Figure 1).

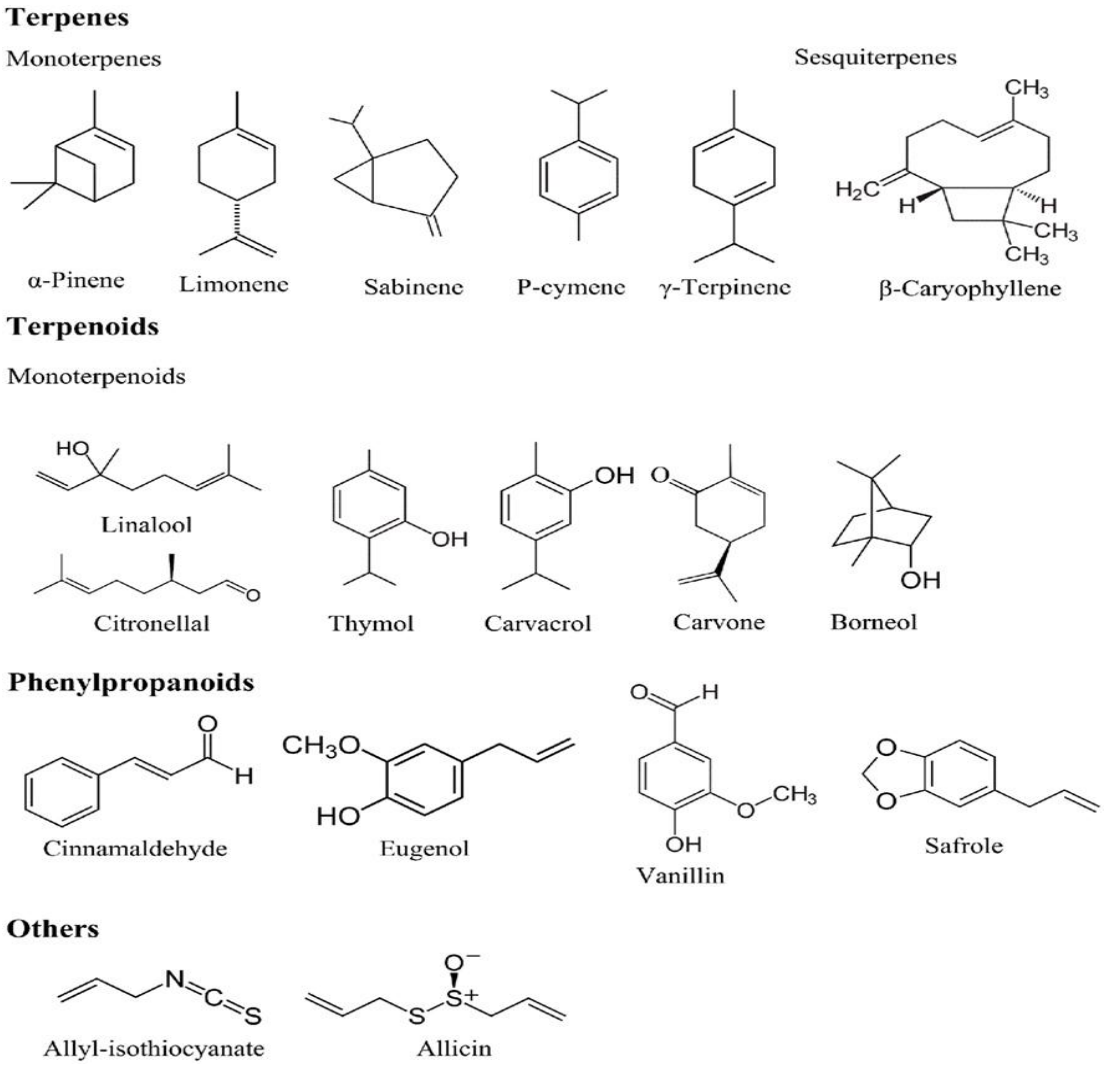

Figure 1 Structure of major bioactive compounds responsible for potential health benefits of essential oils

Fragrant EOs are often colorless or yellowish liquids that can be easily ignited. The viscosity of most Eos is comparable to that of water or alcohol, while others can be rather thick. Aromatic EOs are sometimes misunderstood because of their misleading name, which suggests that they are composed of fats or oils. Unlike water, EOs completely dissolve or almost completely dissolve in all organic solvents. Eos get thicker, darker, and more acidic when exposed to air for extended periods (Sakkas and Papadopoulou 2017; Chandran 2021a; Kumar et al. 2022b). It is challenging to determine the boiling point of these complex mixtures due to the large number of chemicals involved; nonetheless, fractional distillation is effective at separating these compounds because their boiling temperatures often fall within the range of 150 to 280°C (Pandey et al. 2017; Omonijo et al. 2018). EOs from various plant species can include anywhere from 20 to 60 different biologically active compounds, with only a subset (20-70%) playing a significant role and the rest present only in minute quantities. It is possible for there to be a big difference in the amount of biologically active substances present depending on the plant parts utilized, the season they were collected, and the region they were cultivated (Hoffmann 2020; Puvača et al. 2022).

3 Potential beneficial effects of EOs on the health of poultry

The major potential benefits of EOs on the health and production of poultry are depicted in Figure 2.

3.1 Effect of EOs on feed intake, feed utilization, and growth performance

According to Zhang et al. (2014), EOs are used as growth enhancers in poultry feed because of their stimulating effect on poultry health and production (Amad et al. 2011). *Origanum vulgare*/ *O. heracleoticum* is a member of the Lamiaceae family of plants and is the accepted scientific name for this herb. The phenolic chemicals found in the oils of this plant are plentiful (69.55% carvacol and 4.09% thymol respectively). Monoterpene hydrocarbons, including cymol, are also mentioned (Mathlouthi et al. 2012; Aziz et al. 2018; Salehi et al. 2018). In comparison to birds on a basal diet, the oregano EO in those birds given a high-fat diet had more growth. Synergy essence, a commercial product containing oregano EO (500g/ton), decreased lipid peroxidation in minced chicken breast (Bozkurt et al. 2014a). Recent studies have shown that including EOs in a broiler's diet causes the birds to consume less because of the EOs' offensive odor, which makes the feed less palatable and may cause the birds to stop eating altogether (Bozkurt et al. 2014b; Leyva-López et al. 2017; Hoffmann 2020).

Feed intake was shown to be reduced in birds given 10 g/kg of oregano herb, but was equal in birds given 1 g/kg oregano EO. Both the rosemary EO-supplemented and control groups consumed the same amount of feed. Feed intake was also shown to rise significantly when either oregano EO (300, 500, or 700 mg/kg) or

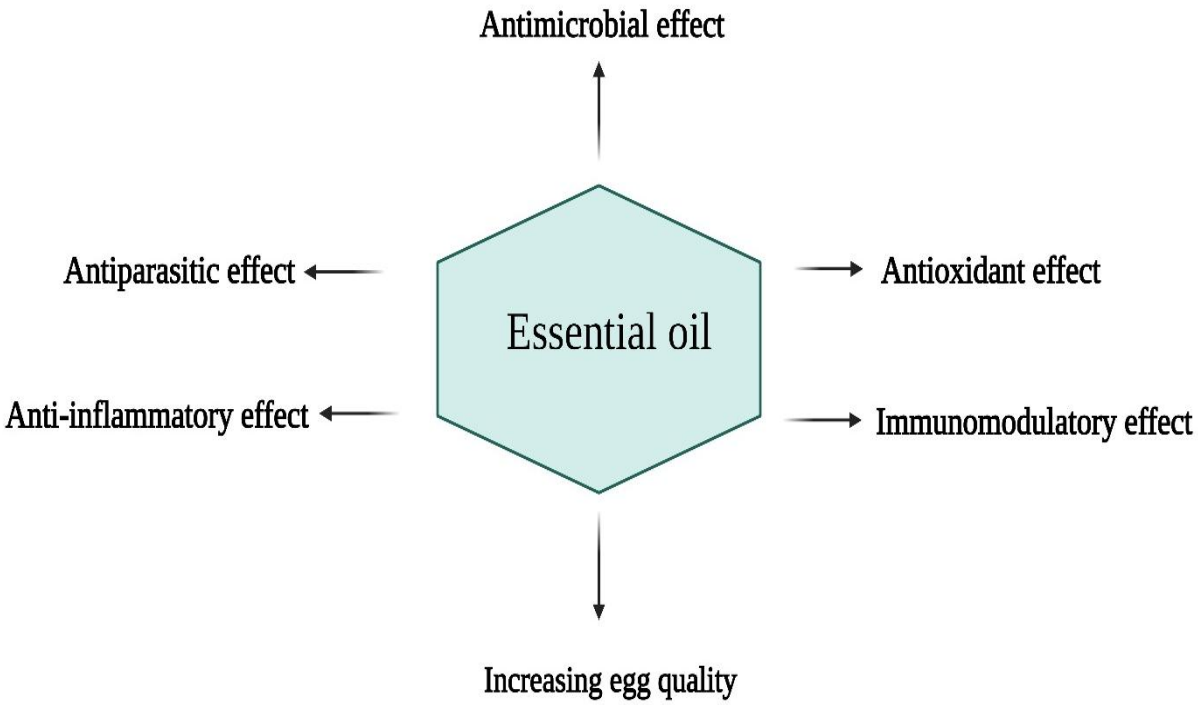

Figure 2 Potential health and production benefits of essential oils in poultry

thyme EO (100, or 200 mg/kg) was added to the feed (Faleiro et al. 2003). Feasting poultry on a diet containing 100 mg/kg of the drug did not influence the birds' food consumption. Poultry fed EOs at 25 or 50 mg/kg demonstrated no observable effects on feed consumption (Jang et al. 2007; Prakash et al. 2021a). Supplementing the diet with 18 mg/kg of oregano, eucalyptus, thyme, and cinnamon EO led to an increase in daily feed consumption (Leyva-López et al. 2017). EOs were thought to have improved feed intake as a whole by helping the gut microbiome return to a more stable state. The feed intake of broilers improved after the EO blend of oregano, anise, and clove was given to their diet at 200 and 400 mg/kg for three weeks (Yitbarek 2015). Potentially responsible for the enticing impact of the EO blend and, thus, the increased feed intake, are chemicals like carvacrol, thymol, anetole, and eugenol (Prakash et al. 2021b).

Thymol is a herb used as a feed additive for livestock, fish, and poultry. It improves performance indicators and feed utilization through structural activation and alteration in the digestive system, among other methods. Strong antibiotic action has been shown in vivo and/or in vitro to improve nutrient absorption and metabolism, alter gut flora and prevent harmful chemicals and free radicals from interacting with cellular biological components (Faleiro et al. 2003; Leherbauer and Stappen 2020). Microorganisms with antibiotic resistance, including methicillin-resistant *Staphylococcus aureus* (MRSA), are present in a wide variety of pathogens (bacterial, viral, fungal, and parasitic) (Lejaniya et al. 2021a; Lejaniya et al. 2021b; Kumari et al. 2022a). Coccidiostat medications are effective against helminths and leishmaniasis. The bacterial enterotoxins A, B, and hemolysin produced by several *Staphylococcus* spp. can be inhibited by thymol. Feeding broiler chicks with thymol was found to have no significant influence on growth performance metrics such as ileal content of microbiota, live body weight, and feed utilization, suggesting that it could be explored as a unique and new medicinal chemical that combats leishmania (Ezzat et al. 2016; Giovannini et al. 2016). The anticoccidial effect of EOs in poultry is depicted in Figure 3.

Certainly, adding EOs to the poultry diet has many advantages, but their use is also accompanied by some issues and restrictions. Following their botanical origin, environment, harvest season, and procedures, they may differ greatly in extraction, drying, and storing, which caused inconsistent results to be reported. Some EOs, like carvacrol, have a very strong flavor and odor, which may cause them to alter feed intake in an unfavorable way, which may account for the contradictory results of studies examining the effects of adding EOs to chicken diets on performance metrics (Fernandez-Panchon et al. 2008; Basmacioglu Malayoglu et al. 2010). Mists of EOs like peppermint and thyme are safe for broilers, and peppermint EO may even improve performance metrics and have a major effect on broilers' immune systems. There is not enough evidence in the literature to support the use of EO mists as growth and health boosters. Additional research into the mechanisms of immunological response in broilers exposed to varying concentrations of EO mist under real-world conditions was emphasized by the cited authors (Giovannini et al. 2016; Sandner et al. 2020). EOs of oregano, rosemary, sage, thyme, and yarrow

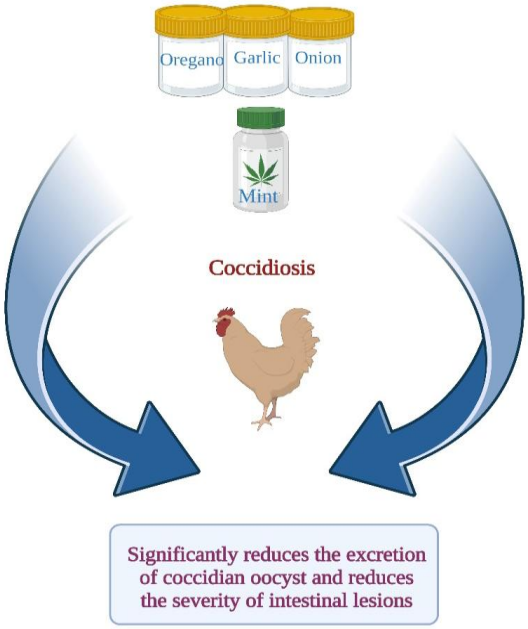

Figure 3 Anticoccidial effect of essential oils in poultry

have been found to improve the health performance parameters of animals, such as increasing egg weight, body weight gain, feed intake, and feed conversion ratio. Sage and garlic EOs, as well as those containing cinnamic aldehyde, thymol, and carvacrol, are immunostimulatory and beneficial to gut microbes. The improvement in animal welfare and hygiene following EO fumigation of chicken houses raises the possibility that EOs can be employed as air disinfectants to get rid of respiratory pathogens (Greathead 2003; Hoffmann 2020; Mucha and Witkowska 2021; Kumari et al. 2022b).

There are undoubtedly many benefits to using EOs in poultry diets and water, but their usage is also associated with some challenges and constraints. Extraction, drying, and storing methods might vary widely according to the plant species, region, harvest season, and other factors, leading to conflicting findings (Salehi et al. 2018; Sharma et al. 2019). The overwhelming aroma and flavor of some EOs, such as carvacrol, may unfavorably alter feed intake, which may explain why studies on the impact of adding EOs to poultry diets have yielded conflicting results (Windisch et al. 2008; Sharma et al. 2019). EO mists, such as those made from peppermint or thyme oil, have no negative impacts on the health of broilers, may improve performance indicators, and have a substantial impact on the immune systems of broilers. As the aforementioned authors emphasized, more study is needed to determine the precise immune response pathways in broilers exposed to varying concentrations of EO mist (Brenes and Roura 2010; Plant et al. 2019). Egg weight, feed conversion ratio, and feed intake are just some of the metrics that have been shown to improve with the addition of EOs from herbs including thyme, oregano, sage, rosemary, and yarrow to the diet. EOs of sage and garlic, as well as those containing cinnamic aldehyde, thymol, and carvacrol, have been shown to have probiotic or immunostimulatory effects. Because of the positive results seen after using EOs to fumigate chicken coops, it is possible that these compounds can be used as air disinfectants to eliminate respiratory infections (Mucha and Witkowska 2021; Chandran et al. 2022). Critical factors affecting the usefulness of essential oil as a chicken feed is presented in Table 2.

3.2 Effect of EOs on feed conversion efficiency

The effects of EOs on chicken production have been proven in several types of research. Their research indicates that EOs can increase weight gain, feed intake, and feed conversion by 2.0%, 0.9%, and 3.0% in piglets, and 0.5%, 1.6%, and 2.6% in poultry (Zeng et al. 2015; Puvaca et al. 2020). Experiments were designed to prove that supplementing broiler feed with 200 ppm of oil extract (cinnamon and thyme) led to a significant increase in feed conversion ratio and weight over six weeks. The ratio of high-density lipids to cholesterol in the blood was also lowered. Herbal plant oils may, then, be useful for boosting poultry output by encouraging faster growth and better digestion.

Anise is the common name for the plant *Pimpinella anisum* L. It is frequently grown in countries like Pakistan, Iran, Turkey, India, and others due to its pleasant perfume and suitable climate. Anethole, along with estragole, eugenol, methyl chavicol, and anisaldehyde, is its active ingredient. The feed conversion ratio (FCR) was shown to be elevated by 12% when anise EO was administered at a dose of 400mg/kg (Bakkali et al. 2008). However, when administered at 100 and 200mg/kg, thyme oil improved the meal conversion ratio. Broiler hens were used in an experiment to measure the effectiveness of thyme oil. There was

Table 2 Critical factors affecting the usefulness of EOs as a poultry feed

Influencing factors	Essential oils / their critical components	Results	References
Dietary composition	Thymol Cinnamaldehyde	Reduced EO effectiveness may be seen with a more easily digested diet	Lee et al. (2003); Abd El-Hack et al. (2022)
Chemical makeup of essential oils	Thyme Oregano Marjoram Rosemary Yarrow	Changes in terpene makeup may account for why different EOs and botanicals produce varying effects	Cross et al. (2007); Abd El-Hack et al. (2022)
Dosage	Oregano	Oregano supplementation induced a quadratic response in feed consumption and weight gain	Abdel-Wareth et al. (2012)
Environment	Caraway Fennel	Fennel and caraway oil appear to alleviate stomach distress	Lee et al. (2003); Zhai et al. (2018)
Age of poultry	Oregano	Oregano oil's effects are minimal at the elite level, but they could be enhanced at lesser levels of competition.	Botsoglou et al. (2002); Zhai et al. (2018)

significant weight gain, an improved feed conversion ratio, a rise in livability, and a rise in turnover in a broiler production system when 100 mg/kg of thyme oil is incorporated into poultry feed (Aziz et al. 2018). *T. vulgaris* is grown for its medicinal properties and also as a spice crop around the world. Extreme heat and dry conditions bring up a widespread appreciation for this herb. This plant's EO is stored in its glandular hairs in a variety of chemical forms and formulas, and it has the unique feature of evaporating in the presence of injury to those hairs. It is the potent fragrance that draws people to the plant, where they can then harvest the oil for use in a variety of applications. Extractions of thyme oil contain between 20 and 55% of the active components namely thymol and carvacol (Wade et al. 2018; Chandran 2021a).

Antioxidant nutrients like polyunsaturated fatty acids (PUFA) are recommended for use in animal feed to delay spoilage and the development of rancid odors. In poultry, increased feed digestibility from EOs has been linked to improved nutritional absorption. There is a lack of consistent data on the effects of botanicals on digestive processes such as bile and mucus production, saliva secretion, feed digestibility and transit time, and enzyme activity (Bakkali et al. 2008; Fernandez-Panchon et al. 2008; Miguel 2010; Pisoschi and Pop 2015). PUFA aids better nutrient absorption because it increases the surface area available for absorption. The high reactivity of EOs is a further obstacle to their direct application and absorption into food and feed products, and an understanding of their mode of action is also required. Keeping their biological activity stable, regulating their responsiveness, and reducing the impacts of expressing organoleptic characteristics are challenging tasks (Franz et al. 2010; Miguel 2010; Calo et al. 2015). The stability and bioactivity of EOs can be impacted by factors such as production environment temperature, light, metal content, and access to water and oxygen. The temperature of 58°C during feed pelleting, for instance, resulted in a low recovery of the indicator compounds (Maenner et al. 2011). The high reactivity of EOs is a barrier to their direct application and absorption into food and feed products; it is also required to understand their mode of action. Controlled release of EOs is possible thanks to the development of new delivery technologies like encapsulation, which protect EOs' bioactivity and volatile components from oxidation and degradation during feed processing and storage and from the varying conditions in poultry gut (Stevanovi et al. 2018). The antiparasitic effect of EOs in poultry is depicted in Figure 4.

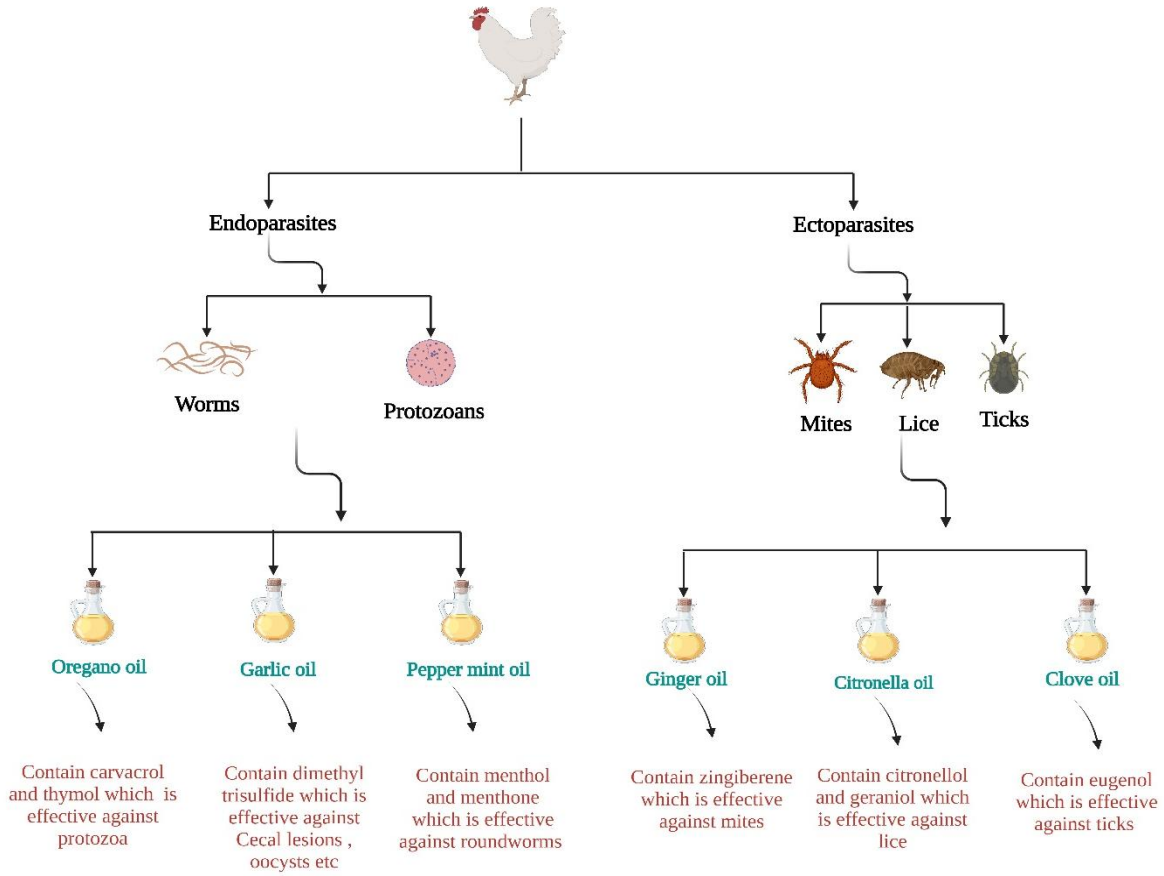

Figure 4 Antiparasitic effect of essential oils in poultry

3.3 Immunomodulatory effect of EOs

EOs are a concoction of various volatile, organic, and aromatic compounds isolated from plants. New studies have shown that the immunomodulatory actions of these drugs are responsible for some of their benefits (Valdivieso et al. 2019). The immune system regulates immunity and disease resistance, and immunomodulator effects specifically alter the immune system. It is essential in poultry because it affects the birds' health, growth, disease resistance, FCR, weight increase, and defense mechanisms. There are many kinds of immunomodulators, such as prebiotics, probiotics, vitamins, adjuvants, polysaccharides, and botanicals (Asif et al. 2020). The primary objective of immunomodulation is to improve the host's ability to resist infection from both external and internal sources. Immunomodulators can be used in place of antibiotics, antimicrobials, and other drugs to achieve the same immune system boosting effects. Furthermore, they enhance immunological and feed molecular qualities, expanding all possible routes for disease avoidance and homeostasis preservation. Since they are not commonly used or added to chicken feed, more work needs to be done to promote their use (Valdivieso-Ugarte et al. 2019; Das et al. 2020). Immunology is a promising new area of study for the prevention and treatment of inflammatory illnesses of the skin, lungs, stomach, central organs, and joints. Immunomodulators regulate the immune system to help patients regain protective immunity during treatment (Patil et al. 2012; Deepak et al. 2020a; Deepak et al. 2020b). Immunomodulation aims to increase the body's natural defenses against invasion by microorganisms and other infectious agents. The primary goals of immunomodulation in poultry are to elicit a strong immune response that lasts for a long time against disease-causing microorganisms; enhance the development of both specific and general immunity during the neonatal period; and reduce susceptibility to disease in poultry (Dhama et al. 2014; Asif et al. 2020). To counteract the immuno-depleting effects of stress and environmental pollution, EOs are beneficial in enhancing local protective immune responses in vulnerable areas of the digestive system, strengthening the immune response after vaccination, and monitoring the body's defenses (Platel and Srinivasan 2004; Das et al. 2020).

Immunostimulants and immunosuppressants are the two most common categories of immunomodulators. Immunosuppressants can be effective in treating autoimmune illnesses and other conditions characterized by an abnormal immune response by dampening the body's natural defenses (Muir et al. 2000; Dhama et al. 2018). Immunostimulants are mediators that help the body's defenses perform better against infection. As an immunotherapeutic treatment, and in immunocompromised individuals, immunostimulants boost immune system baseline levels. Immunostimulant drugs are useful in the treatment of a wide range of medical conditions, including viral infections, cancer, autoimmune illnesses, and immunodeficiency (Hadden 1996; Patil et al. 2012). The Labiatae family, which includes oregano, has received a lot of attention for its potential as a chicken feed ingredient. Broilers' European production efficiency factor improved after being supplemented with 300 ppm of oregano EO (OEO). Based on estimated body weight, viability, FCR, and duration of the trial, it was shown that while 300 ppm OEO in the meal increased secondary total antibody titers, RBCs, and immunoglobulin G (IgG) titer, it did not affect the primary antibody titer. In contrast, another study found that Newcastle disease and avian flu-vaccinated broilers responded positively to 500 and 1000 ppm OEO supplementation, respectively. Treatment of birds with 300 ppm OEO decreased stress markers, namely serum heterophil counts and the heterophil to lymphocyte ratio (H/L ratio) (Huang and Lee 2018).

Chemical feed additives can be replaced with phytochemicals. Broiler dry matter and crude protein digestibility were both greatly improved by the addition of phytochemicals, as were pancreatic and intestinal enzyme secretion (Rice-evans et al. 1995; Jamroz et al. 2006). The immune system's ability to respond and its resistance to outside stressors were both improved by including phytogenic in the diet. Aromatic terpenes and aliphatic terpenoids are two types of phytochemicals made in plants by different mechanisms. This is why phytochemicals suppress inflammatory cytokines and moderate the immune response in chickens (Hadden 1996; Brenes and Roura 2010; Negi 2012). Adding cinnamon to the diet of broilers has been proven to improve growth rates and reduce coliform bacteria in the jejunum and the large intestine. A higher FCR, lymphocyte percentage, and hemoglobin level were seen when either 0.4% or 0.8% cinnamon was introduced to broiler diets. It has been found that cinnamon EO has antioxidant effects (Huang and Lee 2018). Thyme EO is extracted from the thymus plant and contains the active ingredients thymol, carvacrol, p-cymene, and terpinene. Leaf powder and EOs are popular choices as dietary supplements. The EOs raised the amount of Lactobacillus and Bifidobacterium in the ileum and reduced the quantity of *Escherichia coli* while enhancing the cutaneous basophil response to phytohaemagglutinin (Ultee et al. 2002; Plant et al. 2019). In general, a healthier immune system is associated with a lower H/L ratio. Poultry immunity relies heavily on the bacteria in their intestines. Broiler weight gain and FCR were both boosted by thyme. This means that thyme is an effective feed additive for the chicken and egg industries (Brochot et al. 2017; Das et al., 2020). The majority of research looked at this phytogenic, which is found in ground-up turmeric roots (TRP). TRP dramatically boosted blood IgA in 42-day-old broilers exposed to sheep RBCs, while decreasing IgM and IgG levels and increasing the number of monocytes. Broilers subjected to heat

stress therapy and supplemented with 0.2% TRP had a lower H/L ratio and a higher total secondary antibody titer against SRBCs. Also, by supplementing their diet with TRP (0.33%) and/or 1.0% (1.16% curcumin), broilers saw sustained increases in FCR. When TRP was administered at dosages of 0.33%, 0.66%, and 1.0%, both abdominal fat and serum triglyceride levels dropped significantly. Therefore, curcumin in the poultry diet may regulate the immune response and boost growth (Huang and Lee 2018).

EOs are a vital aromatic component used as a substitute for antibiotic growth boosters in poultry feed. These are effective against certain infectious microorganisms and parasites. It also promotes the production of digestive enzymes, boosts immunity, and stimulates the appetite (Corbo et al. 2009; Kumar et al. 2022d). Carvacrol, or cymophenol, is a monoterpoid phenol that has a strong, characteristic odor. Carvacrol is found in the EOs of several different plants, including oregano (*Origanum vulgare*), thyme, pepperwort, and wild bergamot (Brochot et al. 2017). The immunomodulatory effects of dietary cinnamaldehyde in broilers were studied by analyzing global gene expression profiles, and the results pointed to antigen presentation, the humoral immune response, and inflammatory illness (Ultee et al. 2000). It was found by *in vitro* testing that cinnamaldehyde treatment of chicken spleen lymphocytes (at doses ranging from 25 to 400 ng/mL) significantly increased cell proliferation compared to the control group. Nitric oxide generation in macrophages was found to increase between 1.2 and 5.0 ng/mL of cinnamonaldehyde, whereas chicken tumor cell growth was inhibited between 0.6 and 2.5 ng/mL and between 10 and 100 ng/mL of cinnamonaldehyde (Brenes and Roura 2010; Sharma et al. 2019). Similarly, adding cinnamon aldehyde to the diet of hens infected with *Eimeria acervulina* or *E. maxima* led to a greater increase in the birds' body weight. The amount of cinnamaldehyde employed in this research was, admittedly, quite small. Animal studies have demonstrated that thymol, the primary component of both thyme and oregano EOs, has anti-inflammatory properties similar to those of the carvacrol isomer. Thymol also inhibits dendritic cell maturation and promotes T cell proliferation *in vitro*. The transcription factor NF-κB was drastically reduced in the jejunum of broilers that were supplemented with thyme powder, which contains the EOs such as thymol (50.48%), terpinene (11.03%), p-cymene (9.77%), and carvacrol (4.3%). The levels of pro-inflammatory cytokines were also reduced in broilers given thyme powder. This is consistent with previous research and lends credence to the idea that thyme's anti-inflammatory capabilities justify its usage in this context (Calo et al. 2015; Huang and Lee 2018).

3.4 Antimicrobial effect of EOs

The development of antibiotics, which had its heyday in the 1950s and 1970s, was made possible by the realization that germs cause a range of ailments in the late 19th century. Despite this, no new antibiotic classes had been discovered (Calo et al. 2015). On the other hand, improper antibiotic use led to the evolution of germs that are resistant to treatment. One of the options is to employ antibiotics as antimicrobial growth promoters for poultry; therefore, it is crucial to create suitable alternatives for antibiotics to preserve the efficiency of the poultry sector as it is currently run, and one promising method is the use of EOs. Most people believe that EOs are safer and more effective than antibiotics since they are all-natural and chemical-free (Dhama et al. 2018; Zhai et al. 2018; Wińska et al. 2019; Ebani and Mancianti, 2020). One of the main factors that may affect the yield of laying chickens is the antibacterial activity of EO. For example, cinnamaldehyde, an EO extracted from the cinnamon plant, inhibits the growth of pathogenic microorganisms. It has been documented that cinnamaldehyde strongly inhibits the growth of *Clostridium perfringens* and *Bacteroides fragilis*. EOs have been shown to fight dangerous microorganisms, although how this happens is not well understood (Brochot et al. 2017; Asif et al. 2020; Chandran 2021a). The effectiveness appears to depend on a variety of factors, including the pH, chemical make-up of the active substances, population, and kind of impacted intestinal bacteria. Coumarin and alkaloids, as well as terpenoids, phenolics, metal chelation by phenol, and flavonoids, all have antimicrobial inhibitory effects that may cause cell membrane damage (Widodo 2020).

Antimicrobial properties of EOs have been studied in vitro multiple times, and carvacrol and thymol have been found most efficient against pathogenic enteric bacteria like *E. coli* and *Salmonella typhimurium* (Tassou et al. 1995; Dhama et al. 2014). Hydrophilic components of the outer membrane may account for the similarities between EOs and gram-positive bacteria. Alterations in the immune response influenced the levels of pathogens (Negi 2012). Antibody titers against infectious bursal disease and Newcastle disease in layer chickens rose when EOs was introduced into their diet. When properly prepared, ginger has antibacterial properties (Dorman and Deans 2000; Asif et al. 2020). It is also possible that ginger-containing feed additions benefited the antioxidant and immune systems of broilers. Given the presence of microbial toxins, this may be due to ginger's powerful antioxidant effects. Several substitutes have been employed as antibacterial agents in poultry production (Brochot et al. 2017). Using ginger extract as a feed supplement would increase the production of low-cholesterol eggs, which are preferred by consumers and clients because cholesterol is a marker for cardiovascular diseases. Studies on poultry have consistently shown that the effects of ginger and its derivatives are on par with antibiotics. Not only are the correct dosage and method of administration essential when utilizing these antibiotic alternatives, but so is the correct use of the antibiotics themselves (Calo et al.

2015). When administered together, many EOs have enhanced antibacterial action. The bactericidal MICs for eugenol, thymol, and carvacrol against *Listeria innocua* were 150, 250, and 450 mg/kg, respectively. A mixture of 62.5 mg/kg thymol and 75 mg/kg carvacrol completely suppressed the growth of *Listeria innocua*, as did a mixture of 75 mg/kg carvacrol, 31.25 mg/kg thymol, and 56.25 mg/kg eugenol. To lessen the financial and experiential costs of using antimicrobial preservatives, combining EOs may be an option (Cannas et al. 2016). Numerous studies have shown that EOs can inhibit the growth of bacteria and other microorganisms, but the mechanisms underlying this effect remain unknown. Several hypotheses have been proposed to explain the mechanisms of action of the chemical components of EOs. Because of the complexity of their composition, the antibacterial effects of EOs cannot be isolated to the action of a single element. Many studies have linked the antibacterial activity of EOs to the lipophilic properties that allow them to penetrate bacterial membranes and exert inhibitory activity on the cell's functional capabilities once inside (Calo et al. 2015; Valdivieso-Ugarte et al. 2019). Components present in certain EOs that have been shown to have antimicrobial effects are depicted in Table 3.

3.5 Anticoccidial effect of EOs

Coccidiosis is a common parasitosis in chickens caused by protozoa of the genus Eimeria, and it manifests itself through malnutrition and decreased performance. The incubation time of coccidiosis varies from four to six days, depending on the number of hosts present and other risk factors such as contact with faeces. Parasites cause malabsorption, diarrhoea, and poor weight gain by establishing a colony in the intestinal cells of the host and triggering the host's immune system to attack these parasites (Cannas et al. 2016). Vaccines against coccidiosis are effective in several studies. Live vaccines are the most common and convenient method of improving host immunity, and they are effective against infectious species of Eimeria. However, due to the lengthy period during which broilers are raised, live vaccines

Table 3. Select essential oil components with demonstrated antimicrobial activity

Plant	Components in EOs	Composition percent	References
Coriandrum sativum	Linalool E-z-decanal	26% 20%	Delaquis et al. (2002); Mahfuz et al. (2021)
Coriandrum sativum	Linalool E-z-decanal	70%	Delaquis et al. (2002); Mahfuz et al. (2021)
Cinnamomum zelandicum	Trans cinnamaldehyde	65%	Lis-Balchin et al. (1999); Zhai et al. (2018); Mahfuz et al. (2021)
Origanum vulgare	Terpinene p-cymene	52-64% 2-5%	Demetzos et al. (2001); Marino et al. (2001)
Rosemarinus officinalis	α-pinene Bornyl acetate Camphor 1-8 cineole	2-25% 0-17% 2-14% 3-89%	Daferera et al. (2003); Zhai et al. (2018); Abd El-Hack et al. (2022)
Thymus vulgaris	Thymol Carvacrol γ-terpene	10-64% 2-11% 2-31%	Juliano et al. (2000); Mahfuz et al. (2021)
Salvia officinalis L	Camphor α-pinene β-pinene 1-8-cineole α-thujone	6-15% 4-5% 2-10% 6-14% 20-42%	Marino et al. (2001); Abd El-Hack et al. (2022)
Syzgiuma romanticum	Eugenyl acetate Eugenol	75-85% 8-15%	Bauer et al. (2008); Zhai et al. (2018)
Bubonium imbricatum and *Cladanthus arabicus*	Oxygenated monoterpenes cis-chrysanthenyl acetate Thymol Isobutyrate Monoterpenes hydrocarbon Sabinene Beta-pinene Alpha-pinene	61.4% 31.4% 3.4% 75.8% 31.1% 16.7% 12.3% 5.3%	Aghraz et al. (2018); Hoffmann (2020)

might produce reactions that can negatively affect flock performance in the event of inadequate management. This shortcoming led to the development of attenuated immunizations with reduced pathogenicity, but their production is expensive (Valdivieso-Ugarte et al. 2019). EOs and their constituents have been studied extensively for their potential to reduce the prevalence of avian coccidiosis. Botanical compounds, especially EOs, added to feed can serve as excellent replacements for anticoccidial drugs, lowering or eliminating the requirement for medication in the treatment and control of avian coccidiosis (Idris et al. 2017). Chicks that were treated with EOs had much lower rates of intestinal lesions and coccidian oocyst discharge (Zhai et al. 2018).

EOs have direct anticoccidial actions, including modifying the ultrastructure of mitochondrial membranes, halting the formation of glycoproteins, and blocking the activity of the cysteine protease cruzain enzyme. EOs help the host by protecting against free radicals and immunomodulating the immune system by scavenging reactive oxygen species (ROS) produced during parasite invasion (Valdivieso-Ugarte et al. 2019; Prakash et al. 2021a). EOs are potent botanical substances that block and destroy parasitic invasion by either directly disrupting parasite metabolism or indirectly doing so by bolstering the antioxidant defenses and immune response of the host. EOs are potent botanical agents that can successfully limit and eradicate parasitic invasion by interfering with parasite metabolism in two ways: directly and indirectly (Idris et al. 2017; Hoffmann 2020).

Poultry that had been artificially infected with *Eimerella tenella* had a lower oocyst mass per gram of excreta when oregano EOs (300 mg/kg) were administered. An indicator of the severity of avian coccidial infection was the number of *Eimeria* oocysts found in bird poop (Cannas et al. 2016). Extensive studies on the anticoccidial effects of oregano oil showed that it was effective against *Eimeria tenella*, *E. maxima*, and *E. acervulina*. It has been claimed that using EOs and coccidial vaccinations together is an effective alternative for avoiding avian coccidiosis. Coccidia-infected birds continue to grow more slowly than healthy birds, even when both groups consume the same diet. Oregano EO's major components are carvacrol and thymol, which have anticoccidial activity against infections caused by *E. tenella* and a mixture of *Eimeria* spp (Krishan and Narang 2014). Beneficial effects against intestinal parasites, especially Eimeria species, have been demonstrated for extracts and EOs from the plant's *Origanum vulgare*, *Allium cepa*, *Echinacea purpurea*, *Chenopodium ambrosioides*, *Mentha* spp., and *Allium sativum*. Many studies have shown that *Origanum vulgare* EOs have anticoccidial action (Idris et al., 2017). Eimeria oocyst release in avian faeces is often considered the major sign for predicting coccidial infection. Treatment of infected birds with EOs and bioactive plant extracts from certain herbs reduces the damage caused by the discharge of coccidial faecal oocysts on the intestinal epithelium. Researchers found that fewer coccidial oocysts were found in the litter when broiler chicks were given oregano EO and were also vaccinated (Bozkurt et al. 2013; Prakash et al. 2021b)

3.6 Antioxidant effect of EOs

Antioxidants are used as food additives to protect perishables from oxidative damage produced by free radicals. Spices have been utilized for thousands of years, and their antioxidant qualities have been well-known for centuries. Industrial processing makes use of synthetic antioxidants to lengthen the storage life of foods. Toxicologists have expressed concern about the possible carcinogenic properties of two synthetic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). There has been a growing backlash against chemical additives in food. This has sparked a surge in interest in discovering whether or not certain natural additives can function as antioxidants (Amorati et al. 2013; Dhama et al. 2014; Dhama et al. 2018). Antioxidant activity in three different commercial cinnamon extracts was found to be comparable to that of the synthetic antioxidant BHT. Researchers used rosemary, olive leaf extract, and several types of tea to show off the antioxidant powers of their respective ingredients (Brenes and Roura 2010; Valdivieso-Ugarte et al. 2019). Vitamins are a type of nutrient that, to aid in a wide variety of metabolic processes and the efficient utilization of other nutrients, must be ingested daily, albeit in extremely minute quantities. The constituents of animal meals contain them naturally, although supplementation is still required especially antioxidants found outside the body, such as vitamins E and C. According to research conducted on chickens, they have a significant role in the bird's ability to cope with oxidative stress. Vitamin E, to provide just one example, is crucial in maintaining the integrity of cell membranes. Broilers and ducks fed with different EOs (0.2% of the diet) gained and matured at similar rates. Also, a vitamin E supplement of tomato pomace (30%) added to the chickens' diet for 21 days did not influence their weight gain. A synthetic antioxidant containing vitamin C is offered to poultry, even though there is not much research on vitamin C (Cavani et al. 2009; Righi et al. 2021). Antioxidant properties of plant-derived EO components are represented in Table 4.

3.7 Antiparasitic effect of EOs

EOs have shown promise as prophylactic treatments against several arthropod ectoparasites, including lice, mites, and ticks, and this area of study continues to expand. The presence of the latter ingredient provides evidence that their mode of action involves

Table 4 Antioxidant constituents in essential oils derived from plants

EOs extracted from plants	Components of EOs	Procedure used	Antioxidant effect	References
Bay leaf, black pepper, coriander, cumin, garlic, ginger, mustard, onion, and turmeric	Coriander and cumin seed oil, linalool, p-coumaric acid	DPPH method	Cumin 163.50 (µg/mL), coriander 150.62 (µg/mL), mustard 155.16 (µg/mL)	Bag and Chattopadhyay (2015)
Deracocephalum moldávica and *Melissa officinalis* and	Citronellal, citral and thymol (in *M. officinalis*); geranial, geranyl acetate geraniol, and neral (in *D. moldavica*)	DPPH method	Both EOs had a stronger action than ascorbic acid in the preservation of β-carotene molecules.	Bag and Chattopadhyay (2015); Sharifi-Rad et al. (2021)
Lovage, basil, parsley, and thyme	β-phellandrene, γ-terpinene, β-myrcene, and α-pinene were the major compounds	DPPH method	*Bacillis cereus* MIC (minimum inhibitory concentration) values: Basil - 10.8 µL/mL Thyme - 0.56 µL/mL *Staphylococcus aureus* MIC values: Basil - 2.45 µL/mL Thyme - 0.06 µL/mL. *Psuedomonas aeruginosa* MIC values: Basil - 10.80 µL/mL Thyme - 0.27 µL/mL Salmonella Typhimurium MIC values: Basil - 22.68 µL/mL Thyme - 0.56 µL/mL	Semeniuc et al. (2018)
Ruta chalepensis	β-linalool, 2-undecanone, linalyl acetate, and 2-nonanone were the major compounds	DPPH method	Percentages of inhibition for *R. chalepensis* collected from Jerusalem and hebron were 6.9 ± 0.94 µg/mL (69.56%) and 7.8 ± 1.05 µg/mL (61.53%)	Jaradat et al. (2017)
Achillea millefolium L., *Anethum graveolens L.*, and *Carum copticum*	*A. millefolium*: thymol, carvacrol, borneol, and limonene; *A. graveolens*: thymol, limonene, α-pinene; and C. copticum: thymol, sabinene, and borneol	DPPH, FRAP and BCBT assays	In all tests performed, *A. millefolium* showed the highest antioxidant activity	Kazemi (2015)
Cupressus macrocarpa and *Corymbia citriodora*	Terpinen-4-ol (23.7%), α-phellandrene (19.2%), α-citronellol (17.3%), and citronellal were the major constituents of *C. macrocarpa*, and α-citronellal (56%), α-citronellol (14.7%), citronellol acetate (12.3%), isopulegol, and eucalyptol were the primary constituents of *C. citriodora*	Standard butylhydroxytoluene	The concentration of *C. citriodora* was greater than that of the reference compound butylhydroxytoluene but less than that of the positive control	Salem et al. (2018)
Fagopyrum esculentum, *Fagopyrum tataricum*, and *Fagopyrum Cymosum*	*F. esculentum*: Nonanoic acid (7.58%), (E)-3-hexen-1-ol (6.52%), benzothiazole (5.08%), 2-pentadecanone (18.61%), and eugenol (17.18%); *F. tataricum*: 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester (13.19%) and (E,E)-farnesylacetone (7.15%); *F. cymosum*: Eugenol (12.22%), (E)-3-hexen-1-yl acetate (8.03%), linalool oxide (7.47%), 1-hexanol (7.07%), and benzothiazole (6.72%)	DPPH and BCBT assays	The antioxidant potential of three EOs is comparable in both tested procedures	Zhao et al. (2018)

EOs extracted from plants	Components of EOs	Procedure used	Antioxidant effect	References
Mentha spicata	Limonene, 1,8-cineole, and carvone were the major compounds	DPPH method, reducing power, chelating power, and BCBT assays	DPPH IC50 3.08 ± 0.07, reducing power EC50, 2.49 ± 0.07, chelating power IC50, 6.33 ± 0.12, and BCBT 6.4 ± 0.07	Snoussi et al. (2016)

a neurotoxic rather than just a mechanical pathway. Immersion, skin contact with treated surfaces, and inhalation of the vapors produced by these oils have all been demonstrated to be toxic. However, because EOs are so volatile, there is a common misconception that their effects wear off quickly. It is not known whether the purported superiority of EOs over standard ectoparasite treatments is attributable to neurotoxicity or mechanical asphyxiation (Ellse and Wall 2013). Several plants and their EOs have been studied for their potential antiparasitic effects. Mint (Mentha spp.), onion (*Allium cepa*), garlic (*A. sativum*) EOs, and seeds, for example, have been reported to be effective against gastrointestinal parasitism. Coccidia-infected birds grow more slowly than healthy birds, even after being fed the same diet. Anticoccidial activity of thymol and carvacrol, the main components of oregano EO, has been demonstrated against both mixed *Eimeria* spp. infection and *E. tenella* infection. Further *in vivo* and *in vitro* tests demonstrated that phenols, in particular, can be used as oocysticides to kill *Eimeria tenella* (Krishan and Narang 2014; Kumari et al. 2022b; Kumar et al. 2022c).

Lice, mites, and ticks are just some of the many ectoparasites that are particularly vulnerable to several EOs. Many different methods of exposure, such as submersion and skin contact with EO-treated surfaces, have demonstrated EO's effectiveness. It was observed that even low concentrations of them in spray form were highly effective (Chandran 2021a; Kumari et al. 2022a). The oils' neurotoxic and suffocative effects on parasites account for their insecticidal effects. Tea tree oil contains a monoterpenoid called terpinen-4-ol, and it blocks the acetylcholinesterase enzyme, which is essential for the transmission of action potentials in arthropods. In addition to their neurotoxic effect, oils' hydrophobic properties may have mechanical impacts on the parasite, which may disrupt the cuticular waxes and clog their spiracles, killing the parasites (Raza et al. 2022; Kumar et al. 2022c). Poultry ectoparasites are big business, especially *Ornithonyssus bursa* (a mite) and *Menopon gallinae* (chicken lice). Layers may not die from them directly, but they do suffer annoyance, weakness, and reduced egg production. In particular, the EOs found in ginger and citronella (lemon grass) are more efficient at lessening the prevalence of these parasites. Chemical constituents and active components of terpenes and oleoresin in ginger and citronella are mostly responsible for this outcome (Vigad et al. 2021). Several plant-based EOs have been used to treat *Dermanyssus gallinae*, and these EOs have proven to be successful. Commonly used EOs against chicken red mites include those derived from the plants such as clove (*E. caryophyllata*), coriander (*C. sativum*), and cade (*Juniperus oxycedrus*) (Abbas et al. 2018; Vigad et al. 2021).

3.8 Anti-inflammatory effect of EOs

Inflammation is a natural protective reaction triggered by tissue injury or damaged or dead host cells to fight off and eliminate foreign invaders (Mohammadi and Kim 2018). It has been proven that phenolic compounds found in a variety of aromatic plants can help strengthen the immune system. The anti-inflammatory characteristics of phenolic compounds have garnered a lot of attention because of their potential to reduce inflammation by blocking the production of pro-inflammatory prostaglandins and nitric oxide. The phenolic components of EOs are employed as feed additives because of their anti-inflammatory properties. Immune function is altered by phenolic compounds in several ways, including increased phagocytosis, secretion of cytokines, and interferon, and increased production of immunoglobulins (Greathead 2003; Hoffmann 2020; Mahfuz et al. 2021). EOs contain terpenoids and flavonoids, two chemicals with anti-inflammatory properties that block prostaglandin absorption. Another molecule presents in EOs that has been shown to alleviate pain, reduce swelling, and reduce inflammation is 1,8-cineole, which is found in eucalyptus oil, and linalool, which is found in lavender oil. Other plants having anti-inflammatory qualities include marigolds, anise, chamomile, and licorice (Wilkinson et al. 2003; Franz et al. 2010). Turmeric, also known as *Curcuma longa*, goes by several other names. The rhizome of this plant is an important source of curcuminoids, curlone, and zingiberene like active components. Anti-inflammatory, anti-cancer, and anti-hepatotoxic characteristics are the principal impacts of this herb on oxidation (Dosoky and Setzer 2018; Raza et al. 2022).

Other alkaloids, such as isoquinoline and acetylsalicylic acid, have been shown to have an anti-inflammatory impact by lowering the production of inflammatory cytokines. These phenolic compound-rich alkaloids can affect gut health by modulating the inflammatory cascade and blocking NF-κB activation. The cellular and humoral immune response was enhanced in broilers that were given thymol and carvacrol EOs (Greathead 2003; Hoffmann 2020). Plant flavonoids genistein (5 mg/kg) and hesperidin (20 mg/kg) were administered to broilers that had been exposed to lipopolysaccharide (LPS), and the animals showed enhanced immune activation and an improvement in gut architecture. Reduced production of IFN-γ, IL-4, IL-13, and IL-18 in LPS-

challenged broilers fed phenolic-rich diets is evidence that plant polyphenols have immunomodulatory effects. Broilers' cell-mediated and humoral immune responses could benefit from a diet of 150 mg/kg of saponins, a phenolic substance derived from soapnut (*Sapindus mukorossi*) (Mahfuz et al. 2021). Proof of carvacrol's anti-inflammatory effects in lipopolysaccharide-challenged broilers was found when the phenolic component carvacrol EOs was fed to the animals (Liu et al. 2019).

The increased monocyte counts in laying hens treated with fennel EO at 300 mg/kg and the enhanced lymphocyte counts in hens treated with thyme powder at 0.2% are both signs of good health. The increased production lifespan and thorough immunization regimens of the laying chickens from day old to curved age may inadvertently improve immune function thanks to dietary phenolic substances. Antibody response to the ND vaccine was enhanced in laying hens fed a combination of 200 mg/kg of EOs from *Mentha piperita*, *Thymus vulgaris*, *Anethum graveolens*, and *Rosmarinus officinalis* (Mahfuz et al. 2021). Non-volatile secondary metabolites like rosmarinic acid, oleanolic acid, and ursolic acid have been identified as the primary anti-inflammatory agents in EOs from the *Origanum* species (Shen et al. 2010). EOs can be used to counteract the effects of free radical scavengers. They can serve as anti-inflammatory drugs because an oxidative burst is a sign of inflammation. The anti-inflammatory effects of several EOs have been the subject of scientific study. For example, chamomile EO has been used for generations to alleviate the discomfort of eczema, dermatitis, and other skin irritations by acting as an anti-inflammatory. Anti-inflammatory preparations have also made use of a wide variety of other plants (pine, clove, and myrrh) and EOs (eucalyptus, rosemary, lavender, and millefolia) (Mohammadi and Kim 2018).

3.9 Effect of EOs on digestive and respiratory systems

Studies have shown that EOs from spices and herbs can aid digestion, hence these ingredients are commonly utilized in cooking. They stimulate the production of digestive enzymes such as trypsin and amylase (Jang et al. 2007). Capsaicin, curcumin, and piperine are just a few of the pungent principles found in EOs that have been shown to increase digestive enzyme activity in the intestinal mucosa and pancreas (Gopi et al. 2014). Researchers found that the spices or their constituents triggered the secretion of bile salts. EOs, alone or in combination, can be used to stimulate growth in broiler chickens. The body weight of broilers increased more when they were fed a diet containing peppermint. EOs improves chicken growth performance by increasing the production of digestive enzymes and balancing the microbes in the chicken's digestive tract. Feed consumption in broilers was drastically cut back when they were given a mixture of herbal EOs. This study provides strong evidence that supplementing with an EO mixture during the stage of development increases productivity, possibly by altering gut architecture and increasing digestive enzyme levels in broiler chicken (Botsoglou et al. 2002; Botsoglou et al. 2005; Yang et al. 2018). For this reason, EO may eventually replace the antibiotic growth promoter in the commercial chicken business. Antibacterial, antioxidant, and antiseptic activities have all been attributed to thymol and carvacrol, two components of thyme and oregano, respectively (Lee and Ahn 1998; Yang et al. 2018). Supplementing with EO improves intestinal microbiota and digestive enzymes, which may contribute to improved growth performance, but the mechanisms by which this occurs are still unknown (Helander et al. 1998). At 50mg/kg, the EO blend significantly increased both the total and specific pancreatic activities of trypsin. Proximal maltase activity was significantly higher in birds given 50ppm of EO compared to those fed a basal diet or a diet containing antibiotics and 25ppm of EO (Jang et al. 2007).

EOs optimizes the microbiome in the intestines, prompt the production of digestive enzymes, and boost the health and productivity of chickens. Broilers that were given a blend of herbal EOs showed a marked decrease in their need for food. The effects of EO components on broiler chicks' growth performance and digestive enzymes have not been the subject of many randomized controlled trials. Regardless, studies demonstrate that a particular EO combination can stimulate the body's natural production of digestive enzymes in hens (William 2001; Botsoglou et al. 2002; Botsoglou et al. 2005). The research was conducted to determine how different EO meal components affected the growth performance, digestive enzyme activity, and macronutrient digestibility of female broiler chickens. There is evidence that dietary EOs decrease blood cholesterol in hens, thus we also measured the plasma lipid level and the fatty acid composition of adipose tissue (Yu et al. 1994). Virus infections are among the most serious challenges facing the chicken business today. Mycotoxin exposure, viral illnesses characterized by immunosuppressive symptoms, ineffective vaccinations, and the inappropriate use of various antibiotics and drug growth boosters are only a few of the causes (Galal et al. 2016). Experimental testing of the commercial EO product AROMAX® demonstrated that indications of respiratory illness were reduced and humoral and local immune responses were improved in broilers with respiratory diseases and respiratory issues due to insufficient treatment.

3.10 Other beneficial effects (egg and meat quality/ biochemical/ hematological) of EOs

Witkowska and Sowinska (2013) conducted an inspiring investigation that demonstrated the efficacy of air sanitizers using

either thyme or peppermint oil alone in enhancing hygienic conditions in a chicken house. Seven different doses (0.025, 0.050, 0.0100, 0.0200, 0.0600, and 0.625g/kg) of a plant feed additive containing a mixture of EOs from black cumin, thyme, rosemary, fennel, and anise were tested on the growth performance, eggshell quality, biomechanical characteristics, and bone mineralization of egg-laying fowls. Findings suggest that laying hens benefit from dietary EOs at low to medium concentrations, but that these values are negatively impacted at higher concentrations (Olgun 2016; Cuppett and Hall 1998).

Significant increases in egg production, feed efficiency, and a decrease in cracked egg percentage were all seen after the EO mixture was added to the diet at a dose of 24mg/kg (Cabuk et al. 2006). A recent randomized controlled trial looked at how changing the chickens' habitat and supplementing them with essential oils (rosemary and cinnamon) affected egg production and quality. The hens were housed in cages and on floors while the effects of three different oils on blood biochemistry, hematological parameters, egg quality, immunological status, oxidative level, and layer production were examined. Egg production and performance were not significantly different between housing types; however the former had a positive effect on both. Results showed that treated groups (with EOs) had significantly higher and better blood cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), immunity, antioxidant, feed conversion, calcium, phosphorus, feed intake, and urea levels compared to control groups (Botsoglou et al. 2002; Botsoglou et al. 2005; Abo Ghanima et al. 2020; Hoffmann 2020). Lavender oil at doses of 300 and 600mg/kg was included in the feed throughout a 42-day experiment. Improvements in gut flora, development rate, and intestinal mucosa were observed at a dose of 600 mg/kg lavender EO. The antioxidant status of the serum and the liver improved (Greathead 2003; Hoffmann 2020).

Lavender EO in the diet is a superior substitute for AGPs for production growth (Barbarestani et al. 2020). A new study suggests that the orange EOs could be used as a chicken meat preservative. The oxidation of lipids in meat (chicken) is decreased by this procedure without any noticeable changes to the meat's color, flavor, pH, or quality (Bozkurt et al. 2014a). The use of thyme and its EOs in feed was the subject of an experiment. The effects of 5, 10, and 15 mg/kg of thyme and 0.5, 1.0, and 1.5g/kg of its EOs on growth performance, antioxidant status, blood picture, and immunological response in broiler chickens were evaluated. Numerous chicken metrics benefited greatly from this investigation (Rice-evans et al. 1995; Ismail et al. 2019).

Broilers that were given feed containing EOs such as *Lippie rotundifolia* and lemon grass were subjected to an experiment in which their histopathological lesions, hematological, and liver function were evaluated. There was a statistically significant difference between the MCV and MCH of the negative control group and the treatment group (broilers). Histopathology scores were greater in broilers given *L. rotundifolia* oil supplements, but these changes were seen in all groups (Santos et al. 2019). EOs of lemon grass and *L. rotundifolia* are sometimes used as a substitute for increasing performance because of their antimicrobial qualities (Souza et al. 2015; Assis et al. 2017; Azevedo et al. 2017). The goal of this study was to determine if broiler chicks benefited from being fed varied amounts of anise, powdered curcuma seeds, and fenugreek. The estimations also included feed efficiency, productive performance, carcass characteristics, and a handful of blood components. The data showed that the FCR, live body weight and overall gain all saw significant improvements. There was no change in feed consumption despite the nutritional enhancement of these oils (Amein et al. 2019). By enhancing the activity of enzymes like protease, lipase, and amylase, curcumin improved digestion and metabolism when given to animal feed (Platel and Srinivasan 2000). In addition to lowering blood glucose, triglyceride, and LDL cholesterol levels, curcumin improves liver function (Gandhi et al. 2011).

The growth, carcass quality, and blood profile of birds were found to be enhanced when the ginger powder was administered as a natural supplement in a study (day-old broilers). In this study, we employed doses of 0%, 0.25%, 0.40%, and 0.60%. According to the findings of this study, there was no statistically significant difference in the amount of weight that the various groups of birds gained. The feed conversion rate, however, was higher than that of the control group. There were no noticeable changes between the groups in terms of biochemical values or hematological values. Therefore, it was concluded that the amount of ginger employed in this study's experimental trial was not enough to stimulate the growth of chicks (Hassan et al. 2019). Soybean oil is commonly used in broiler diets, but for this experiment, linseed oil (high in omega 3) and pomegranate peel extract were substituted. The lipid profile, fatty acid contents, avian performance, flavonoids, and phenols were just some of the many factors evaluated. By changing the diet to include pomegranate peel extract and linseed oil, it was able to lower the amount of fat in poultry meat. Linseed oil was also found to reduce the total cholesterol and triglycerides fatty acid levels in the serum of broiler chickens. Protein, fat, and carbohydrate enrichment in the diet increased phenol and flavonoid content in chicken (Kishawy et al. 2019). In a separate investigation on the properties of Oregano EO, the oil was included in the diet of commercial laying hens. The researchers wanted to see if any shifts in metabolic profile would indicate a problem with liver function or lipid/protein metabolism. Oregano essential oil doses of 0mg/kg of feed, 50mg/kg of feed, 100mg/kg of feed, 150mg/kg of feed, and 200mg groups given doses of 150

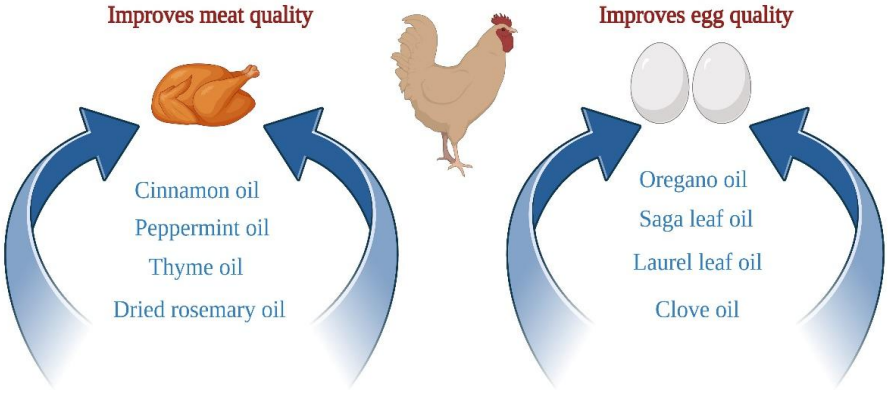

Figure 5 Various essential have potential impact on enhancing the meat quality and egg quality in the poultry industry

and 200mg/kg showed elevated levels of globulins and proteins (Migliorini et al. 2019). Additionally, broiler hens exposed to a high concentration of EO from the herb oregano had elevated levels of cholesterol in their blood serum (Basmacioglu Malayoglu et al. 2010). Various EOs that have beneficial effects on enhancing egg and meat quality in poultry is represented in Figure 5.

4 Side effects of usage of EOs

Although EOs serve several purposes, they are also to blame for the host's toxicity and unwanted side effects. Volatile oils relax the membranes of organelles and the cytoplasm, including the peroxisome and mitochondria. The mitochondrial membrane is disrupted by EOS, affecting the cell's capacity to depolarize via alterations in ion channels and hence ATP generation (Hoffmann 2020). The hydrophobic and lipophilic characteristics of EOs like thymol and carvacrol make them toxic to the intestinal cells and the mucosa layer that lines them. EOs derived from Egyptian and Chinese plants have also been related to the development of fumigant toxicity. It is important to remember that essential/volatile oils and the metabolites they produce can cause hypersensitivity reactions and symptoms in certain people (Jamroz et al. 2006; Raza et al. 2022).

However, while several EOs have been found to have beneficial anti-oxidant qualities, their use in meat and meat products is not without its downsides. To begin, some EOs' interactions with food's specific components and structural makeup can reduce the food's nutritional value (Lambert et al. 2001; Fernandez-Panchon et al. 2008; Migliorini et al. 2019; Sharifi-Rad et al. 2021). When applied to meat and meat products, the effects of EOs may be greatly attenuated by the presence of lipids, carbohydrates, proteins, and salts in food systems, as opposed to what is observed in vitro. Ham brushed with canola oil has the same amount of fat as pate. Herbal EOs like mint and cilantro are largely ineffective (Basmacioglu Malayoglu et al. 2010; Dhama et al. 2014; Dhama et al. 2018). When compared to synthetic antioxidants, the antioxidant power of most EOs is lesser and their price is higher. There may be inconsistency in EO quality and composition because of potential influences from harvest season and plant species. Further, their application as food preservatives has been limited because of the need for their presence in extremely high concentrations to exert sufficient activity (Juliano et al. 2000; Giovannini et al. 2016). The aroma and flavor of food can be ruined by the use of some EOs. The potent scents of these EOs, even at low absorption rates, may have been a major factor in turning off potential buyers. So, they are best reserved for usage with fiery dishes that feature strong seasonings or herbs (Jayasena and Jo 2014; Leherbauer and Stappen 2020).

5 Conclusions and future prospects

New research into the creation of safe and effective growth enhancers has been motivated by growing pressure on the chicken industry to limit or eliminate the use of antibiotics in feed as growth enhancers. Some of the most cutting-edge innovations in the feed additives industry include extracts of herbs and essential oils. Herbs, spices, and bioactive compounds have been used therapeutically for thousands of years, and their benefits to human health have been documented in a wide range of culinary traditions and scientific studies. Some bioactive chemicals found in a variety of plants could serve as useful additives to poultry feed. Extracts from common herbs and spices are said to affect the chicken's performance, oronasal somatosensing, digestion, lipid metabolism, prevention of tissue oxidation, and control of microbial populations. As a result, EOs probably affect not just the microbiota but also peripheral chemosensing and animal metabolism. It may be helpful to have a better grasp of the mechanism of action and effects of individual compounds before attempting to formulate mixtures of chemicals to boost efficacy.

More research into the specific class of EOs is required to better understand their practical applications for the poultry sciences. This includes looking at dose-responses, effects in combination with different commercial feed formulations, and the impact of chicken genetics and raising circumstances. Since these extracts are likely to be employed as natural additives in the food and feed industries, it is important to provide analytical methods for tracking the presence of active components in foods and animal feeds. Unfortunately, there is not yet a reliable analytical method for detecting and measuring EO residues in animal feed and other food sources. These testing procedures are necessary for achieving feed traceability and decreasing residual levels in carcasses, eggs, and milk. The problem of the instability of particular EO compounds in feed processing requires additional attention. A method for identifying and standardizing the functional components. Since the inclusion of a single herb or its extracted EO in meals may not always have the same effect on broiler performance, it is crucial to analyze the chemical quality of a plant extract to discover optimal compositions of secondary plant compounds for future reference. More study is required to determine if and how EOs react with the chemicals and additives used in feed. The intricacy of the number and diversity of bioactive compounds and their interactions slows the rate of progress in the study of raw plant materials and plant extract preparations. Taking use of the synergistic effects amongst EOs may boost their antibacterial activity while decreasing the concentrations required to obtain the same result. Interactions with other feed additives, such as organic acids and probiotics, should also be explored.

In vitro studies have shown that EOs and their constituents have potent antibacterial, antioxidant, immunomodulatory, and anti-inflammatory effects. It is widely agreed that these chemicals are safe to use as feed additives. Only in cases where resistance to or unpleasant side effects from already available approved medications or chemical substances, like antioxidants or antimicrobials, might the EOs and their derivative compounds are considered. Since there is such a wide variety of EOs, it may be necessary for researchers to zero in on certain compounds, including carvacrol, thymol, eugenol, alicin, and cinnamaldehyde, to see whether or not they can serve as substitutes for antibiotic growth promoters in poultry diets. These chemicals and their mixtures need more research on their chemical and biological properties and activities. We can conclude that EOs and related constituents can be employed as natural, non-antibiotic growth boosters in poultry diets from the available in vivo data. However, there is a dearth of credible information showing how this improves poultry's ability to digest nutrients. Applications of EOs in poultry can be successful in a variety of ways. The main factors to think about are the variations in feed inclusion levels and active components, animal genetics, and overall diet composition. An obvious prerequisite for the design of highly effective EOs-based products is the improvement of knowledge and understanding of the complex ecosystem of the gastrointestinal tract of diverse animals. In general, EOs are beneficial, but our understanding of how they might be used in animal nutrition is still sketchy, thus additional study is needed to determine its precise mode of action, as well as the optimal supplementation dosage and duration.

EOs are used for *in vitro* as well as *in vivo* testing on chickens. EOs have been shown to increase the productivity of broiler chickens by reducing their feed consumption, causing them to gain weight more quickly, and boosting their immune and general health. Therefore, there is a constant supply of new chicken preparations containing powerful bacteriostatic EOs. One of the greatest advantages of using EOs is that there have been no reported occurrences of bacteria developing antibiotic resistance as a result of using its components. Another advantage of EOs, beyond their use in immunization, is that they can be administered in a wide range of doses.

Researchers are utilizing cutting-edge technology in their quest to find solutions to the current challenges associated with using EOs as feed in the chicken industry. Encapsulating EOs in edible and biodegradable polymers for coatings or sachets, or combining their volatile components into films or edible coatings, are two examples of how this might be done to improve results. Additionally, a synergistic effect can be generated without compromising the antioxidative capabilities of EOs by combining them at lower quantities with other antioxidants and/or preservation methods. Researchers have shown that chicken farming is especially vulnerable to changes in the surrounding environment. There needs to be a thorough analysis of how volatile EOs influences the ecosystem. The immunomodulatory and anticoccidial effects of EOs have come into greater prominence in recent years. A thorough understanding of the molecular structures of EOs is crucial for extracting their full therapeutic potential.

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Techniques of Bioremediation using bacteria for the treatment of polycyclic aromatic hydrocarbons: A Review

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ABSTRACT

The term "hydrocarbon" is self-explanatory and refers to solely carbon and hydrogen compounds. Hydrocarbons play an important role in our everyday lives. Hydrocarbons, particularly polycyclic aromatic hydrocarbons, harm biota. The relatively fast introduction of xenobiotic compounds, as well as the enormous movement of natural materials to various environmental compartments, can often overwhelm the self-cleaning capabilities of the recipient ecosystem, resulting in pollution and accumulation of hazardous or even lethal levels. Bacteria capable of hydrocarbon degradation are frequently used in the bioremediation of fuel oil-contaminated sites. Presently, multiple sophisticated methodologies, transcriptomics, proteomics and are effectively utilized for the depiction of hydrocarbons degrading microorganisms. These expertises are highly developed, and its integration with bioinformatics tools makes it even more efficient. Though health science and biological science are the major relevant areas for molecular docking, it has been effectively used to explore the process of bio-degradation in ecological remediation in recent years. This review focuses on the sources, fate of PAHs, human exposure, various computational aspects associated with PAHs, and some approaches of synthetic biology related to pollutant degradation and PAH-degradation by genetically engineered microorganisms.

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1 Introduction

Now in these days pollution related to hydrocarbon is the most serious concern. Various factors like waste disposal, unintentional spills, pesticides, and losses during shipping and storage, are some ways through which hydrocarbons come into the environment. Unfortunately, oil spills are seriously associated with the hydrocarbon contamination of agricultural fields, which kills vegetation and the biodiversity connected with it (Mishra 2020). PAHs are a type of chemical compounds that are usually colorless, pale yellow, or white in colour. PAHs are a family of chemicals that are found in almost every environment and consist of hundreds of chemically related compounds. These chemicals can survive in the environment for many years and have a wide range of structures and detrimental effects (Patel et al. 2020). Further, these chemicals have demonstrated a variety of negative health impacts on the human system. Hydrocarbons are the most frequently used energy and fuel resources on the planet because of the energy they create. Spills that appear to be unavoidable during ordinary operations of crude oil production, refining, and distribution, as well as a result of severe accidents, have aroused interest in this sector (Patel et al. 2020). Since during oil field incidents, leak from oil pipes and storage space reservoir, oil tanker, and seepage calamities, and renovations of refineries and petrochemical fabrication apparatus are all widespread causes of petroleum hydrocarbon spills and discharges (Petrov 2012).

Despite the difficulty of treating oil pollution, microorganisms that can degrade petroleum hydrocarbons have progressed as a consequence of living in close contact with biologically occurring environmental petroleum hydrocarbons. These organisms could be used to clean up oil pollution (Lea-Smith et al. 2015). Therefore identification of the bacteria which can digest left-over products from the food, farming, biochemical, and medicinal industries is a necessary step of bioremediation. Due to less cost and eco-friendly character, the usage of bacteria to contract environmental toxins has become a potential tool in today's time (Guerra et al. 2018). The advancement of molecular tools, as well as a piece of improved knowledge about microbial metabolic and genetic organizations and activities, has expedited the spread of recombinant DNA engineering strategies to improve bioremediation for the elimination of contaminants from the atmosphere. Molecular docking is a simple and low-priced method for accurately understanding the bioremediation of the PAH mechanism of enzymes or proteins through Ligand. The present review article aimed to study the mechanism of PAH degradation by some recent approaches of synthetic biology related to pollutant degradation and PAH-degradation by genetically engineered microorganisms (Figure 1).

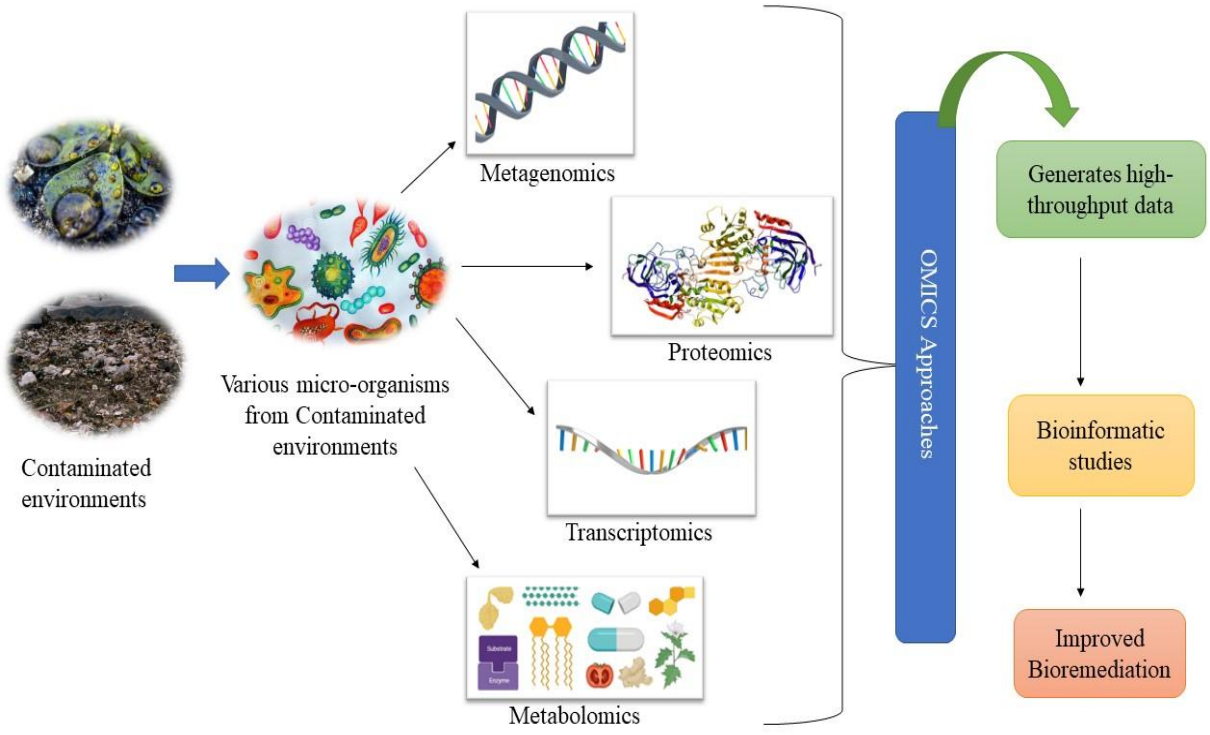

Figure 1 Graphical representation of an integrated strategy for biodegradation of xenobiotic substances using sophisticated technologies (Tanveer et al. 2018).

1.1 Hydrocarbons

Oil production and shipping cause accidental contamination of petroleum hydrocarbons in the soil. Every year, approximately 8.8 million metric tonnes of oil are spattered on land (Jawaid 2022). These hydrocarbons might be carcinogens or neurotoxic organic pollutants when they are available in higher quantities. Hydrocarbons pollute the soil, air, and freshwater (both surface and groundwater), particularly polycyclic aromatic hydrocarbons (PAHs), which have sparked a public concern since many PAHs are lethal, mutagenic, and carcinogenic (Shukla et al. 2022). The toxicity of PAHs has been reported to increase with increasing molecular weight and alkyl substitution in the aromatic ring (Jesus et al. 2022). The hydrocarbons reach horizontally on the surface of groundwater and contaminate it extensively. The lower boiling-point portions, particularly those in the C10–C19 range, have a strong correlation with petroleum hydrocarbon toxicity. Aromatic, aldehydes, ketones, carboxylic acids, fatty acids, etc. were among the metabolic components that led to the biota toxicity of petroleum pollution. Earthworm and seed germination assays can be used to assess the toxicity of organically contaminated soil (Tang et al. 2012). TPH (total petroleum hydrocarbon) is an intricate combination of substances that includes saturated hydrocarbons, aromatic chemicals, asphaltenes, and resins. High quantities of total petroleum hydrocarbons (TPH) in the severely polluted soil impeded seed germination and created nutrient-deficient circumstances in which the plants could not survive. For successful rhizo-degradation, certain soil environmental variables (e.g., oxygen permeability and water availability) must be increased (Haider et al., 2022). The major sources of hydrocarbon pollutants are crude oil, used engine oil (spent oil), and diesel.

1.2 The Elementary components of Hydrocarbons

Polyaromatic hydrocarbons degrading bacterial microbes are using enriched naphthalene (20 isolates), phenanthrene (25 isolates), or anthracene (6 isolates) as the sole carbon and energy source (Mangwani et al., 2021). Three isolates, N6, A3, and P3, that were found to use naphthalene, phenanthrene, and anthracene better than the other isolates, were subjected to additional characterization (Al-Thani et al. 2009). Cycloparaffins are a solitary cause of carbon that has proven to be ineffective. The coordinated occurrence of a diverse microbial population on cyclohexane has been revealed, implying that commensalism is involved in the microbial degradation of cycloparaffinic hydrocarbons (Sime-Ngando et al. 2018). Microorganisms are a very dependable approach to biodegrade petroleum-derived materials. Even *Bacillus subtilis* inoculum completely converts the synthetic and semi-synthetic vehicle lubricant oils and changes the color of DCPIP (2,6-Dichlorophenolindophenol) from blue to translucent (Bidoia et al. 2010). According to a study on the isolation of *Cladosporiunz resinae* from soil and air samples, it was reported that *C. resinae* strains can easily develop a deep culture on C10 to C14 n-alkanes. Other strains thrive on alkanes ranging from C9 to C18 (Jun et al. 1973).

2 PAHs and their sources

PAHs are obtained from a variety of sources, including human activities as well as natural sources. PAHs are formed primarily as a result of thermal alteration, incomplete burning of organic matter, combustion of fossil fuels, and industrial wastewater. Natural sources of PAHs include volcanic eruptions, oil seeps from crude oil reservoirs, bush burning, and erosion of ages' sediments, whereas anthropogenic sources include thermal alteration, incomplete burning of organic matter, combustion of fossil fuels, and erosion of ages' sediments (Okoro et al. 2020). Petrogenic, pyrogenic, and biological methods are the three main ways by which PAHs are delivered into the environment. Petrogenic PAHs are those that are generated during crude oil preparation and other related events. Petrogenic PAHs are formed primarily by petroleum products and are the most common sources of PAHs due to the widespread use of various modes of transportation, as well as the widespread storage of crude oil and its products in the environment. The major sources of petrogenic PAHs are oil spills from marine and freshwaters, tank leaks from petroleum products stored beneath and above ground, and the retention of various quantities of discharge of gasoline, motor oil, and other substances. Pyogenic PAHs are produced during pyrolysis. Pyrolysis is the non-oxygenated putrefaction of organic matter at elevated temperatures (350°C to >1200°C). The harsh condensation of coal into coke and coal tar, as well as the heated splitting of oil by-products into smaller hydrocarbons, are common examples of pyrogenic sources of PAHs that exist naturally in the environment. Inadequate combustion of fuels in automobiles and trailers, partial burning of wood in forests, and a variety of other pyrolysis processes contributed to the creation of PAHs. Biologically produced PAHs are produced from a range of biological activities, as well as the bio-synthesis of some plants, microorganisms, as well as plant breakdown (Hussein et al. 2016).

3 Bacterial degradation of Polycyclic aromatic hydrocarbons

The removal of PAHs from the environment has been studied using a variety of bioremediation methods during the past 30 years. In terms of PAHs decomposition, bacteria have shown the best and most economical methods when compared to fungi, algae, and plants. Bacteria have evolved, developed, and adopted a technique to digest several environmental contaminants for more than three billion years (Ghosal et al. 2016). Numerous researchers have also established many metabolic pathways in bacteria that are involved in the breakdown of PAHs (Govarthanan et al. 2020). The majority of the time both aerobic and anaerobic conditions are used for the bacterial-based PAHs breakdown techniques. Oxygen, which also

Table 1 Bacteria and their respective degradable PAH

PAH degraded	Bacteria	References
Phenanthrene	*Pseudomonas, Acinetobacter, Arthrobacter, Mycobacterium,*	Li et al. 2021
Pyrene	*Rhodococcus, Bacillus, Xanthomonas, Mycobacterium, Pseudomonas*	Shahsavari et al. 2019
Naphthalene	*Acinetobacter, Alcaligenes, Pseudomonas, Sphingomonas*	Rabani et al. 2022
Fluoranthene	*Pseudomonas, Arthrobacter, Mycobacterium*	Fathepure 2014
Anthracene	*Alcaligenes, Rhodococcus, Beijernickia, Mycobacterium, Sphingomonas, Janibacter*	Shahsavari et al. 2019

acts as a co-substrate for the aromatic compounds' hydroxylation and oxygenolytic ring cleavage, is the final electron acceptor in the aerobic type of PAH breakdown. The bacteria in anaerobic PAHs degradation take completely different routes to break down the aromatic ring based on reductive processes (Mallick et al. 2011).

The aerobic mode of PAHs degradation has received the greatest attention from researchers for several decades, but in recent years interest in the research on the anaerobic technique have been increased. The majority of anaerobic catabolism occurs in aquatic sediments, submerged soils, and aquifers, where ferric ions, sulfate, and nitrate serve as the bacteria's ultimate electron acceptors (Foght 2008). Additionally, the aerobic environment is favorable for the breakdown of PAHs as demonstrated by oxygenase-mediated metabolism carried out by monooxygenase or dioxygenase enzymes. The aerobic decomposition of PAHs is the hydroxylation of an aromatic ring, which is carried out by the action of two enzyme's dioxygenase and dehydrogenase, and these work on a cis-dihydrodiol and rearomatized to a diol. Further, cleavage of these diol intermediates by extra- or intra-diol ring-cleaving dioxygenases via the ortho- or meta-cleavage mechanism produced catechols by the TCA cycle (Mallick et al. 2011). Numerous studies have demonstrated how effectively certain bacterial species can use PAHs as their only carbon source.

Employing fungi and bacteria under a co-cultivation technique for PAHs biodegradation is more promising than employing monoculture. According to Arun et al. (2008), significant outcomes demonstrating the value of co-cultivation were seen when *Basidiomycetes* fungus and *Pseudomonas* spp. were co-cultured. *Coriolus versicolor* and *Fomitopsis palustris* co-cultivated with *Pseudomonas* spp. also demonstrated 93.7% effectiveness in the degradation of pyrene (Table 1).

4 Polycyclic aromatic hydrocarbons: computational aspects

4.1 Bioinformatics

Bioinformatics fostered a modern set of computational tools that combines information technology (IT) with biology. This cutting-edge technology collects data from a variety of high-throughput biological systems and stores it all, allowing researchers to explore and regulate the association between organic molecules such as biochemical and metabolic conduits, expression of proteins, structures, metabolites, and macromolecular sequences structures (Shekhar et al. 2020). Protein, DNA, and RNA sequences provide enormous volumes of information that must be carefully carried out; as a result, bioinformatics has resulted in the creation of specialized computing tools to assess such enormous amounts of biological data (Bhatt et al. 2021). As a result, bioinformatics-related technologies are critical for understanding harmful pollutant bioremediation. The molecular, genetic, and cellular origins of xenobiotic breakdown and detoxification are better understood using bioinformatics (Huang et al., 2020).

Bionemo, a database created by the Spanish National Cancer Research Center's structural computational biology department, provides information on particular genes and proteins involved in the bio-degradation reaction and metabolic routes (Carbajosa et al. 2009). Unified bioinformatics-based approaches are used to uncover the inherited and functional characteristics of unlike soil microbial populations utilizing MetaPhlAn, LEfSe, XLSX, and KEGG bioinformatics databases for metagenomics-based categorization of soil microbial populations of diverse soil sites (Kumar et al. 2016). Degradation of contaminants by microbial populations is a favorable potential and suitable remediation skill, and there is no specific source available that contains all of the data about environmental toxins, microorganisms, and their bioremediation capabilities. As a result, these databanks combining comprehensive insight into the type of contaminants, their enzymes, metabolic processes, catabolic genes, and protein-expression reports can be a valuable tool for enlightening upcoming investigations in the area of bioremediation.

4.2 Quantum mechanical techniques

The researchers gathered PAHs that interact with NDO and their reaction outcomes in a systematic manner. The relevance of in silico bioremediation through DFT investigations signifies that thermodynamically all the polycyclic aromatic hydrocarbons can associate with the active binding sites of NDO. Only steric hindrance governs whether compounds (enzymes and PAHs) can optimally react with NDO or not. Some researchers used the exact relevance of the structure/reactivity correlation to enhance the understanding of PAH-enzyme interactions (Librando and

Alparone 2007). The PAH degradation efficiency was predicted using a combination of ab initio and functional density theoretical models for a range of dimethyl naphthalene (DMN) isomers. These findings aid the idea that electronic polarisability, in addition to performing a crucial task in the biodegradation of DMNs and stipulating a foundation for Farnet's hypothesis, could be an effective method for calculating the biodegradation tendencies of a set of molecules. The approach was integrated with docking procedures to successfully illustrate PAH–NDO connections.

The process of PAH-enzyme interactions is not entirely understood, and experimental procedures are unlikely to reveal it. Recent research using a quantitative structure activity relationship (QSAR) methodology implies that some investigational methods could be very useful in this regard (Ha et al. 2019). The binding relationships between PAHs and diverse compounds such as DNA or oestrogen receptors were explored by Li et al. (2012). The binding action was connected to van der Waals volume, molecular size, shape outline, hydrogen bonding, polarisability, hydrophobicity, electron topological state, and p–p interactions, according to the descriptors used in the QSAR models (Gbeddy et al. 2020). In these investigations, QSAR has been used to assess for mutations that progress enzyme activity. Furthermore, QSAR was also used to give proper details regarding the toxicity of PAHs and degradation arbitrates (Kobeticova et al. 2011), revealing that toxicity and lipophilicity (Kow) have a significant relationship, implying that non-polar narcosis is the most common lethal consequence of the tested PAHs. This is since toxicity, which is specifically linked to hydrophilicity for biological membranes (i.e., non-polar narcosis), is typically determined by the quantity of the molecule accumulating in the same membranes.

4.3 Database approach

The massive volume of information on the interaction between the environment and degradation makes it difficult to discover the proper reaction, degradation products, and so on. As a result, a database strategy could be beneficial. The incorporation of different sources of bioremediation information is one problem connected with the database strategy. In this regard, Pazos et al. (2005) provided a fine-tune 'metarouter,' which is a valuable tool for evaluating the ecological future of compounds and creating bio-derivative methods for such groups. It provides information regarding the name, molecular weight, the image of chemical structure, chemical formula, SMILES code, canonical 3D structure in PDB format, physicochemical properties, and connections to other databases are provided for chemical compounds. For reactions, the following information is provided: substrates, products, catalyzing enzymes, and relationships with other databases. The drawback of metarouter's is that it concentrates on the biochemical features of biodegradation instead of the type of biomolecules that perform the processes. Carbajosa et al. (2009) presented Bionemo, a novel database that complements current biodegradation databases, in a recent study. Bionemo was created by integrating data (manually) from available articles and the biodegradation literature in general into a fundamental biochemical network. In addition to transcription regulation information for more than 100 promoters and transcription factors, Bionemo now offers sequence data for 324 processes. Meanwhile, current biodegradation databases tie reactions to protein sequences in databases identified by EC codes. This strategy, however, may be inappropriate. Many reactions, for example, have the same EC code even though they utilize various substrates and produce numerous products. Bionemo is a databank that includes metabolic, genetic, and regulatory data. Enzymatic complexes are the database's principal entries. These are associated with biological reactions that convert substrates to products.

4.4 Docking studies

PAHs such as anthracene, phenanthrene, pyrene, and benzopyrene are made up of two or more benzene rings and include both hydrogen and carbon. PAHs are also characterized as electroneutral, non-polar compounds retrieved from oil and coal that lack auxiliary branching substituents on their ring structures. Inadequate combustion of wood, coal, oil, tobacco, and organic polymer mixtures produces them. These are major pollutants in the environment as well as a source of food contamination. Although physical and chemical approaches can be used to adsorb, decompose, and attenuate PAHs but these are associated with some environmental issues like air, soil, and groundwater pollution (Xu et al. 2012). Biodegradation as an alternative approach might be a highly efficient way to convert PAHs into H_2O and CO_2 (Ukiwe et al. 2013). Biodegradation is aided by a variety of microorganisms. The mechanisms of degradation have previously been discovered at the molecular level. However, there is a need to put attention to receptor-ligand interactions from an enzyme or protein standpoint (Jin et al. 2015). The efficiency with which PAHs are removed varies from species to species.

In an attempt to improve degrading potentials, the mutants could increase the formation and lessen nuclear repulsion by simply changing steric interactions (Librando and Pappalardo 2014). Similarly, while addressing fluoranthene breakdown and bond structure based on the active site of ring-hydroxylating dioxygenase in *Microbacterium paraoxydans* JPM1, researchers found the fluoranthene molecule was surrounded by hydrophobic residues. Two oxygen atoms and a mononuclear iron atom formed a triangle with the terminal of Asn207, forming two hydrogen bonds.

Even though the number of benzene rings has an impact on the bonding energy, the width/height ratio of the substrate is also an important physical characteristic. When molecules are afar than the

Table 2 Various docking software and their algorithms

Software	Algorithm	References
PythDock	Particle swarm	Chung et al. 2011
MolDock	Guided variance evolution	Thomsen and Christensen 2006
GOLD	Genetic	Verdonk et al. 2003
Molegro Virtual Docker	MolDock SE	Storn and Price 1997
AutoDock	Lamarckian Genetic	Morris et al. 2009
FlexX	Incremental creation	Schellhammer and Rarey 2004
Surflex-Dock	Molecular resemblance-based search	Jain 2007
AutoDock Vina	Broyden-Fletcher-Goldfarb-Shanno	Trott and Olson 2010

width/height ratio threshold, they have complexity in interacting alongside the active site of the enzyme. Various docking software are used for the biodegradation of PAHs, information regarding the available docking software is available in table 2.

5 Synthetic Biology-Based Alternatives for Microbial Remediation of PAHs

5.1 Genetic and Metabolic engineering

The term "genome editing" was proposed by Enrquez (2016) and it is used rational genetic manipulation at a local (gene) or global (genome) level to assure precise addition, removal, or substitution of DNA fragments. Transcription activators such as zinc finger nucleases and effector nucleases (TALEN) are mostly used in this gene editing. According to Kanchiswamy et al. (2016), CRISPR_Cas is the most proficient and reliable method for gene editing methods. Genome editing helps in accelerating the rate of the bioremediation process. Further, TALEN is a sequence-specific DNA-binding component that is specific to the particular host genome (Utturkar et al. 2013). When TALEN attaches to DNA, it worked on the target sequence and creates sticky ends, and produced DSB (Double-stranded break) in the target sequence. On the host genome's target site, the Fok1 cleavage domain produces DSBs. Another method of using hybrid nucleases made up of TALENs and ZFNs nucleases was devised to address the molecular complexity. On the other hand, the CRISPR-Cas system offers distinctive sequence specificity and sophisticated gene editing as unique action properties. These gene-editing methods produce knock-in and knock-out effects and are currently being tested in bioremediation investigations (Kumar et al. 2018). According to the latest details, the CRISPR-Cas system is primarily used and executed by scientists working with ideal organisms such as *Pseudomonas* (Nogales et al. 2020) or *Escherichia coli* (Chen et al., 2018). In the field of bioremediation, new insights into CRISPR toolkits and creating gRNA for the manifestation of function-specific genes relevant to remediation in non-model organisms (e.g., *Comamonas testosteroni*, *Rhodococcus ruber* TH, and *Achromobacter* sp. HZ01) are also being proposed (Liang et al. 2020). Pollutant-inhabited bacteria are the best applicants for gene editing and metabolic engineering since they are acclimated to surviving and harboring in the presence of diverse toxic and non-degradable xenobiotics. Furthermore, interpreting metabolic pathways appears to be valuable in exploring microbial bioremediation (Plewniak et al. 2018), such as the decontamination of pyrethroid from the soil using the fenpropathrin biodegradation pathway studied in *Bacillus* sp. DG-02 and the bioremediation of hazardous pollutants via the manufacturing pathway of haloalkane dehalogenases (Chen et al. 2014). Metabolic engineering involves modifying an existing route to improve the bioremediation method (Michel et al. 2007). This strategy chiefly wraps the analysis of microbial enzymes, i.e., oxidases, oxidoreductases, esterases, phenoloxidases, and monooxygenases participating in diverse degradation stages. Enzymatic bioremediation is a simple, quick, and ecologically favorable approach for microbial removal and decomposition of relentless xenobiotics (Sharma et al. 2018). With the limitation of lower productivity, isolation and characterization of microorganisms by enzymatic potentials have been carried out. Organophosphates (OP) and organochlorines (OC), two of the main components of pesticides, accumulate in agricultural soil and get into water bodies through agricultural runoff (Panelli et al., 2017). It has been demonstrated that genetically altered bacteria can efficiently bioremediate methyl parathion (OP) and hexachlorocyclohexane (OC) (Gong et al. 2016). Additionally, using genetically altered *P. putida* KT2440, organophosphate, and pyrethroid bioremediation has been carried out (Zuo et al. 2015). The catabolism and destruction of numerous persistent substances have been observed since the advent of metabolic engineering.

5.2 Artificial Genetic Circuit and Microbial Biosensor

The insertion of the artificial genetic circuit necessitates the use of chassis. The Recombinant DNA Advisory Committee has

designated *P. Putida* as the Host Vector Bio safety strain. It's GRAS (Generally recognized as safe) when it comes to environmental discharge. Because it had a high tolerance to fluctuating circumstances such as pH, temperature, toxins, solvents, osmotic, and oxidative stress, it is excellent to be used for the future creation of synthetic biology framework panel. *P. putida* also has a flexible metabolism and minimal nutritional prerequisites (Pabo and Nekludova 2000). These characteristics formulate this organism as the ideal bacterial prototype for bio-remediation purposes in the environment (Tanveer et al. 2018). Recently, a synthetic genetic circuit was created for *P. putida*, which has specific promoter genes for persistent chemical degradation, and the expression of these genes depends on the available pollutant (Adams 2016). For the construction of synthetic genetic circuits, serine integrases were used. Microbial cells benefit from the processes of the biological system that regulate cell growth and responses to environmental stimuli such as light, temperature, pH, and oxygen (Tropel and Van Der Meer 2004). The amounts of various persistent chemicals present will affect how microbes living in the surrounding environment at a contaminated site respond. Whole-cell biosensors for detecting the occurrence, and biodegradation ability of xenobiotic chemicals (paraffin, pharmaceutical residues, PAHs, and PCBs, among others) existing in ecological samples are gaining popularity (Patel et al. 2019). When a transducer detects specific pollutants, the reporter proteins acting as microorganism produces a color signal (Zhang and Liu 2016). A biosensor designed for recognition and bioremediation should have increased microbe-contaminant interaction (Dhar et al. 2019). This allows bacteria to modify their biological processes in reaction to external environmental circumstances and codes the genes needed to use resistive substances as a substrate (Skinder et al. 2020). Since genetic circuits can be built in contrast to an exogenous environmental toxin, the strategies of synthetic biology are conceivable for detoxifying a specific harmful chemical. Two-component regulatory system (TCRS) synthetic genetic circuits were also developed for the bacteria (Ulu Seker et al. 2017). In a bacterial TCRS, histidine kinase (HK) and response regulator are present (RR). Here HK is a sensor domain protein with an extracellular loop that is a part of the membrane. HK features a transmitter domain, a highly conserved domain, in the last cytoplasmic transmembrane. As a result, using TCRS-based synthetic biology to construct biosensors for cell-mediated recognition and bioremediation could be a significant step forward.

6 PAH-degradation by Genetically engineered microorganisms (GEMs)

Although bioremediation for PAH-degradation has recently gained prominence as a field of study, it can occasionally be quite slow because of several biotic and abiotic variables. In lab tests, microbial species have the best capability to degrade PAHs, but in field trials, they do not perform well. Even though some microbial species may utilize PAHs as a source of carbon, improving their catabolic efficiency is necessary for total PAH degradation. The enzymatic activity of these microbial species could be improved to increase their capacity for PAH-biodegradation by genetic engineering. Therefore, the use of genetically modified or engineered microbes has the potential to degrade numerous types of pollutants, including PAHs. The breakdown of PAHs by GEMs with unique metabolic activity has received substantial attention. By modifying or manipulating the genetic traits of microorganisms, GEMs are created utilizing genetic engineering (Filonov et al. 2005). Various genetic engineering approaches, like DNA recombinant technology, genetic transposition, gene duplication, etc., have been created for the construction of new microorganisms for the removal of environmental toxins (Figure 2). To achieve high catalytic activity under environmental stress, these strategies induce controlled gene expression. Occasionally, local microbial species cannot survive in polluted environments. In comparison to laboratory size experiments, the degrading efficiency of such microorganisms may be lower in contaminated natural ecosystems. In cases when native microbial species are unable to digest PAHs, GEMs may be utilized as a substitute.

The use of GEMs in the bioremediation of PAHs has entered a new phase of the study. Numerous studies have demonstrated the tremendous potential of engineered microbes as PAH-degrading agents. Numerous aliphatic, aromatic, and polyaromatic hydrocarbons can be broken down by genetically modified multi-plasmid microbial strains (PAHs). This multi-plasmid microbial strain simultaneously oxidizes hydrocarbons while plasmid-specified breakdown pathways can be triggered in the presence of the right inducer. It was observed that this multi-plasmid bacterium grows on crude oil more quickly than microorganisms with a single plasmid (Sakshi et al. 2020). *P. putida* strain KT2442 has been altered using plasmid pNF142::TnModOTc. This recombinant strain of *P. fuorescens* HK44 with naphthalene degrading capabilities was the first GEM that was used for field application in the USA (Sayler and Ripp 2000). According to Filonov et al. (2005), the genetically altered *P. putida* KT2442 (pNF142:: TnMod-OTc) is more stable and exhibits more specific growth on naphthalene. The bioremediation activity of *Pseudomonas* sp can be improved through genetic engineering employing various molecular approaches as per several studies (Zhao et al. 2011). The use of GEMs could potentially improve pollutant dissolution. The GEM *P. putida* KT2440-rhlABRI was created by cloning the rhlABRI cassette containing the rhamnolipid-producing genes from *P. aeruginosa* strain BSFD5 into a random transposon vector. The rhlABRI cassette could be strongly expressed by this GEM, producing rhamnolipid. It was discovered that *P. putida* KT2440-rhlABRI can promote pyrene

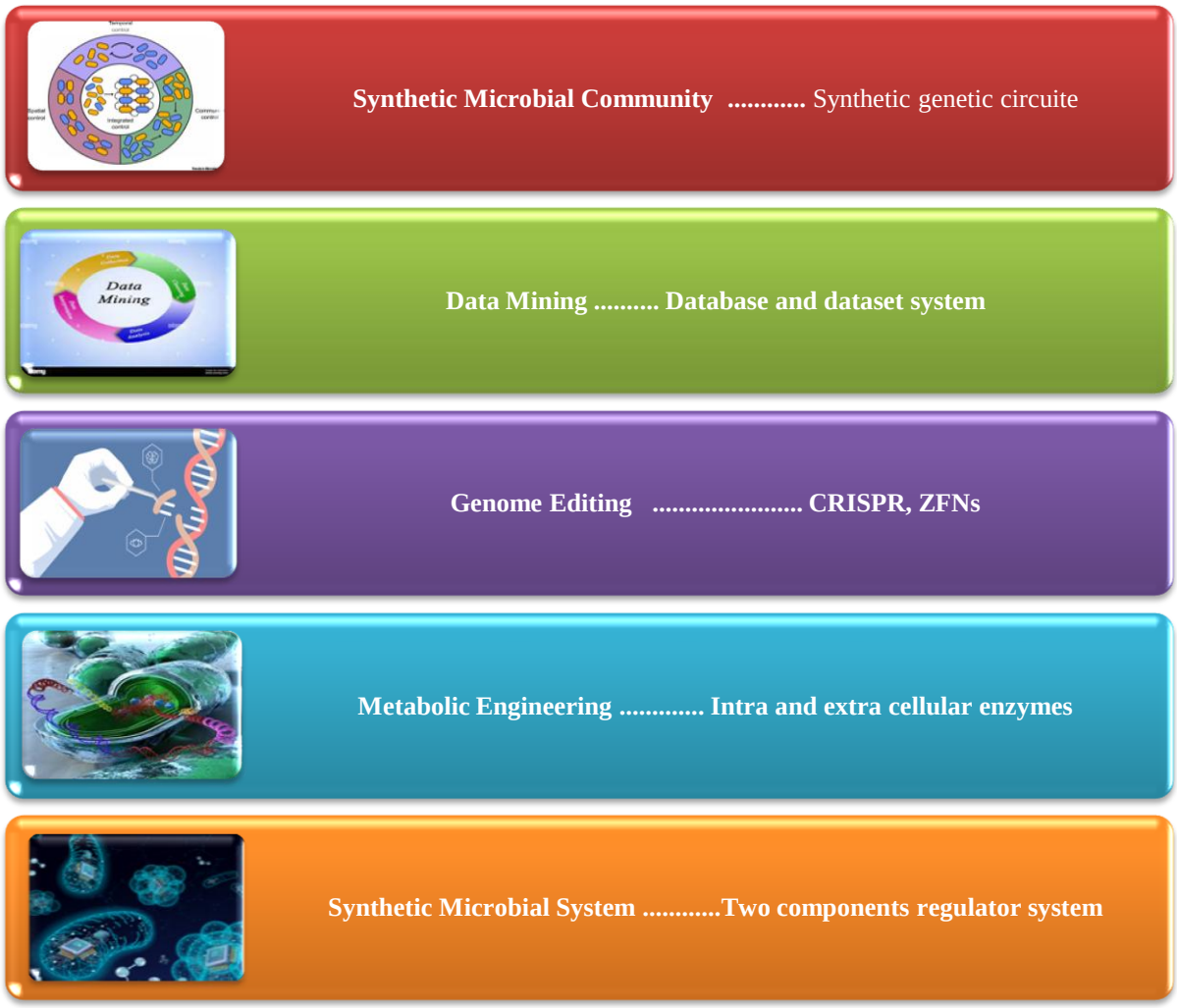

Figure 2 The building fundamentals of synthetic biology used for bioremediation studies

breakdown by native microbial species in natural soil. As a result, *P. putida* KT2440-rhlABRI may increase the remediation of soils contaminated with PAHs (Cao et al. 2012).

For effective PAH-degradation increased catabolic enzymatic activity in GEMs. The GEM is made from *Pseudomonas* sp. CGMCC2953, a bacterium isolated from an oil-contaminated soil, demonstrated improved phenanthrene degradation capabilities. The PAH degradation gene C23O (catechol 2,3-dioxygenase) is present in this developed GEM. Effective cloning of the biodegradative C23O gene into plasmid pK4 was created from plasmid pRK415. Given that C23O is the primary enzyme involved in the breakdown of phenanthrene, *Pseudomonas* sp. CGMCC2953-pK was shown to be extremely effective. To effectively remove PAH (phenanthrene), it is, therefore, effective to increase the activity of important enzymes such as C23O (Wei et al. 2013). Using a gene encoded by catechol 2,3, -dioxygenase (C23O), the genetically modified dioxygenase-producing bacteria *P. putida* was created (nahH). The recombinant vector pUC18-nahH was effectively transferred into *P. putida* after this gene was cloned into the plasmid pUC18. When compared to biodegradation by the natural type, a significant improvement in PAH (phenanthrene and pyrene) biodegradation by genetically modified *P. putida* was seen (Mardani et al. 2017). A PAH-degrading microbial consortium built with two genetically modified fungal strains of *Aspergillus niger* exhibited significant levels of tolerance and high effectiveness of LMW and HMW PAHs degradation. These GEMs express the fungus *Phanerochaete chrysosporium's* LiP (lignin peroxidase, LiP+5 strain) and MnP (manganese peroxidase, MnP+7 strain) genes. These GEM strains demonstrated increased HMW-PAHs degradation and longer life in PAH-polluted soil (Zafra et al. 2017). Nevertheless, various studies have demonstrated that genetic engineering can be useful for pollutant biodegradation and offers a substantial opportunity for utilizing native microorganism capabilities for the creation of GEMs that are well adapted to polluted environments and more effective for

PAH-degradation. Unfortunately, these GEMs are only useful in favorable laboratory settings, and their potential for use in PAH-bioremediation at the field scale is constrained by many obstacles, including obtaining permission to release GEMs into contaminated environments and determining their fate as well as their monitoring, control, and risk to ecosystems and human health. Therefore, the key to achieving comprehensive and secure pollutant removal is genetic modification of the appropriate microbial species with rapid growth and effective biodegradation skills without any damage to the environment. To increase the reclamation of contaminated environments, further study is required into bioremediation using various microbes as well as GEMs.

Concluding Remarks

The most serious environmental issues in the world are hydrocarbon contaminants in the ecosystem. The investigation revealed that there has been an overuse of hydrocarbon pollutants, posing a severe environmental concern. Petroleum hydrocarbons rank among the toxins that are most dangerous to human and environmental health. Bioremediation utilizing microorganisms that degrade petroleum hydrocarbons is largely recognized as an ecologically beneficial and effective approach. A considerable number of bacterial species having the ability to degrade petroleum hydrocarbons have been identified and utilized in bioremediation. It is impossible to overstate the importance of developing strains that could be effective in the bioremediation of hydrocarbon-polluted locations. But it has been found that many problems slow down the effects of biodegradation. PAH removal from the environment is a difficult task. As a result, a thorough understanding of the mechanisms underlying the various remediation processes is critical. Enzymes, in contrast, play a critical function in biodegradation. Investigations of enzyme-substrate interactions and alterations are useful in guiding related experiments. Because of its convenience and inexpensive cost, molecular docking was extensively used in numerous research-based domains. Molecular docking, in particular, is capable of predicting and accounting for the procedure of biological reactions. Molecular docking can be utilized as a pre-experiment for investigating the characteristics of these contaminants and providing theoretical data for further research. The remediation techniques for the detoxification of xenobiotics and associated substances in the environment are also covered by synthetic biology.

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Conflicts of Interest

The authors declare no conflict of interest.

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Functional, and phylogenetic analysis of maleylacetate reductase of *Pseudomonas* sp strain PNPG3: An in-silico approach

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ABSTRACT

Shrinking freshwater ecosystems are under tremendous pollution threat due to anthropocentric activities. Para nitrophenol (PNP), a well-documented priority pollutant extensively used in dyes, petrochemical, pharmaceutical, explosives, pesticides, leather industries, and agrochemicals, is responsible for contaminating aquatic ecosystems globally. It is highly toxic and has carcinogenic and mutagenic effects on living organisms like humans and several animal models. Bioremediation approaches mainly involving bacteria are considered the best, most eco-friendly, cost-effective, green, and clean method for effective removal PNP from its contaminated sites. This manuscript highlights the structural and functional analysis of a lower pathway enzyme involved in PNP degradation, maleylacetate reductase (MR), from *Pseudomonas* sp strain PNPG3, which was recently isolated from a freshwater ecosystem. This enzyme plays a role in converting maleylacetate to 3-oxoadipate. Despite its crucial functional role, no model is available for this protein in the protein database (PDB). Therefore, attempts were made for the computational investigation of physicochemical, functional, and structural properties, including secondary, and tertiary structure prediction, model quality analysis, and phylogenetic assessment using several standard bioinformatics tools. This enzyme has a molecular weight of about ~37.6 kDa, is acidic and thermostable, belonging to a member of iron-containing alcohol dehydrogenase. Moreover, this study will benefit the scientific community in deciphering the prediction of the function of similar proteins of interest.

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1 Introduction

P-Nitrophenol (PNP) considered a priority environmental pollutant, is one of the most abundant chemical pollutants, which has been thoroughly and extensively used in the manufacturing of dyes, explosives, plastics, medicines, and a diverse range of chemical products (Bhushan et al. 2000; Kuang et al. 2020). Besides, PNP is reported to be released into the environment from parathion-based pesticides and several other agrochemicals resulting in possible high levels of PNP contaminations in several ecosystems. It is highly toxic and is reported to have carcinogenic and mutagenic effects on several animal models, causing dysfunction of the kidney, liver, and other crucial physiological life processes of animals (Kuang et al. 2020).

Since genomic DNA is the central repository of all the information for an organism, the study of genome sequences of PNP degrading bacterium (especially in this omics era) may provide a blueprint of the full potential of these bacteria (Koonin et al. 1996). To execute these, rational approaches involving wet laboratory-based experimental and computational work are necessary (Munn et al. 2017). It is now possible to hypothesize and speculate the possible potential of a bacterium with the current tools and methods available in public servers and databases (Booth et al. 2013).

Enzymes perform a significant role in the biodegradation of toxic nitroaromatic compounds. Maleylacetate reductase (MR) is one of the essential enzymes in aerobic bacteria for the degradation of PNP and other chloroaromatic compounds (Vikram et al. 2013). This enzyme catalyzed NADPH or NADP-dependent reduction, converting the carbon-carbon double bond (maleylacetate) to 3-oxoadipate (Vikram et al. 2013). Currently, for the vital enzyme MR, no protein model is available at PDB.

Considering the significance and application of MR, particularly its importance in the biodegradation of toxic compounds like PNP, the current study was undertaken for its computational analysis. In these contexts, we carry out in-silico bioinformatic analyses of MR (selected by genome mining) from the genome sequence of a bacterium *Pseudomonas* sp strain PNPG3. Attempts were also undertaken to predict the secondary structure, modelling the tertiary structure, and analyses of MR of the strain using various standard tools to intimate the scientific community about the structure-function details of this particular vital protein.

2 Materials and Methods

2.1 Sequence retrieval and phylogenetic analysis

The sequence of MR was retrieved from NCBI manually in FASTA format [(locus tag - MJ643_19190; protein id-MCK2122717.1; GenBank accession no of node 31 - JALLKV010000031; Whole Genome accession no- JALLKV010000000) or RAST accession no. - fig|286.4486.peg.3636]. This genome sequence was retrieved from a PNP degrading aquatic bacterium *Pseudomonas* sp strain PNPG3, isolated from the Ganges river, Chinsura, Hooghly district, West Bengal, India (Biosample Id SAMN26116625). This sequence was used later for computational investigation, including molecular modeling.

NCBI-BLAST_P search was performed to find similar protein sequences. FASTA formatted sequences were retrieved and the phylogenetic tree was constructed using MEGA11 (Tamura et al. 2021) (based on the Maximum Likelihood tree method). The evolutionary distance was computed by the pairwise distance method. The enzyme P-nitrophenol-4-monooxygenase (6AIN_A) of *P. putida* strain DLL-E4 was used as an outgroup.

2.2 Protein localization

PSORTb forecasted localization of the protein– 3.0 (Yu et al. 2010) and CELLO (Yu et al. 2004) (http://cello.life.nctu.edu.tw), CELLO2GO (http://cello.life.nctu.edu.tw/cello2go/) (Yu et al. 2014). PSORTb provides the localization of bacterial protein most accurately. CELLO is user-friendly and simple, and no other algorithm is required for this tool. This tool provides better performance than the PSORT-B tool (Yu et al. 2004). The signal peptide sequence was determined using Signal P - 6.0 Server (Teufel et al. 2022). Information about the transmembrane protein was determined using TMHMM v 2.0 (Krogh et al. 2001).

2.3 Primary sequence analysis

Primary sequence analysis, including the physicochemical characteristics of the protein of interest, was predicted by the Expasy ProtParam Tool (Gasteiger et al. 2005). A variety of parameters like the composition of amino acid, isoelectric point (pI), molecular weight (MW), aliphatic index (AI) (aliphatic side chains occupying a relative volume of protein), and Grand average of hydropathicity (GRAVY) data of the protein were obtained from this server.

2.4 Secondary structure prediction

Prediction of the secondary structure of the protein was done by Netsurf-P (Klausen et al. 2019). Here solvent accessibility, the number of helices, strands, coils, and structure disorder can be determined. Another tool was used for this purpose- Chou and Fasman Secondary Structure Prediction Server (CFSSP) (Kumar 2013). Secondary structural elements, like the beta sheet, alpha helix, and turns from the amino acid sequence could be determined in a lined sequential view by this server (Kumar 2013).

2.5 Structural modelling and evaluation

The same protein of strain PNPG3 was nominated to forecast the tertiary structure. The I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Yang et al. 2015) was used for modeling the tertiary structure of MR of strain PNPG3. The modules like ERRAT, PROCHECK, and Verify 3D under the SAVES Server (http://services.mbi.ucla.edu/SAVES/) were used to evaluate the quality of the prepared structural model of the protein. The .pdb file obtained from I-TASSER was used to obtain the Ramachandran plot from the Structural Analysis and Verification Server (SAVES) (http://services.mbi.ucla.edu/SAVES/ Ramachandran/). Ramachandran plot helps to invent the energetically favourable regions which were connected to backbone dihedral angles (ψ and φ) of amino acid residues of the interested protein. The tertiary structure of the predicted model was visualized by UCSF chimera (http://www.cgl.ucsf.edu/chimera) (Pettersen et al. 2004).

2.6 Prediction of ligand binding sites and active sites

After validation of the tertiary structure of MR, protein-ligand binding interactions were analyzed using the COFACTOR module (https://zhanglab.ccmb.med.umich.edu/COFACTOR/) (Roy et al. 2012) integrated into the I-TASSER server for protein structure prediction and structure-based function annotation (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Yang et al. 2015). Important amino acids which were involved in ligand binding sites and active sites were also predicted.

2.7 Functional analysis

The STRING server (Szklarczyk et al. 2019) (http://string.db.org) was used to predict functionally interacting proteins. MR of *P. fluorescens* F113 was selected as the query sequence for this interactome analysis. Functional motifs of the protein sequence were identified by the tool known as MOTIF search (http://www.genome.jp/tools/motif/). Highly conserved regions among the MR were investigated by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

3 Results and Discussion

3.1 Sequence retrieval and Phylogenetic analysis

Homologous amino acid sequences of MR from other strains of *Pseudomonas* were retrieved from NCBI. To compare evolutionary relationships among the MR of strain PNPG3 with the close relatives, a phylogenetic tree (based on the maximum likelihood method) was constructed using MEGA11. From this analysis, it was evident that the MR of strain PNPG3 was placed within similar sequences from various strains representing the genus *Pseudomonas*. It also formed a cluster together with *Pseudomonas* sp. strain PDS-7 (AWB99095.1). This cluster was closely related to MR of strain NyZ402 (ACZ51383.1) and the latter formed a well-defined lineage. Also, the remaining MR sequences from the representative 8 strains were well separated (Figure 1), indicating a robust probable correlation among these taxa based on their protein sequences.

3.2 Localization of Protein

Both PSORTb and CELLO data revealed that MR is a cytoplasmic protein. The cytoplasmic score for PSORTb and CELLO is 8.46 and 3.362, respectively. CELLO2GO tool also supported the result of the cytoplasmic nature of this protein (Figure 2). According to Grasso et al. (2021), the subcellular localization of protein determines the environment where it operates, and it could be strongly correlated with the determination of said protein function. The function of a protein could be correlated to the localization of

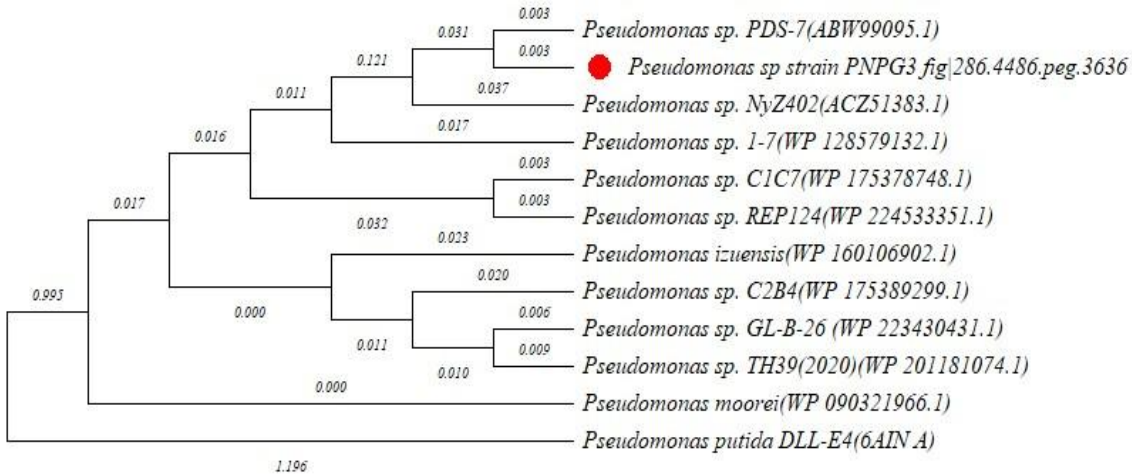


Figure 1 Phylogenetic analysis (using maximum likelihood method) of amino acid sequences of maleylacetate reductase (MR) of different strains of *Pseudomonas* sp. Values at the internodes indicate branch lengths

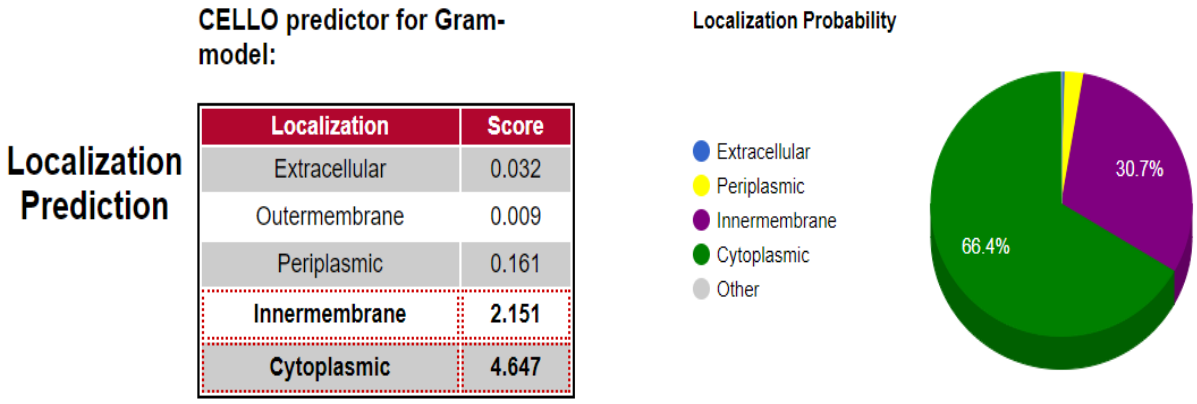


Figure 2 Localization prediction for the protein MR by CELLO2GO

the protein. SignalP - 6.0 server indicated that this protein has no signal peptide sequence. TMHMM-2.0 server indicated that no transmembrane helices are present within this protein. These results predicted that the MR protein is functionally active in the cytoplasmic region of the cell.

3.3 Primary sequence analysis of protein

Physicochemical characterization plays an important role in knowing the protein's nature (Pramanik et al. 2017). The Expasy ProtParam tool indicated the proportion of alanine (15.5%) to be higher than the other amino acids, indicating the stable nature of the protein (Figure 3). The protein consisted of 355 amino acid residues having a molecular weight of ~37.6 kDa. Molecular weight is one of the crucial criteria for the functional characterization of proteins (Prabhu et al. 2020). Theoretical pI is 5.81, indicating the acidic nature of this protein. For purification and isoelectric focusing in the development of buffer, pI prediction plays an important role (Prabhu et al. 2020). The aliphatic index of the protein was very high (95.83), indicating the protein is thermostable, indicating this protein can be used for industrial or research applications. The general concept is that less thermostable enzymes generally have limited applications in industry, that's why researchers are actively engaged in searching for highly thermostable proteins for industrial applications (Delgove et al. 2018). The grand average of hydropathicity (GRAVY) was low (0.063), indicating this protein has better interaction with water. Similar types of results were reported in the case of *Rhodococcus rhodochrous* while working with alkane-1-monooxygenase (Pal and Sengupta 2022).

3.4 Secondary structure prediction

Netsurf-P server prediction revealed that there are 14 helical structure and 9 strands within this protein (Figure 4). Prediction of secondary structure by CFSSP showed that the protein has alpha-helix 73.2%, sheets 33.5%, and turns 12.7%. From this secondary

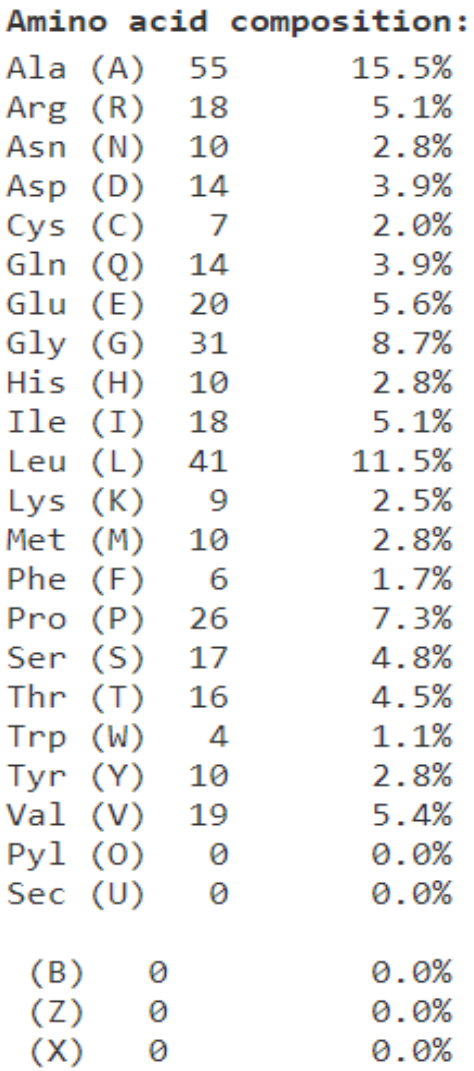


Figure 3 Amino acid composition of the sequence of MR determined by Expasy protparam tool

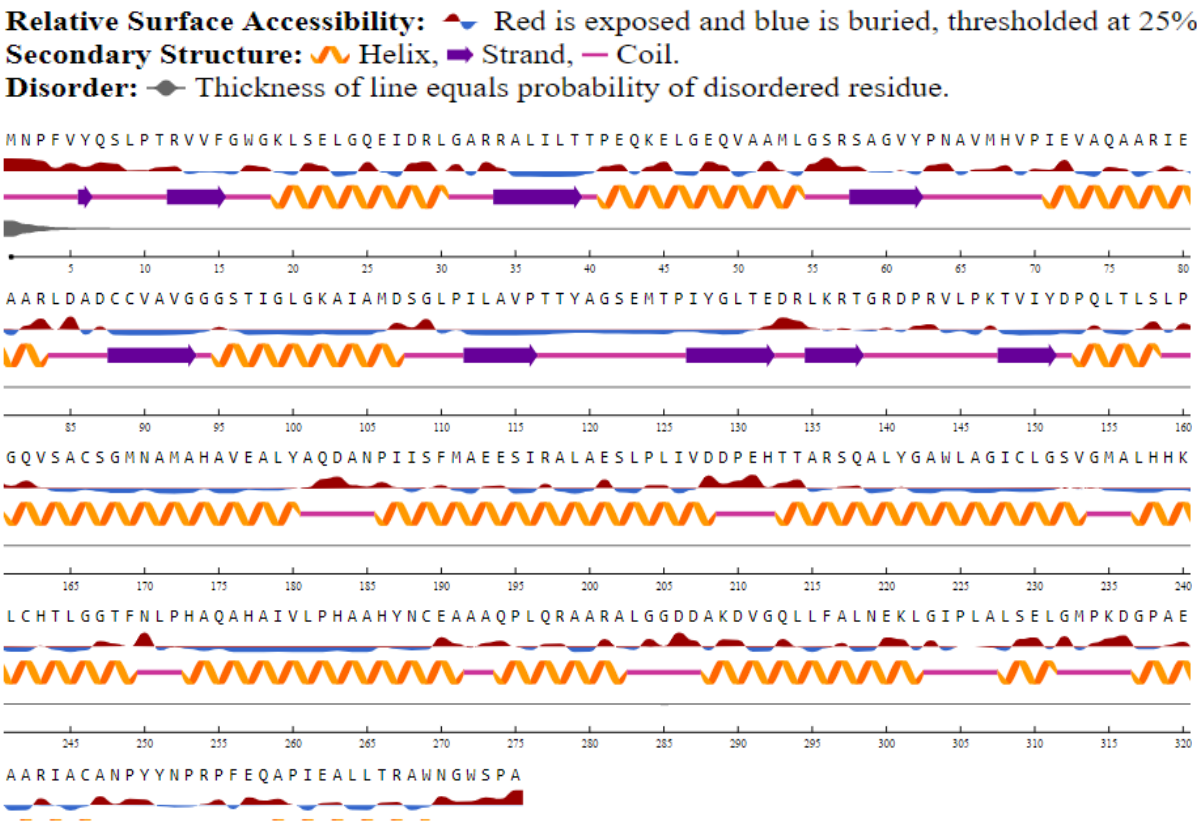


Figure 4 Secondary structure of the enzyme was determined by Netsurf-P

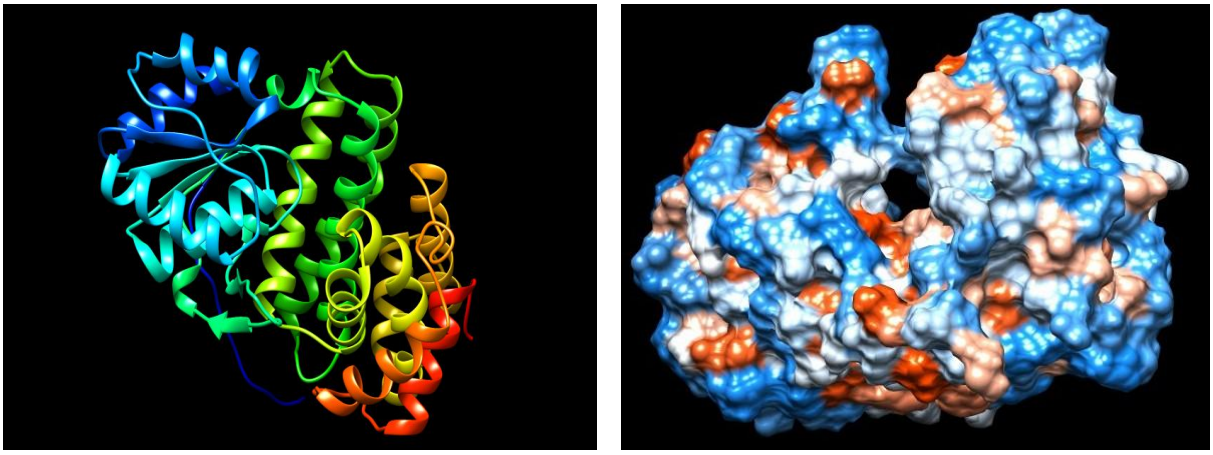


Figure 5a Predicted ribbon structure of MR visualized by UCSF chimera; Figure 5b Predicted hydrophobicity surface view of MR, showing major and minor grooves

structure prediction, it was evident that the proportion of alpha helix was much higher than the other type of protein conformations (turn and sheet). Protein-protein interactions are often mediated by an alpha helix. The higher content of alpha-helices indicated that the protein is thermostable because such secondary structures are participated to resist the high temperature in thermophilic organisms (Kumar et al. 2000; Yakimov et al. 2016).

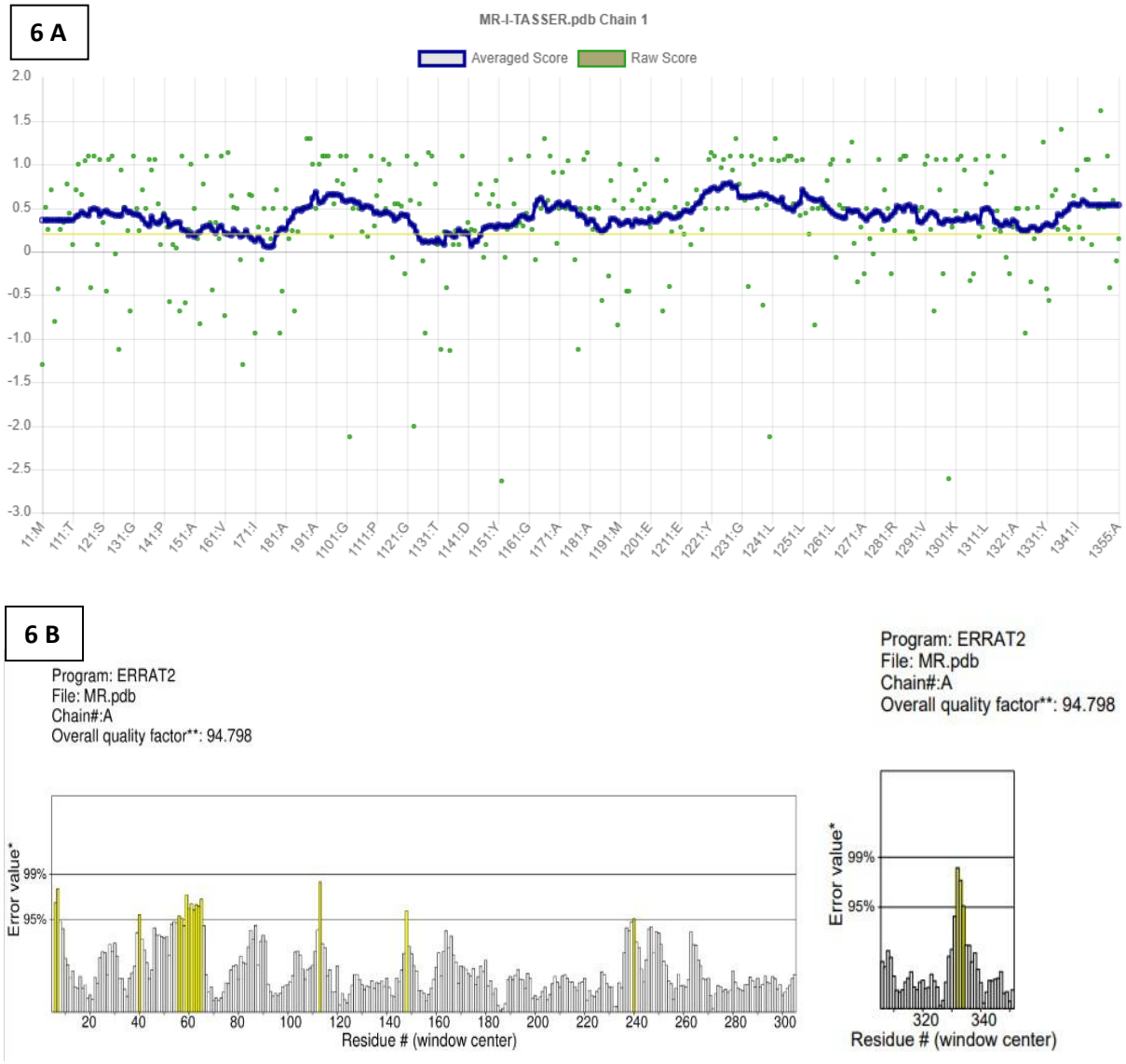
3.5 Structure analysis and model quality assessment

The I-TASSER server predicted the high-quality tertiary structure of a protein. The server generates only one model for which the C-scores is 0.81. The positive C-score of MR protein secures high confidence for the generated model. The Estimated TM-score of 0.82±0.08 and estimated RMSD of 4.9±3.2Å show a positive

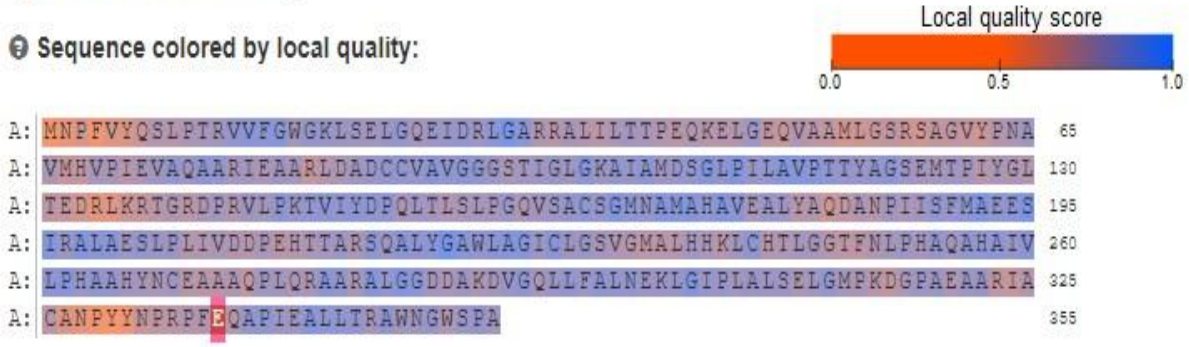
correlation with its native protein structures. The chosen template proteins like 3W5S, 6SCI, 3JZD, 1VLJ, 5BR4, 7FJG, and 6JKO by the I-TASSER server are different by origin but functionally similar i.e., oxidoreductase. From these parametric values of TM-align, the structural model of MR protein is certainly similar to the following proteins like 6SCI, 3JZD, 3BFJ, 3OWO, 4FR2, 7DAG,3ZDR,3HL0, and 2BI4 that have Oxidoreductase function. Thus, based on structural similarity with other proteins having oxidoreductase function, it can be predicted that in biological system MR protein may also have oxidoreductase properties. The helix, sheet, and turns of the predicted model was visualized by UCSF chimera, shown in Figure 5a, whereas the hydrophobicity surface was visualized in Figure 5b. Blue patches were indicated by hydrophilic area and red patches were hydrophobic in nature.

The confidence score (C-score) of the predicted model was 0.81. C-score generally ranges from [-5,2], where the higher values indicated the model was high confidence and vice-versa. The estimated TM and RMSD was reported 0.81±0.8 and 4.9±3.2Å respectively. All these values indicated that the model was highly significant. The model was selected for further analysis.

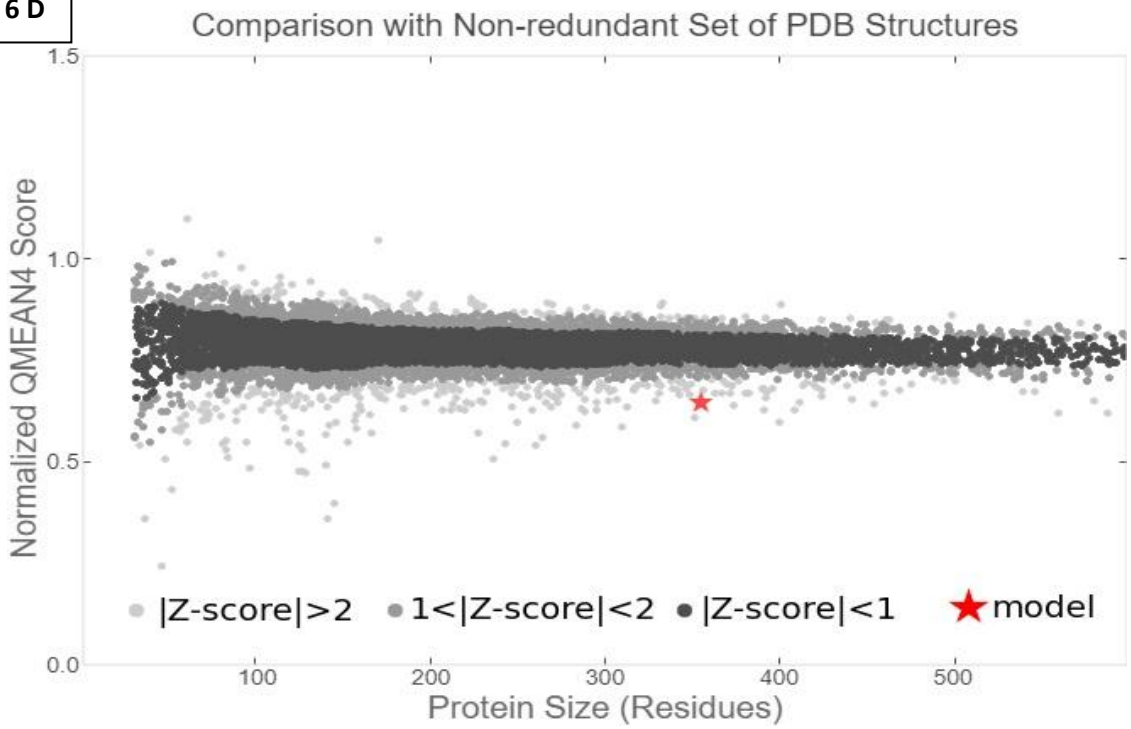
The quality of the build 3D model (.pdb) was assessed by PROCHECK, Verify 3D, ERRAT, and QMEAN Programmes. The average 3D-1D score of the protein model showed that 91.83% of residues have ≥ 0.2 scores in Verify 3D server; whereas the threshold value to attain the best fit and acceptable model is 80% (Laskowski et al. 1993) (Figure 6a). So, it could be concluded that the model was accepted and passed through Verify 3D server. The ERRAT value for this protein model was 94.7977 for both chains



6C



6 D



(Figure 6b). A higher score of ERRAT value indicates the good quality of this model. The accepted threshold value for a high-quality model is >50 (Pal et al. 2021). QMEAN is a scoring function that calculates the global as well as local per residue quality analysis of the protein of interest, based on one single model (Benkert et al. 2011). The QMEAN score close to zero denotes the good quality of the model. QMEAN4 score for this predicted protein of MR was -3.64 (Figure 6c). The QMEAN score of less than -4 is an indication of a low-quality model (Pal et al. 2021). The Z score of the query protein sequence (MR of strain PNPG3) was within an acceptable range, i.e., 1< [Z score] <2 in comparison with a non-redundant set of PDB structures (Figure 6d).

The Ramachandran plot was constructed to find the locations of amino acid residues of the protein of interest. According to the PROCHECK result, Ramachandran plot data (Figure 6e, Table 1) showed 87.2% residues were in the maximum favored regions; whereas 10.8% and 2% residues existed in the additional allowed regions and the generously allowed regions respectively. All these values were somewhat nearer to the expected value and statistically significant. Generally, a good quality model usually shows more than 90% residues in the favored regions (Berman et al. 2000; Yadav et al. 2013). A similar type of model validation was reported by Pramanik et al. (2018) and Pal et al. (2021).

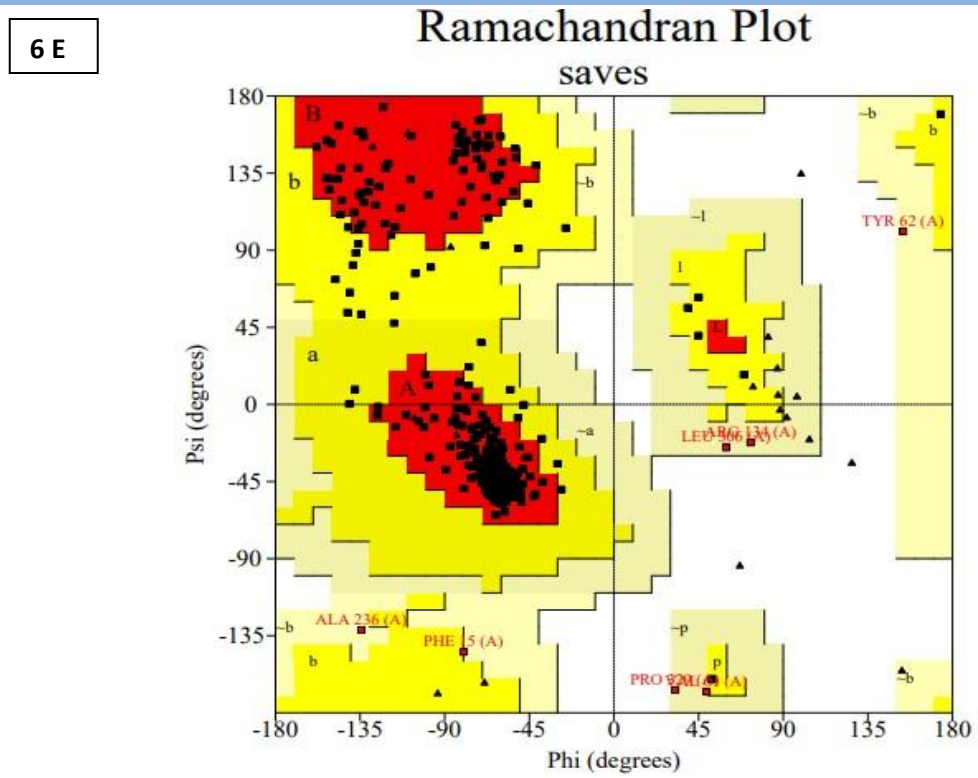


Figure 6(a-e) Evaluation of protein model of MR of *Pseudomonas* sp strain PNPG3; Figure 6a Average 3D-1D score of the protein model determined by SAVES server; Figure 6b The ERRAT value for this protein model determined by SAVES server; Figure 6c QMEAN4 score for this predicted protein of MR; Figure 6d The Z score of the predicted protein of MR; Figure 6e Ramachandran plot of the predicted protein model of MR

Table 1 Statistics of Ramachandran plot

Statistics	Number of amino acid residues	Percentage
Residues in the most favoured regions [A, B, L]	258	87.2%
Residues in the additional allowed regions [a, b, l, p]	32	10.8%
Residues in the generously allowed regions [~a, ~b, ~l, ~p]	6	2%
Residues in disallowed regions	0	0%
Number of nonglycine and nonproline residues	296	
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	31	
Number of proline residues	26	
Total number of residues	355	

3.6 Ligand binding sites and active sites determination of the protein

Ligand binding sites of a protein provide significant information related to their functional studies. The I-TASSER server indicated that NAD, Zn, and ADP were the ligands for this protein molecule; whereas COFACTOR indicated NAD, and Cobalt (Co) were the ligand. Threonine, Glutamine, Methionine, Histidine, Glycine, Glycine, Serine, Threonine, Threonine, Alanine, Serine, Threonine, Isoleucine, Lysine, Leucine, Serine, Leucine, Proline, Valine, Serine, Asparagine, Histidine were identified as ligand binding residues (determined by I-TASSER) at 40,43, 67, 68, 94, 95, 96, 117, 118, 120, 122, 125, 127, 136, 155, 158, 159, 160,163,167, 170, 253 positions respectively for NAD (Figure 7a). Similarly, Asparagine, Histidine, Histidine, and Histidine have been identified as ligand binding residues at 170, 174, 239, and

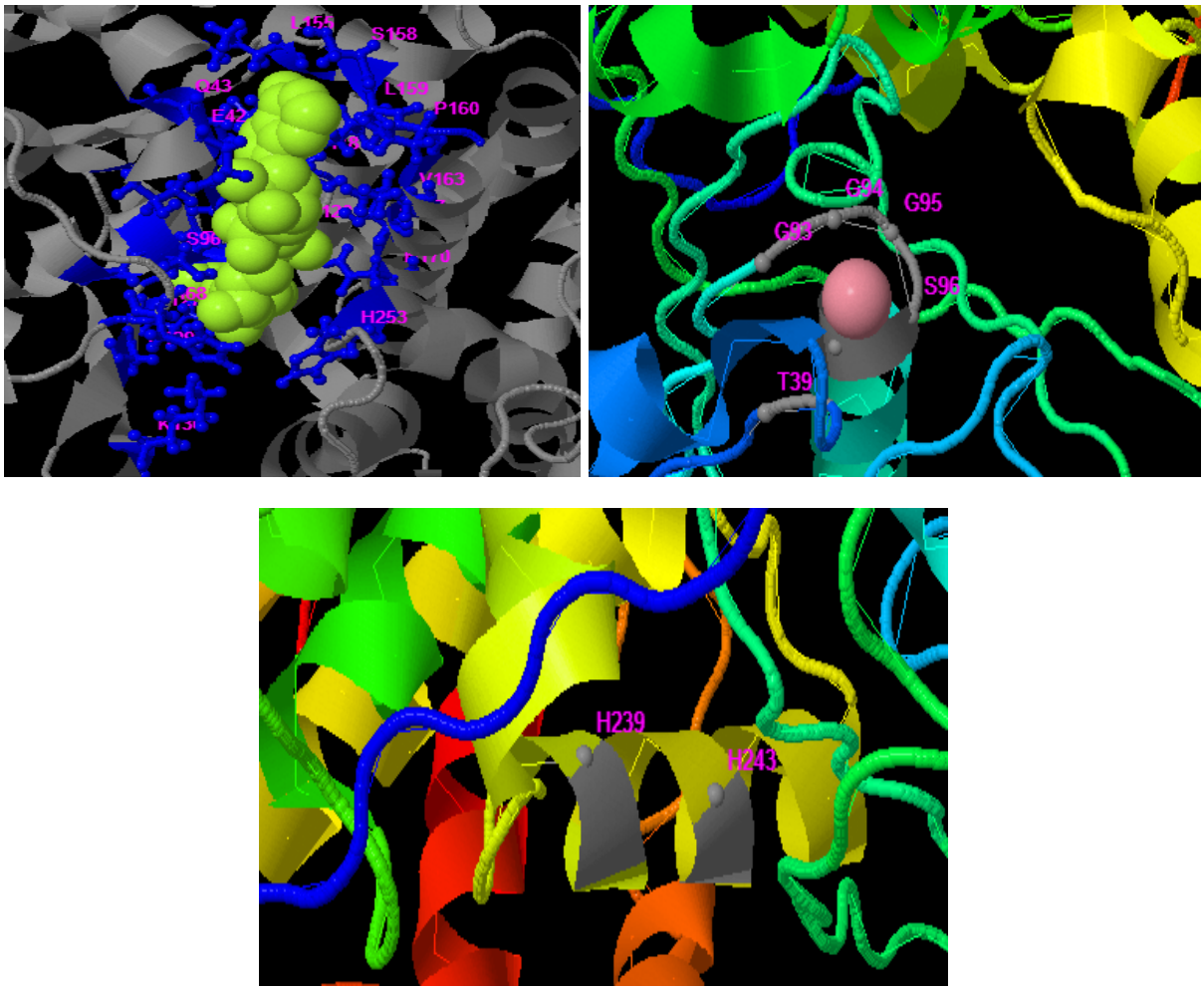
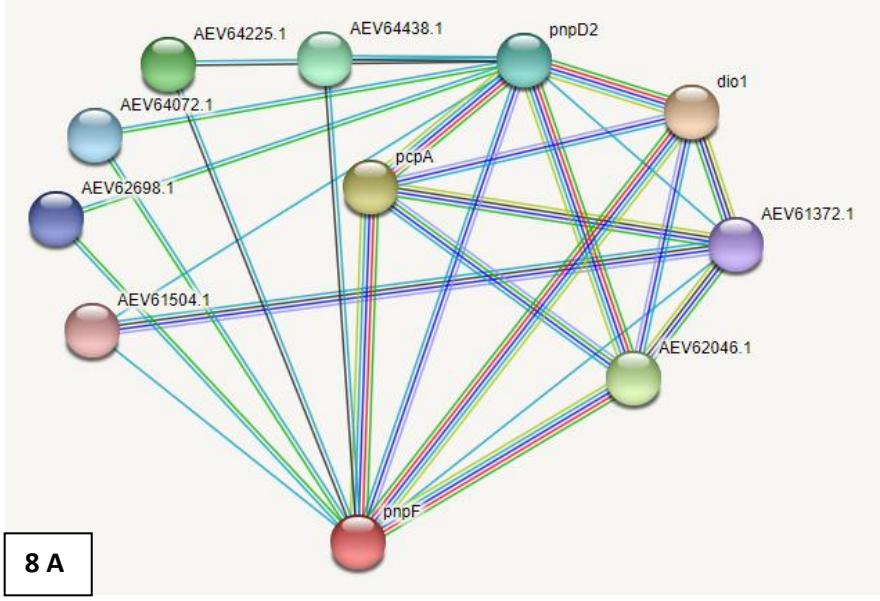


Figure 7(a-c) Ligand binding side of the protein MR; Figure 7a Ligand binding sites of NAD at the protein MR; Figure 7b Ligand binding sites of Zn at the protein MR; Figure 7c found amino acid Histidine[239] and Histidine[243]

253 respectively for Zn (Figure 7b). Threonine, Glycine, Glycine, Glycine, Serine, and Threonine were identified as ligand binding residues (determined by COFACTOR) at 39, 93, 94, 95, 96, and 97 respectively for Co. Active sites of the predicted protein were determined by the COFACTOR server. According to this server active sites of this model were found to be Histidine[239] and Histidine[243] (Figure 7c).

3.7 Functional analysis

Ten potential interacting partners of pnpF encoding protein MR were revealed by STRING analysis. The pnpF protein was MR, metal ion binding, cation transmembrane transported activity, dio1 was the closest protein with the shortest node, having catechol-1,2-dioxygenase activity, while the distantly interacting protein was recorded to be AEV61504.1 and AEV61372.1 both having 3-oxoadipate enol-lactonase activity (Figure 8a, 8b). Two functional motifs were detected by the motif finder tool (Figure 9). Both motifs were found to the member of iron-containing alcohol dehydrogenase. Pramanik et al. (2017) reported that the alkaline phosphatase of *P. aeruginosa* PA01 contains two motifs. The multiple sequence alignment among the eleven MR protein sequences revealed several stretches of conserved amino acid sequences (designated as*). Such conserve sequences are – YQSLPTRVVFGWGKLS-RLGARRALILTTPEQ-LGEQVAA-AVMHVP-EVAQAAR-EAARL-ADCC-GGGSTIGLGKAIAMDSGLPI-PTTYAAQDANPII-FMAEESIR-QALYGAW-AGICLGS-GMALHHK-CHTLGGTFNLPHAQAHIVIPHAAHYN-EAAA-RAARALGG-KLGIPLAL-GPAEAA-IACA-PYYNPRPFEQ-PIEALL (Figure 10). Pramanik et al. (2018) while working on myo-inositol hexakisphosphate phosphohydrolase of *Enterobacter*, revealed that DG-DP-LG was a conserved sequence of that enzyme. *Aspergillus niger* 3-phytase A and 3-phytase-B have conserved sequences like RHGXRXP-HD as explored by a multiple sequence alignment study by Niño-Gómez et al. (2017).



8 B

Your Input:

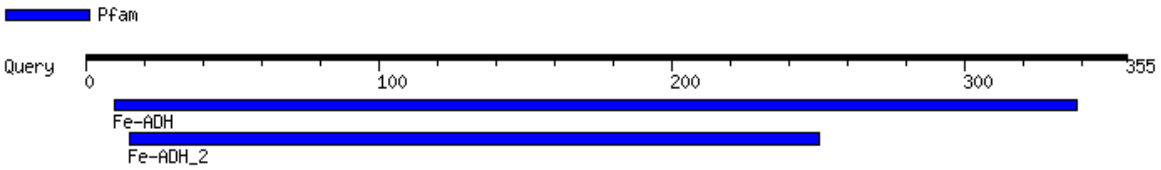
		Neighborhood	Gene Fusion	Cooccurence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
pnpF	PnpF; Maleylacetate reductase; Protein involved in maleylacetate reductase activity, metal ion binding, cation transmembrane transporter activity, oxidation reduction, cation transport and transmembrane transport (355 aa)									

Predicted Functional Partners:

dio1	Dio1; Catechol 1,2-dioxygenase 1; Protein involved in catechol 1,2-dioxygenase activity, ferric iron binding, hydroxyquinol 1,...	●	●	●			●	●		0.990
pcpA	PcpA; Catechol 1,2-dioxygenase; Protein involved in oxidoreductase activity, acting on single donors with incorporation of ...	●	●	●			●	●		0.977
AEV62046.1	Hydroxyquinol 1,2-dioxygenase; Protein involved in catechol 1,2-dioxygenase activity, ferric iron binding, oxidation reductio...	●	●	●			●	●		0.975
AEV64225.1	Dienelactone hydrolase family protein; Protein involved in carboxymethylenebutenolidase activity, phenylpropanoid catabo...				●		●			0.903
AEV64438.1	Dienelactone hydrolase family; Protein involved in carboxymethylenebutenolidase activity, aromatic compound catabolic p...				●		●			0.903
pnpD2	PnpD; Iron-containing alcohol dehydrogenase; Protein involved in iron ion binding, maleylacetate reductase activity, fatty a...			●			●		●	0.902
AEV64072.1	annotation not available	●					●			0.902
AEV62698.1	annotation not available	●					●			0.901
AEV61372.1	Beta-ketoadipate enol-lactone hydrolase; Protein involved in 3-oxoadipate enol-lactonase activity, beta-ketoadipate pathwa...						●			0.900
AEV61504.1	3-oxoadipate enol-lactonase; Protein involved in transcription factor activity, 3-oxoadipate enol-lactonase activity, beta-ket...						●			0.900

Figures 8a and 8b Result of STRING based interactome study of maleylacetate reductase

Number of found motifs: 2



Pfam (2 motifs)

Pfam	Position(Independent E-value)		Description
Fe-ADH	10..338(1.4e-81)	Detail	PF00465, Iron-containing alcohol dehydrogenase
Fe-ADH_2	15..250(1.1e-16)	Detail	PF13685, Iron-containing alcohol dehydrogenase

Figure 9 The motif of maleylacetate reductase determined by themotif finder

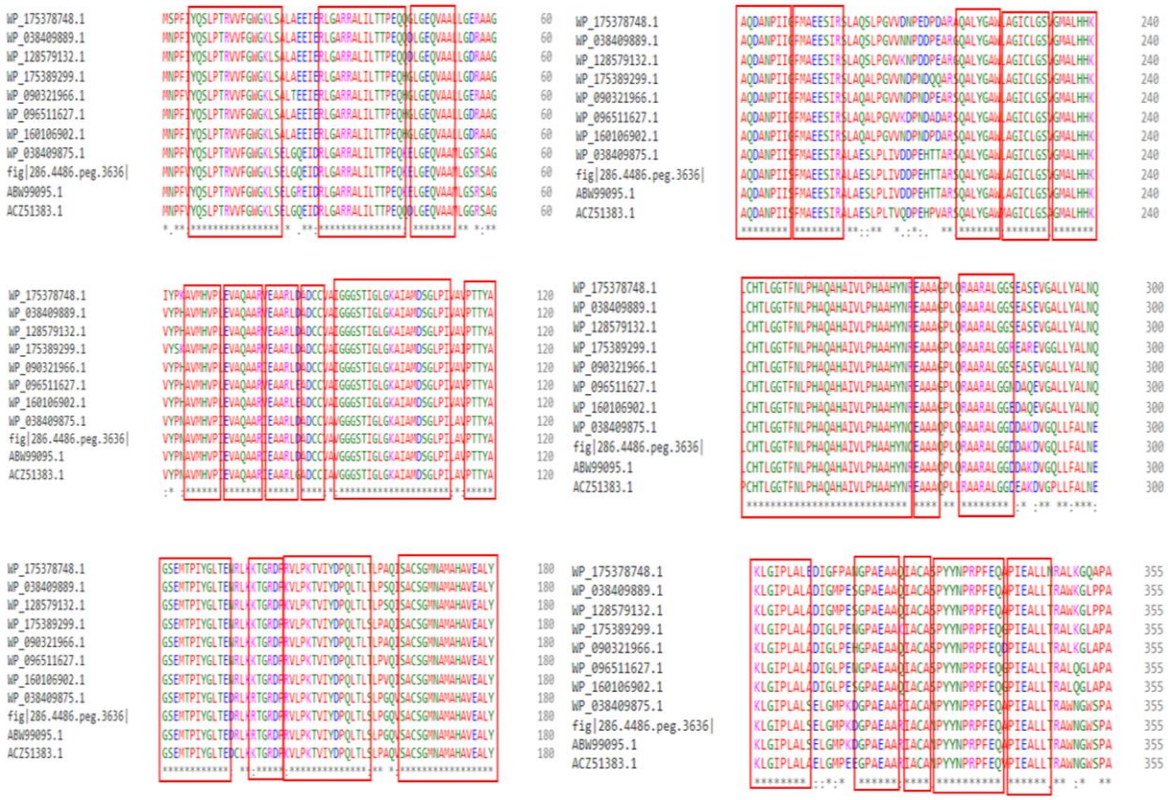


Figure 10 Multiple sequence alignment of 11 MR sequences of *Pseudomonas* spp. showing highly conserved amino acid residues. An * (asterisk) meansfully conserved amino acids, a: (colon) suggests strongly similar type of amino acids and a. (period) conveysweakly similar typesof amino acids. Hence, the hierarchy of conservation is (*) > (:) > (.). The highlighted area in the sequence indicates thehighly conserved region of the protein

Conclusion

Maleylacetate reductase, one of the major lower pathway enzymes found in different aerobic microbes, plays a vital role in the biodegradation and bioremediation of toxic xenobiotic compounds like PNP. However, no model for this protein is currently available in PDB. Thus, theoretical knowledge of this enzyme's structural, functional, and phylogenetic properties are significant to get further insight into biodegradation study by microorganisms. Computational-based analysis of protein structure has nowadays become a very beneficial tool for correlating the structure-function features of the protein as sometimes the crystal structure of the protein is not obtained readily. Here, attempts were made to determine and predict the structural-functional correlation of maleylacetate reductase of *Pseudomonas* sp strain PNPG3. The protein is thermostable and acidic, having a molecular weight of 37.6KDa. The number of alpha helices of this protein is much higher than the other types of secondary structures. Ligand binding sites and active sites of the enzyme was predicted. A model of this protein was generated with a good quality score. Both motifs were shown to be a member of iron-containing alcohol dehydrogenase. Highly conserved regions of the protein were determined by multiple sequence alignment. This work might be helpful to the scientific world of bioinformatics research. Researchers could get information about the protein's physicochemical properties, protein-protein interaction, structure, and conserve domain, and structural motifs essential for detoxification and cost-effective bioremediation in the aquatic ecosystem.

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Ethical Approval

The manuscript does not involve any work or studies with human and or animal participants performed by any of the authors.

Conflict Declarations

The authors declare that they do not have any conflict of interest connected to the manuscript.

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Removal of Lead from Aqueous Solution by *Fusarium oxysporum*: Equilibrium and Phytotoxicity Studies

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KEYWORDS

Adsorbent

Lead

Fusarium oxysporum

Phytotoxicity

ABSTRACT

Lead is a toxic metal of public health concern. The applicability of *Fusarium oxysporum* biomass as a biosorbent for the removal of lead ions from wastewater is assessed in the present investigation. Batch experiments were conducted under different experimental conditions for analysis of the lead biosorption capacity of live and dead biomass of *Fusarium oxysporum.* Lead ions were significantly absorbed at pH 5 with a 2g adsorbent amount at 30^0C. Equilibrium results were analyzed by Langmuir and Freundlich isotherms and found that Langmuir isotherm is the best fit under this condition. A phytotoxicity study revealed that the growth parameters of wheat seeds were significantly increased in the lead solution treated with dead biomass as compared to the live biomass of *F. oxysporum*. Further, dead *F. oxysporum* significantly removed lead within 3 hours whereas live fungal biomass took two days for the complete removal of lead. Therefore, the results of the study suggested that live and dead biomass of *F. oxysporum* can be used as an effective, safe, and economically feasible sorbent for the removal of lead present in industrial effluent or wastewater systems.

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1 Introduction

Increased urbanization, anthropogenic activities, industrialization, and careless management practices have shown adverse effects on the environment by dumping wastes containing heavy metals. Heavy metal pollution is one of the most serious environmental problems due to the long-term persistence, toxicity, and non-degradable nature of metals which impose adverse effects on human health after entering into the food chain (Trikkaliotis et al. 2022). Heavy metals belong to a group of elements characterized by high atomic weight and density of more than 5 g/cm^3 (Ali and Khan 2018). They have been categorized into a group of pollutants known as emerging contaminants (Lodeiro et al. 2019). Heavy metals are toxic, non-biodegradable with a long half-life and easily react with organic substances and form toxic metal-organic complexes and trigger long-term implications on human beings, animals, and environmental health (Hong et al. 2020). Heavy metals cannot be easily eliminated after entering the human body and tend to accumulate which may cause changes in biochemical processes and show chronic effects (Abdus-Salam and Adekola 2018; Hong et al. 2020). Among heavy metals, lead (PbII) is one of the most hazardous pollutants in the environment and it is a serious ecological concern due to its impact on human health, animals, and plants (Wang et al. 2022). Lead is used in > 900 industries such as mining, metal plating, and finishing, battery manufacturing, smelting, fertilizers, pesticides, photographic materials, explosive, ceramic, and glass industries (Shao et al. 2020; Wang et al. 2021). As per the Institute for Health Metrics and Evaluation, the University of Washington, more than nine lakh death and twenty-one million disability cases have been reported worldwide due to lead exposure (Irawati et al. 2022).

As per guidelines of the Environmental Protection Agency (EPA) and World Health Organization (WHO), the permissible limit of lead in potable water is 15 and 50 µg/L, respectively (Mahmud et al. 2016). Lead is extremely toxic even at very low concentrations and causes anemia, hypertension, and headache (Alghamdi et al. 2019) and damage to the kidney, liver, nervous system, and reproductive and gastrointestinal system of human beings (Bouabidi et al. 2018; Kumara et al. 2019).

Different procedures like ion exchange, membrane separation, reverse osmosis, chemical reduction, and electrochemical treatment have been used for heavy metals elimination from industrial effluent. These methods are costly, not effective at large scale, and require expertise and sophisticated equipment. Therefore, obtaining material with desirable sorption properties is still a major challenge for researchers.

Biosorption is a physicochemical process that utilizes biomass for the removal of heavy metals from contaminated medium (Chauhan et al. 2020a). The surface of fungal biomass has high electronegativity which can attract and bind metal ions (Chauhan et al. 2020b). Dead microbial biomass can also be used for the recovery of metal ions as it binds metal ions more efficiently (Kapoor 2022). In comparison to conventional methods, biosorption has many edges like high efficiency, low cost, simple process, stability, high surface area, recovery of metals, and no sludge formation (Sarma et al. 2020).

The study of pertinent literature revealed that the effect of live and dead biomass of *Fusarium oxysporum* has not been studied for the removal of lead and the impact of the treated solution on the growth of wheat seeds. The present study was executed to determine the potential of live and dead biomass of *F. oxysporum* for lead removal from aqueous solution and its effect on the growth of wheat plants. Hence, the development of biosorbent from *F. oxysporum* can be a sustainable approach for lead removal from aqueous solution and environmental restoration.

2 Materials and Methods

Experiments were conducted at Amity Institute of Biotechnology, Amity University Uttar Pradesh, Noida.

2.1 Fungal biomass

F. oxysporum was isolated from soil samples and culture was maintained on Potato dextrose agar medium at 4^{0}C and sub-cultured within fifteen days.

2.2 Growth conditions

One disk of fungal biomass (4 mm diameter) was inoculated into Sabouraud Dextrose medium [Dextrose 40g, Peptone 10g, Distilled water 1 liter] at pH 5.8. Flasks were incubated for a week at 25±2^{0}C. After that, fungal biomass was separated by filtration, washed with deionized water, and dried properly with filter papers for wet biosorbent (Saad et al. 2016).

2.3 Dead fungal biomass preparation

Dead biomass was prepared by boiling *F. oxysporum* with 0.5N sodium hydroxide solution for fifteen minutes and then thoroughly rinsed with deionized water till eluent pH reached neutral. After proper washing, the biomass was dehydrated at 5^{0}C for 24 h and powdered. Dead fungal biomass was stored in a desiccator and used in experiments. Around four grams of live biomass of *F. oxysporum* was equivalent to 0.38 g of dead biomass.

2.4 Batch test for lead removal by *Fusarium oxysporum*

Lead solution (1000 ppm) was prepared by mixing lead nitrate in deionized water and desired concentrations were prepared. *F.*

oxysporum live or dead biomass was added to hundred milliliters of metal solution and lead solution without live or dead *F. oxysporum* was also incubated in the same manner called control. For maximum adsorption of lead, different experimental conditions such as time (1-96 hours), pH (1-6), initial lead concentration (20-140 mg/l), and temperature (10-50^{0}C) were used. Different amount of fungal biomass (0.5-3.5g live and dead biomass) was used for the absorption of lead ions and flasks were kept in a shaking incubator at 150 rpm as per the treatment. After filtration, fungal biomass was removed and the supernatant was analyzed for identification of residual metal (Saad 2015).

2.5 Lead biosorption capacity

The amount of lead ions absorbed at equilibrium q_e (mg/g) shows the metal uptake and it was assessed by the following equation:

$$q_e = (C_i\text{-}C_e)\ V / m$$

V = lead solution volume, C_i and C_e= initial and final lead metal concentrations, m= biosorbent mass.

2.6 Equilibrium Studies

Two isotherm models were applied for the analysis of sorption equilibrium. Different concentrations of lead metal solution were treated with different adsorbent doses of fungal biomasses.

2.6.1 Langmuir isotherm

Langmuir isotherm model predicts the monolayer mode of the adsorption procedure. It also explains that adsorption energy is consistent throughout adsorbed layer on the adsorbent surface at a constant temperature (Bharathi and Ramesh 2013). Langmuir equation can be denoted as:

$$C_e/q_e = 1/q_e\ K_1 + C_e/\ q_m$$

q_e (mg/g) = lead amount adsorbed at equilibrium, q_m (mg/g)= lead amount adsorbed, C_e (mg/l)= lead concentration at equilibrium, K_1 = Langmuir constant associated with a binding capacity of lead on fungal surface.

2.6.2 Freundlich isotherm

Freundlich isotherm reflects solute molecules distribution between aqueous and solid phases at equilibrium (Ng et al. 2002). The Freundlich equation can be reflected:

$$\text{Log } q_e = \log K_f + 1/n \log C_e$$

Freundlich constants like K_f = adsorption capacity, n = adsorption intensity, respectively. n shows nature of process as linear phenomenon (n=1), chemical phenomenon (n < 1), physical process(n > 1).

2.7 Phytotoxicity assessment

The impact of the lead solution was analyzed on the growth of wheat (*Triticum aestivum* var. UP2554) seeds before and after treatment with *F. oxysporum* live and dead biomasses.

2.7.1 Seed germination bioassay test

Fresh and healthy seeds of wheat (*Triticum aestivum* var. UP2554) were procured from the Seed agency of Noida. Seeds of wheat were properly rinsed with tap water to eliminate dirt for five minutes and sterilized with $HgCl_2$ (0.1% w/v) for five minutes to inhibit the activities of microbes and rinsed with deionized water five to six times. Wheat seeds were soaked in lead (Pb II) solution before and after the treatment with live and dead biomass of *F. oxysporum for* up to 4 hours respectively. Wheat seeds were transferred into Petri dishes and irrigated with distilled water or lead solution treated with live or dead *F. oxysporum* biomass as per the treatment. Petri dishes were kept in a seed germinator for 10 days under 75% relative humidity at 26±2^{0}C with 12 hours of photoperiod as per ISTA (2008).

Germination (%): Total number of wheat seeds germinated / wheat seeds taken for germination x 100

Length of the seedling and vigor index was determined in control and treatment after ten days of seedling growth by the following methods:

Seedling length: Length of the radicle and plumule were measured with a measuring scale and denoted in centimeters.

Vigour index: It was calculated by the following formula: Vigour index = Length (radicle and plumule) (mm) x germination (%).

2.8 Statistical analysis

Treatments were arranged as randomized block designs with three replications. Results were assessed by ANOVA in SPSS software. The standard error of the mean was calculated for presentation with tables and the treatment mean was analyzed by using DMRT at $P < 0.05$.

3 Results and Discussion

The optimum conditions for lead elimination were assessed by comparative analysis of live and dead biomass of *F. oxysporum* by changing different parameters such as pH, contact time, lead concentration, adsorbent dose, and temperature through the batch experiments.

3.1 Impact of pH

The effect of pH on lead removal by live and dead *F. oxysporum* is reflected in Figure 1. Results of the study revealed that pH plays a pivotal role in the lead metal biosorption process. For both live and dead biosorbents, Pb(II) uptake level was escalated with an increase in pH from 1 to 6 reaching the maximum sorption at pH 5. The highest 81 and 92% lead was removed with live and dead *F. oxysporum* at pH5 respectively. Sorption ability for both live and dead biosorbents decreased as the pH value was increased beyond 5. Protonation of the biosorbent surface was decreased with a rise in pH, formation of a negative charge showed electrostatic repulsion between biosorbent and lead, and reduced adsorption capacity.

3.2 Effect of the exposure period

The exposure period is an important parameter for the estimation of lead removal by live and dead biomass of *F. oxysporum*. Fungal biosorption capacity was increased by enhancing exposure time (Figures 2 & 3). Maximum lead removal was reported after 2 days with live biomass whereas 3 hours for dead biomass of *F. oxysporum*. Maximum 89 and 93% lead removal was recorded with live and dead biomass of *F. oxysporum*, respectively. However, after the optimum exposure period, the lead removal efficiency was reduced.

3.3 Effect of initial lead concentration

Results presented in figure 4 revealed the effect of initial lead metal concentration on the adsorption of Pb(II)by *F. oxysporum* live and dead biomass. Lead removal was increased by enhancing lead ions concentrations. The results reflected that the adsorption capacity of lead was increased with an increasing initial concentration of lead ions as 73 and 87% lead elimination was observed with lead concentration (100 mg/l) by live and dead *F. oxysporum* respectively. Lead ions generate driving pressure to promote mass transfer resistance of lead ions between solid and liquid sorbent. An increase in the initial lead ion concentration increased interaction between the lead ions in the liquid phase and surface of *Fusarium* and escalated lead absorption by *F. oxysporum*.

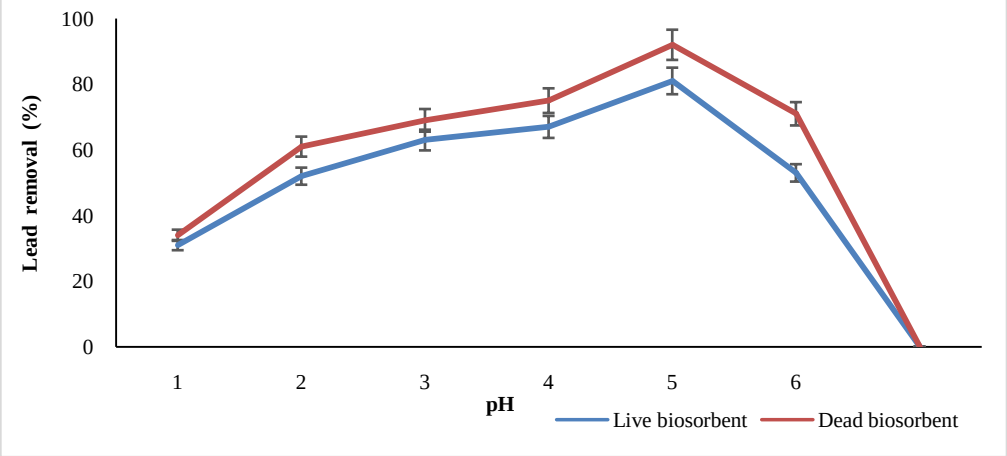

Figure 1 Biosorption of lead by *F. oxysporum* live and dead biomass at different pH; given values are mean ± SEM of three replicates.

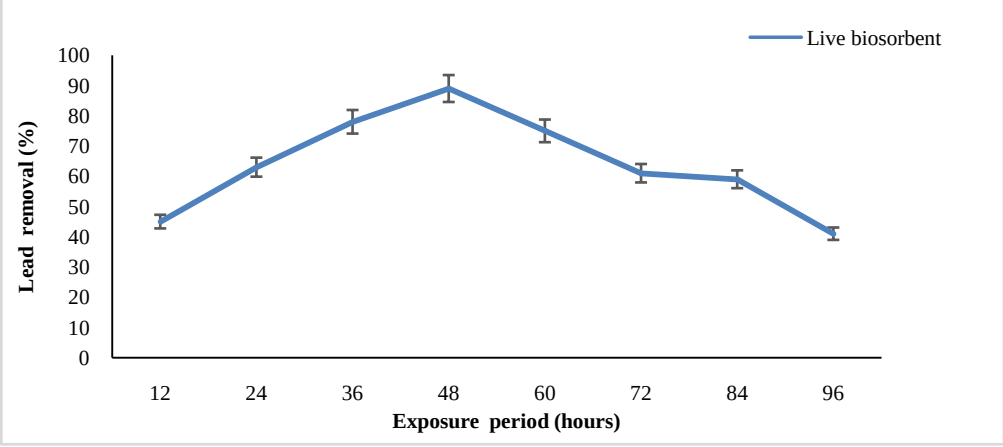

Figure 2 Effect of exposure period on lead removal by live *F. oxysporum* biomass; given values are mean ± SEM of three replicates.

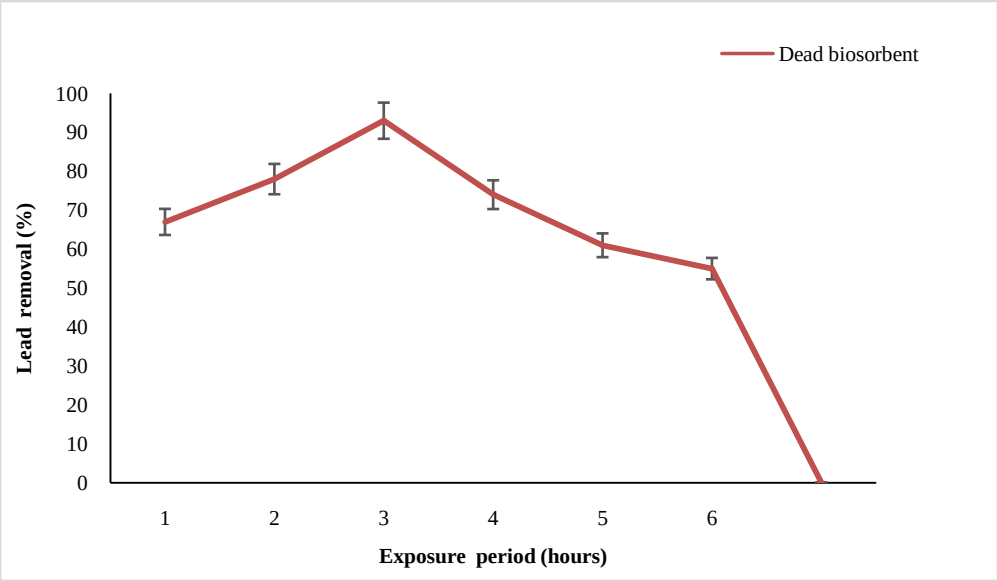

Figure 3 Effect of exposure period on lead removal by dead *F. oxysporum* biomass; given values are mean ± SEM of three replicates

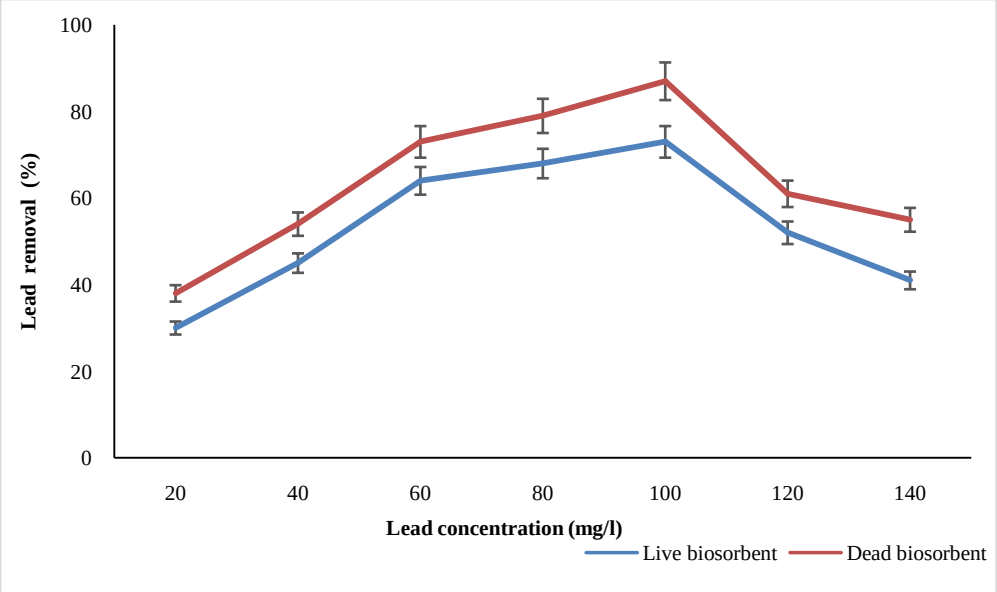

Figure 4 Effect of initial lead concentration on the removal of lead ions from aqueous solution; given values are mean ± SEM of three replicates

3.4 Effect of adsorbent dose

The biosorption rate of lead was escalated with an increase in the amount of *F. oxysporum* (Figure 5). Live and dead *F. oxysporum* absorption ability is reflected in the following trend: 2 > 2.5 > 3> 3.5> 1.5 > 1 > 0.5. The highest lead ions were absorbed with 2 g of both types of fungal biomass. Further, the results of the study revealed that dead biomass was more effective in lead removal as compared to the live biomass of *F. oxysporum* (Figure 5). It might be due to more surface area and availability of more functional groups on the dead biomass in comparison to live biomass. Maximum lead removal (90%) was recorded by 2 g dead biomass where's 81% sorption was observed by the same amount of live biomass of *F. oxysporum* but a further increase in adsorbent dose could alter the uptake of lead ions. This might be because of the non-availability of active sites on fungal biomass and equilibrium establishment between the lead on biosorbent and solution. Results are in agreement with the findings of Sarikaya (2019), who reported that an increase in biosorbent doses enhanced hexavalent chromium sorption by *Agaricus campestris.*

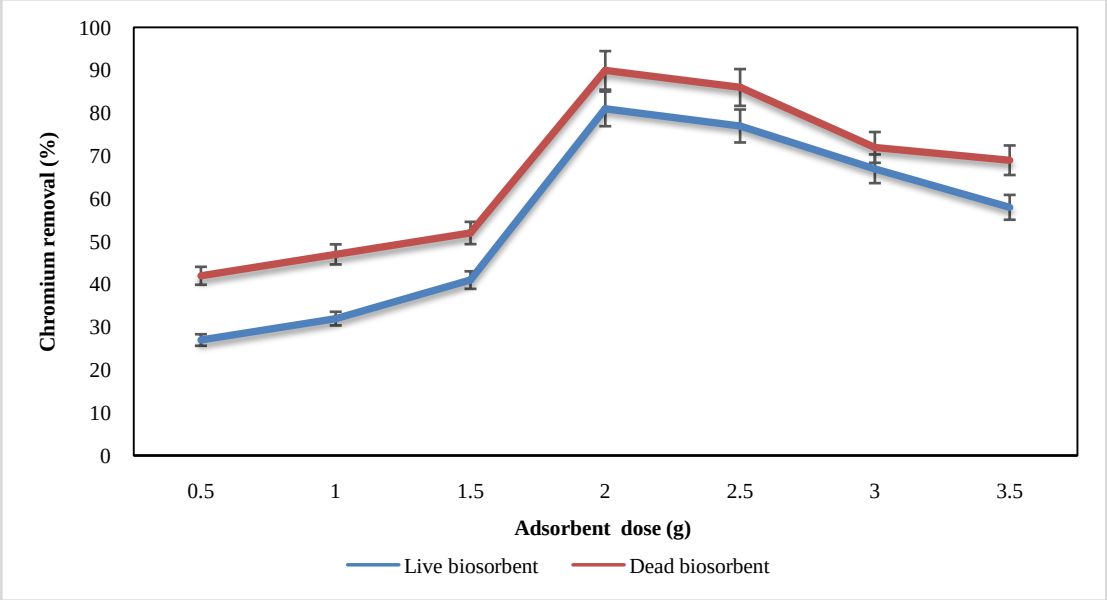

Figure 5 Effect of adsorbent dose on the lead removal; given values are mean ± SEM of three replicates

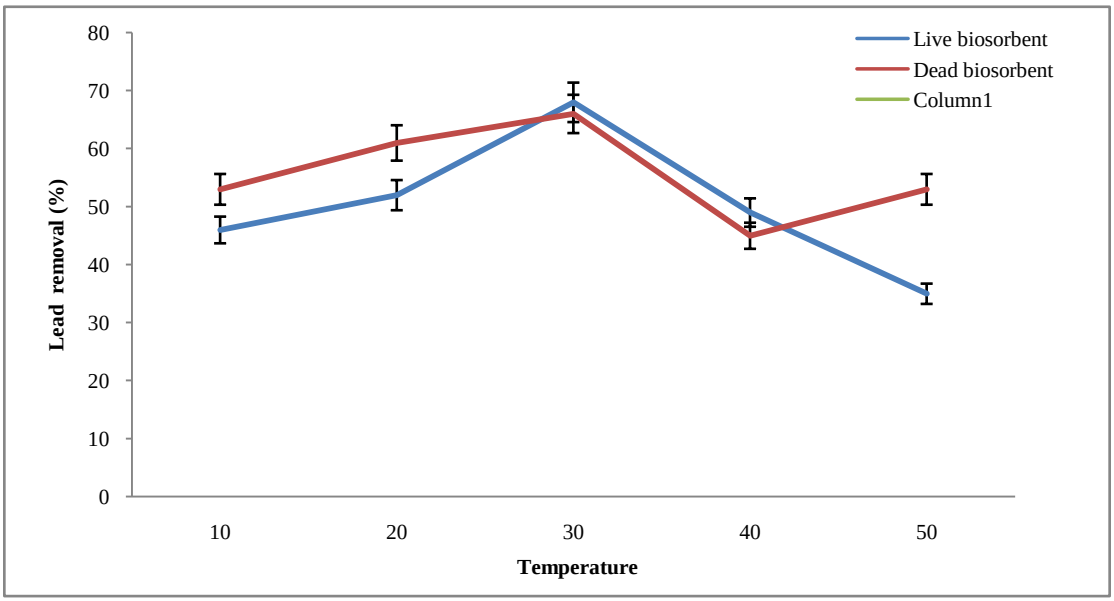

Figure 6 Effect of temperature on the lead removal by live and dead *F. oxysporum* biomass; given values are mean ± SEM of three replicates

3.5 Effect of temperature

Temperature also showed a pivotal role in the biosorption procedure. The biosorption process was enhanced with a rise in temperature as 68% was observed at 30^0 C with live *F. oxysporum* (Figure 6). With the increase in temperature, the biosorption ability of live sorbent declined. The dead biomass did not show any significant alterations in biosorption capacity with a change in temperature as they were resistant to temperature. Results of the study revealed that dead biomass did not modify the functional groups of the biosorbent with a rise in temperature.

3.6 Adsorption isotherm

The adsorption isotherm model explains the mechanism of sorption, surface property, and sorbent capacity. Langmuir isotherm defines the homogeneous distribution of active sites on the adsorbent surface, which adsorb a monolayer with no interaction between sorbed molecules whereas the Freundlich model applies to a heterogeneous system with the interaction between adsorbed molecules. It describes the rise of dye concentration also increases biosorbent, while the energy of sorption reduces on completion of sorption centers of an adsorbent.

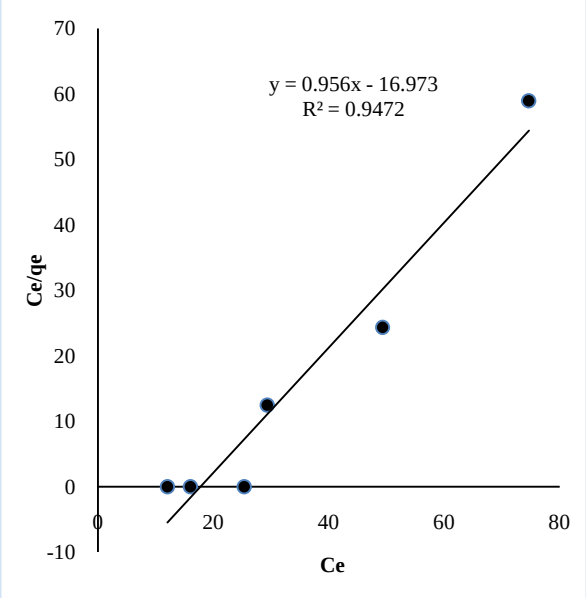

Figure 7(a) Langmuir isotherm for adsorption of lead by dead *F. oxysporum*

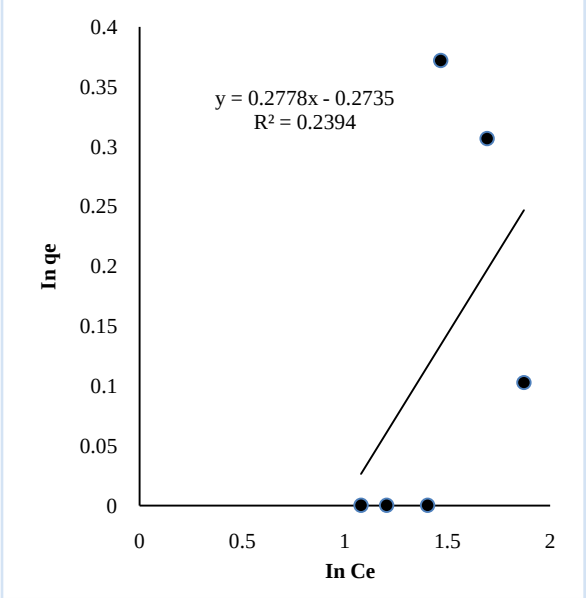

Figure 7(b) Freundlich isotherm for adsorption of lead by dead *F. oxysporum*

Table 1 Isotherm constants for lead sorption by dead *F. oxysporum*

Isotherm	Equation	Plot	Parameters	Value
Langmuir	$C_e/q_e = 1/q_e K_1$ `+ C_e/q_m	A plot Ce/qe versus Ce showed a straight line of slope 1/qm and an intercept of 1/(Ka qm)	qm (mg/g) K (l/mg) R^2	1.046 15.52 0.9472
Freundlich	Log q_e= log K_f +1/n log C_e	K_f and 1/n values were evaluated from the intercept and slope of the linear plot of lnqe versus lnCe, respectively	n K_f (mg/g) R^2	3.599 1.3146 0.2394

Therefore, isotherm results of lead adsorption on *F. oxysporum* was analyzed by Langmuir and Freundlich models (Table 1). Further, the Langmuir isotherm model showed a better fitting model as compared to Freundlich with a high correlation coefficient (R^2= 0.9472). Langmuir constants indicated values: q_m= 1.046 mg/g, k = 15.52 mg^{-1}, R^2 = 0.9472; Freundlich constants were K_f = 1.3146, n = 3.599, R^2 = 0.2394 (Figure 7a and b).

3.7 Phytotoxicity study

A phytotoxicity test was also conducted to compare the effect of lead ions and treated lead solution with live and dead biosorbents of *F. oxysporum* on the growth parameters of wheat seeds. Significant variations were observed under various studied treatments i.e. seed germination and growth characteristics of wheat seeds (Table 2). In control, 97% seed germination was observed whereas lead solution-treated wheat seeds reflected only 21% germination. Further, in the case of radicle and plumule length, it was reported 2.9 and 10.2 cms in control and these parameters decreased to 0.31 and 2.4 cms with lead solution treatment. Wheat seed germination was enhanced by 247.6 and 328.5% with live and dead *F. oxysporum* treatment respectively over the lead metal solution and the growth parameters were significantly enhanced with dead fungal biomass treated lead solution in comparison to living fungal biomass treatment. Seedling length and vigor index of wheat seeds indicated the following trend: Control > dead biosorbent treated lead solution > live biosorbent treated lead solution > lead solution.

Results of the study suggested that lead contamination affected various biochemical processes in plant cells. Lead toxicity reduced wheat seed germination, biomass, and other growth parameters. Lead showed disruption in minerals uptake in plants (Sharma and Dubey 2005). Lead produces reactive oxygen species in plants which reduces plant growth and photosynthesis (Ekmekci et al. 2009). Javaid et al. (2011) used *Aspergillus niger* after treatment with acid and sodium carbonate for the elimination of copper and nickel metals. Rao and Bhargavi (2013) observed that pretreated fungal biomass can be used as an adsorbent for the removal of lead and nickel from the single and binary metal system and found that Zinc metal ions were significantly removed by *Aspergillus flavus* (Mali et al. 2014). Further, Garcia et al. (2016) observed lead biosorption by *Bacillus* species and Mahish et al. (2018) reported a 90% lead adsorption ability of *Penicillium oxalicum.*

Table 2 Effect of live and dead biomass of *F. oxysporum* on germination and seedling length of *T. aestivum* var. UP2554.

Treatment	Seed germination (%)	Radicle length (cms)	Plumule length (cms)	Vigour index
Control	97 ± 0.79[a]	2.9± 0.21[a]	10.2 ± 0.68[a]	12707
Lead solution	21± 0.62[c]	0.31± 0.03[c]	2.4± 0.17[c]	569.1
Lead solution treated with live fungal biomass	73± 0.34[b]	1.2± 0.04[b]	3.5± 0.56[b]	3431
Lead solution treated with dead fungal biomass	90± 0.56[a]	1.7± 0.12[b]	7.9± 0.72[a]	8640

Results are mean ± sem of three replicates; different letters on values have shown significant differences at $P < 0.05$ among treatments as per ANOVA and DMRT

Table 3 Lead removal efficacy of various biosorbent

Metal	Biosorbent	Adsorption capacity (mg/g)	References
Lead	Watermelon-Fe_3O_4 composite	138	Adebowale et al. (2020)
	Polypyrrole-based activated carbon	50	Alghamdi et al. (2019)
	Hazelnut husk	13	Imamoglu and Tekir (2008)
	Apricot stone	21	Mouni et al. (2011)
	Juniperus procera	30	Ali et al. (2019)
	Sugarbeet pulp	71	Pehlivan et al. (2008)
	Sugarcane bagasse	23	Salihi et al. (2016; 2017)
	Fusarium oxysporum	1.046	This study

3.8 Performance of *F. oxysporum* with other sorbents

Results presented in table 3 showed the lead removal efficiency by *F. oxysporum* and other sorbents. Lead elimination ability is in agreement with earlier reports suggesting that lead can be sorbed on *F. oxysporum*. Results of the study revealed that fungal biomass is cost-effective and promising sorbent for lead elimination.

To the best of our information, very few studies have been conducted to check the ability of live and dead biosorbents for heavy metal removal from aqueous solution or wastewater system (Hu et al. 2020). The effect of live and dead biomass of *F. oxysporum* has not been observed previously. Results of the study suggested that dead biosorbents are better alternatives as compared to live sorbents due to several advantages like high efficiency, cost-effective nature, no requirement of nutrients or growth media, and no waste sludge production (Cheng et al. 2015). Further, Paul et al. (2012) suggested that bacterial biosorbent enhanced its heavy metal absorption capacity after heat treatment because of the degradation of cell walls and exposure of binding sites for metal ions. Live biosorbents can transport adsorbed heavy metals into cells and alters heavy metal ion's state into less toxic forms (Yin et al. 2018). Hlihor et al. (2017) reported that biosorbents can easily remove heavy metals even present at very less concentration.

Conclusion

Results of the present investigation can be concluded that lead can be significantly eliminated from aqueous solution with the help of *F. oxysporum*, which is a cost-effective and sustainable option for environmental protection. Further investigations are needed for its application at a large scale for the treatment of industrial effluents containing heavy metals and organic contaminants.

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Conflict of interest

The author declares that there is no conflict of interest.

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Antifertility Potential of n-Butanol and Ethyl Acetate Extracts of *Penicillium oxalicum* OM282858 in Male Albino Rats as Biological Control Agents

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ABSTRACT

Rodents cause significant damage to many crops, spread diseases, and pose a severe risk to public health. Several synthetic contraceptive agents are available for controlling rodents; however, their use is associated with toxic effects on non-target organisms. *Penicillium oxalicum* has several medical properties, but no reports were available on fertility. This study aimed to assess the antifertility potential of n-butanol and ethyl acetate extracts of *P. oxalicum* in adult male albino rats as biological control agents by lowering the population size of rodent pests. Rats were assigned into three groups (n = 36). The first control group (GI) was injected intraperitoneally with 0.5% dimethyl sulfoxide (DMSO). The second (GII) and third (GIII) groups were injected with a single dose of 200 mg/kg body weight (b.wt.) of n-butanol and ethyl acetate extracts of *P. oxalicum* intraperitoneally, respectively, after dissolving in 0.5% DMSO. Further, *P. oxalicum* was identified morphologically and molecularly and then submitted with accession number OM282858 to the National Center for Biotechnology Information (NCBI) GenBank. The antifertility potential of *P. oxalicum* was evaluated after 24 h (the injection period), 96 h, and 168 h (the recovery periods) of treatments. The effects of the treatments on organ weight, testicular histology, histomorphometry measurements, and sperm characteristics were assessed. Both *P. oxalicum* extracts caused changes in reproductive organ weights, testicular histology, histomorphometry measurements, and spermatogenic arrest accompanied by a significant decrease in the count of epididymal sperm and its motility and an increase in the percentage of sperm abnormalities during the injection and recovery periods. Thus, the results suggest that both *P. oxalicum* extract treatments cause suppression of fertility in adult male rats. Therefore, these outcomes are essential for public health, farming establishments, and vertebrate pest control managers.

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1 Introduction

Rodents are a substantial component of the earth's terrestrial ecosystems due to their economic and pathogenic relevance. However, they cause significant damage to many crops and wreak havoc on native fauna. Furthermore, they are critical vectors for transferring various diseases to humans and domestic and wild animals. As a result, rodents have become a significant concern in pest management and the recovery of endangered rodents in their natural habitats (Tobin and Fall 2004; Abdel-El-Azeem 2008).

Traditional strategies such as poisonous baits, anticoagulant rodenticides, trapping, hunting, and pesticides have been adopted to limit rodent damage or lower rodent populations. These traditional methods are ineffective, and the repetitive use of chemicals endangers human health and causes environmental hazards (Taha and Soliman 2019; Taha 2022). Therefore, pest managers have been directed to search for non-lethal approaches to increase the desire for environmentally friendly control methods at low cost and limit the non-target effects (Aktar et al. 2014; Mahmoud et al. 2018; Taha and Soliman 2019). One of these approaches is biological control using natural materials containing bioactive compounds from plants, algae, animals, microorganisms, and marine biota (Asyura et al. 2017), which is effective against rodent pests, phytopathogens, insects, and weeds.

The *Penicillium* genus is the dominant fungus and is well-known because it produces bioactive secondary metabolites (Zhang et al. 2020). After Fleming experimentally discovered penicillin, the bio-prospecting efforts of thousands of *Penicillium* isolates have been tested for their high biological activities (Zhang et al. 2020; Shankar and Sharma 2022; Weng et al. 2022). *Penicillium oxalicum* is one of the most abundant of all the *Penicillium* species (Currie and Thom 1915) that can produce novel bioactive metabolites (Weng et al. 2022), which are used to control bacteria, fungi, and insects (Kubátová et al. 2019). In the current study, the male antifertility capabilities of *Penicillium oxalicum* n-butanol and ethyl acetate extracts were assessed in adult male rats to integrate both fungal extracts as biological control agents in pest management programs.

2 Materials and methods

2.1 Fungal isolation

In this study, the fungal strain QR20 was previously isolated as a mycoflora isolate from rice rhizospheric soil in Egypt by serial dilution technique according to methods used by Johnson and Curl (1971). Fungal colonies were purified and cultured on potato dextrose agar (PDA) media containing potato slices (200 g/l) and 20.0 g/l for both dextrose and agar (Rotem 1994), then were stored at 4 ℃ in slants and in 15% glycerol at -4℃.

2.2 Fungal morphological identification

The fungal isolate QR20 was identified by observing the macroscopic properties, including colony morphology and color on PDA. The fungal microscopic slides were prepared by the slide culture technique using lactophenol cotton blue stain (Vainio et al. 1998), Conidia and conidiophore were observed on the slides using an optical microscope.

2.3 Molecular identification of the isolated fungus

2.3.1 DNA extraction and polymerase chain reactions (PCRs) protocol

All genomic DNA was extracted and purified from a 7-day-old culture of 100 mg wet fungal isolate QR20 spores using the Quick-DNA™ Miniprep Kit per the manufacturer's protocol. Fungal identifications were based on the internal transcribed spacer (ITS) of their ribosomal DNA (ITS1–5.8S–ITS2). Universal primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify ribosomal DNA of *Penicillium* spp. (White et al. 1990). The amplification reactions were performed in a 50 µl reaction volume of COSMO PCR Red Master Mix. The program of the thermal cycler used was as follows: initial denaturation at 95 ℃ for 2 minutes, denaturation at 95 ℃ for 15 seconds, annealing at 50 ℃ for 20 seconds, and extension at 72 ℃ for a minute. This program was followed by 35 cycles of denaturation at 95 ℃ for 15 seconds, annealing at 50 ℃ for 20 seconds, and extension at 72 ℃ for a minute. They were followed by a final extension at 72 ℃ for 5 min.

2.3.2 Alignments and phylogeny

The PCR amplicons were sequenced at GATC Company, Germany, using an ABI 3730x1 DNA sequencer. The obtained sequences and other *Penicillium* species sequences from the National Center for Biotechnology Information (NCBI) GenBank nucleotide database were used to carry out the phylogenetic analysis and tree development. The phylogenetic study was performed using MEGA version 11 software (Tamura et al. 2021). The maximum likelihood (ML) method was used to reconstruct the phylogenetic tree, with bootstrap values calculated after a run of 1000 replications using routines included in MEGA software (Felsenstein 1985). The phylogenetic tree was demonstrated by Fig Tree version 1.4.4 (Rambaut 2020).

2.4 Purification of *Penicillium oxalicum*

To guarantee the purity of *P. oxalicum*, fungal hyphal tips that emerged from previously sub-cultured fungi were taken and sub-

cultured again by putting it on fresh PDA media and incubating for 7 days at 28 ± 2 ℃.

2.5 Inoculum preparation and extraction

Penicillium oxalicum QR20 secondary metabolites extraction was performed according to the procedures of Petit et al. (2009) and Tirumale et al. (2020), with minor modifications for *P. oxalicum* QR20 inoculum preparation. A pure culture of fungus was inoculated on the modified Czapek-Dox broth medium (Dox 1909), which consisted of sucrose as a carbon source (30.0 g/l), KCl (0.5 g/l), $NaNO_3$ (3.0 g/l), K_2PO_4 (1 g/l), $MgSO_4 \cdot 7H_2O$ (0.5 g/l), $Fe(II)SO_4 \cdot 7H_2O$ (0.01 g/l), and pH was adjusted at 7.3 ± 0.2. After fungal inoculation, the culture was incubated at static conditions for two weeks at 28 ± 2ºC. At the end of the incubation period, broth media were first filtered with cheesecloth to remove the mycelial mats and then with Whatman filter paper No. 1 to remove mycelium. The aqueous filtrate was subjected to liquid-liquid extraction with ethyl acetate (EtOAc) solvent three times with an equal volume of culture filtrate (1:1, vol/vol). It was shaken well for 15 min in a separating funnel. The organic layer (EtOAc) was collected, subjected to Na_2SO_4, and concentrated under a vacuum using a rotary evaporator. After evaporation, a crude dried EtOAc extract was obtained. Moreover, the fungal filtrate was extracted with n-butanol (n-BuOH) using the same procedures used for the ethyl acetate extract. Both fungal extracts were prepared to evaluate the antifertility potential.

2.6 Animals

Thirty-six (120–130 g) adult male Wistar albino rats (*Rattus norvegicus*) were obtained from the National Research Center (Cairo, Egypt). Rats were fed *ad libitum* per the standard rodent diet and water intake. The rats were prepared for acclimatization for ten days before the start of the experiment under natural light and dark cycles at 22 ± 2 ℃ and 40- 60% humidity (laboratory conditions). They were humanely handled, and this study protocol was approved by the Ain Shams University Research Ethics Committee (ACUC-FP-ASU RHDIRB2020110301 REC#90).

2.7 Experimental protocol

Rats were randomly assigned into three groups, with twelve rats in each group. Treatments were assigned to each group; the first control group (GI) was administered intraperitoneally with 0.5% dimethyl sulfoxide (DMSO). The second (GII) and third (GIII) groups were injected with a single dose of 200 mg/kg body weight (b.wt.) of n-butanol and ethyl acetate extracts of *P. oxalicum*, respectively (Kaur et al. 2021). Doses were prepared by making a suspension of the fungal extracts, which were dissolved in 0.5% DMSO for intraperitoneal injection.

2.8 Body weight, reproductive organ weights, and sperm characteristics

Rats were weighed, and the obtained weights were recorded at the experiment's beginning and end. Then, the percentage change in body weight was calculated according to the following equation:

Percentage change in body weight = [(recorded end body weight-recorded beginning body weight/ recorded beginning body weight)] x 100

After 24 hours of injection, four rats per group were sacrificed. To assess the reversal or delay effects of antifertility of both fungal extracts, the remaining four rats from each group lasted for 96 and 168 h. They were then sacrificed to test the recovery period.

The anesthetization of rats was carried out with chloroform. First, the male reproductive organs (testes, epididymis, vesicula seminalis, and prostate gland) were autopsied, washed in saline, and then weighed. The organ index weight (relative weights) was recorded as organ weight/body weight × 100. Then, the epididymis was removed and perfused with a modified 2 ml of Tyrode's solution (125 mM NaCl, 2.7 mM KCl, 25 mM $NaHCO_4$, 0.5 mM $MgCl_2$, 1.80 mM $CaCl_2 \cdot 6H_2O$, 05.56 mM glucose, 36 mM $NaH_2PO_4 \cdot 2H_2O$, 100 units of penicillin, and 4 mg/ml BSA) at 34 ℃ to determine sperm parameters and then crumbled by scissors to allow sperm release. The sperm sample was incubated at 38 ℃ for 15 min; then, a hemocytometer was used to count sperm, expressed as 10^6/ml. The same sample was used to evaluate the percentage of sperm motility under a light microscope. A smear of sperm was placed on microscopic slides, and methanol was used to fix sperm and stained with eosin as described by Mahmoud et al. (2018), which was then examined at 1,000x by an objective lens with oil immersion in a light microscope. According to WHO (2000), the sperm were classified as abnormal. The abnormality of sperm was calculated as a percentage of the total spermatozoa count (Taha and Soliman 2019).

2.9 Histopathological evaluation

Before histological analysis under a light microscope, the testis tissue slices were stained with hematoxylin and eosin (H&E) for histopathological examination, as Soliman et al. (2016) described. Testis capsule thickness, epithelial height, and diameter of seminiferous tubules were measured at 400x. The testicular scoring system of Johnsen (1970) was used to assess spermatogenesis in a semi-quantitative manner. The order of maturation was given a degree ranging from 10 to 1 for each sectioned tubule according to the appearance or disappearance of main cell types: complete spermatogenesis (10), spermatozoa present with random spermatogenesis (9), few spermatozoa (8), no spermatozoa but spermatids were present (7), few spermatids were present (6), spermatocytes were only present (5 or 4), presence of

spermatogonia (3), presence of only Sertoli cells (2), and practically empty lumen (1). The mean score was obtained by randomly picking ten seminiferous tubules/rats.

2.10 Statistical analysis

All data values were tabulated and statistically analyzed using one-way variance analysis (ANOVA) and performed by Minitab V17 software. The significant difference was at a p-value ≤ 5%, and the data were expressed as mean ± standard error (SEM).

3 Results

3.1 Morphological and molecular identification of the isolated fungus

The fungal strain (QR20) was morphologically identified according to macromorphology and micromorphology as a member of the genus *Penicillium*. Macroscopic characteristics of fungal growth on PDA media included rapid growth and appearance as white mycelia with a cottony texture that turned bluish-green with a powdery texture due to the production of sexual conidiospores (Figure 1a). Microscopic characteristics of the fungus showed hyphae and long chain conidia with smooth walls (Figure 1b). These results were confirmed by molecular identification.

The molecular identification was carried out using rDNA (ITS1-5.8S-ITS2) region sequencing. In blast similarity analysis, the isolated fungus sequence (ITS-rDNA) revealed a 100% sequence similarity with *P. oxalicum*. The fungal strain was submitted to GenBank with accession number OM282858. Phylogeny was constructed based on rDNA sequences and ITS data of the identified fungal isolate QR20 sequence with the closest relative species sequences obtained from NCBI by Mega11 software. The maximum likelihood (ML) method to study genetic relatedness revealed that the fungal isolate QR20 in accommodate to two taxa (*Penicillium oxalicum* PSF-4 MK720103 and *Penicillium oxalicum* 2-4F MW077049) with 100% bootstrap (BT) support, as shown in Figure 2.

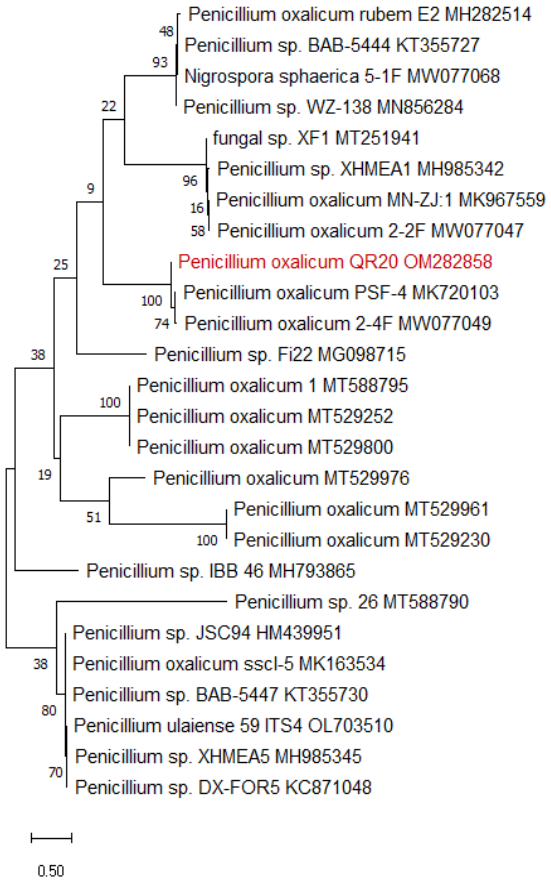

Figure 2 Phylogeny constructed based on ITS sequence dataset of *P. oxalicum* QR20 (OM282858) in red color with other related genes through maximum likelihood analysis. Bootstrap support values (one thousand replicates) are represented at each branch

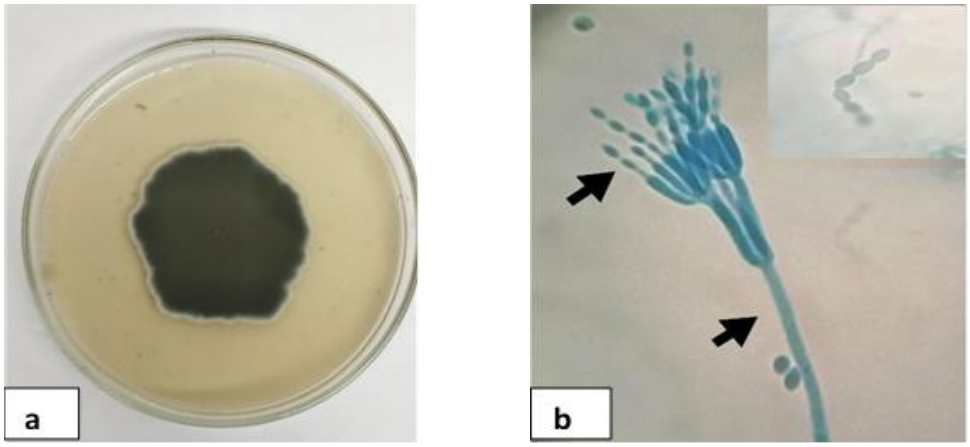

Figure 1 Photograph of *P. oxalicum* isolate (QR20), (a) Colony growth on PDA media after incubation for seven days at 28 ± 2 °C, (b) A microscopic examination showing both conidiophores and conidia (in the inside box) at 400×

3.2 Clinical signs and mortality

There were no toxicity signs in either fungal extract treatment, and all treated rats appeared without abnormalities during both the injection and recovery periods. Additionally, all treated rats had no changes in stool, urine, or eye color. Furthermore, no mortality was recorded in any treated rats during the experiment.

3.3 Effect of *P. oxalicum* extracts on body weight gain and reproductive index organ weights

During the injection period with n-butanol and ethyl acetate extracts, body weight significantly decreased ($p = 0.003$ and 0.018, respectively). On the other hand, there were no significant changes after 168 h in either treatment ($p = 0.189$ and 0.121, respectively) compared to the control rats (Table 1). These results indicated that during the recovery period, body weight could be recovered to the standard (control) range (Table 1).

In GII, there was a significant increase in relative testis weights ($p = 0.044$) and a significant decrease in both relative vesicula seminalis weights ($p = 0.029$) and relative prostate gland weights ($p = 0.008$) after 24 h of treatment (the injection period). Moreover, compared to the untreated rats, relative prostate weights after 96 and 168 hours (the recovery period) showed a highly significant ($p = 0.001$) decrease.

In GIII, there were non-significant changes in testis index weights in treated rats after the injection period ($p = 0.725$) and recovery periods (96 h and 168 h) ($p = 0.387$ and 0.645, respectively; Table 1). There was a significant decrease in vesicula seminalis weight after the injection period of ethylacetate extract ($p = 0.011$). Besides, the relative prostate gland significantly decreased after 96 hours of recovery ($p = 0.008$) compared to the untreated rats (Table 1).

3.4 Effect of *P. oxalicum* extracts on sperm characteristics

During the injection and recovery periods, epididymal sperm count and motility showed a highly significant decrease ($p<0.001$) in comparison to the control rats (Table 2). In the present study, abnormalities found in epididymal sperm were classified as primary abnormalities and identified as the following: (a) abnormalities in the head, which included a compact head, an amorphous head, a headless tail, and a head with a cytoplasmic droplet; (b) abnormalities in the tail, which included a tailless head, a bent tail, and a coiled tail. Total sperm abnormalities demonstrated a significant increase during the injection period of n-butanol extract ($p = 0.021$) and recovery periods of 96 h ($p = 0.019$) and 168 h ($p = 0.013$). Furthermore, in the ethyl acetate extract, total sperm abnormalities revealed a significant increase during the injection ($p = 0.010$) and recovery periods of 96 h ($p = 0.002$) and 168 h ($p = 0.001$) (Table 2).

3.5 Histopathological results

3.5.1 Control group

Untreated rats dissected testes appeared normal in size and color, according to a gross examination. The control group's testicular histology showed a typical histological structure at all phases of spermatogenesis, and the lumen was filled with sperm (Figure 3a and 3b). A thin tunica albuginea entirely encircled the testis (Figure 3a). Testicular tissue contains seminiferous tubules (round or oval) separated by interstitial tissue (Figure 3a). The Sertoli cells are on a thin basal lamina, and a series of spermatogenic cells line each seminiferous tubule (Figure 3b).

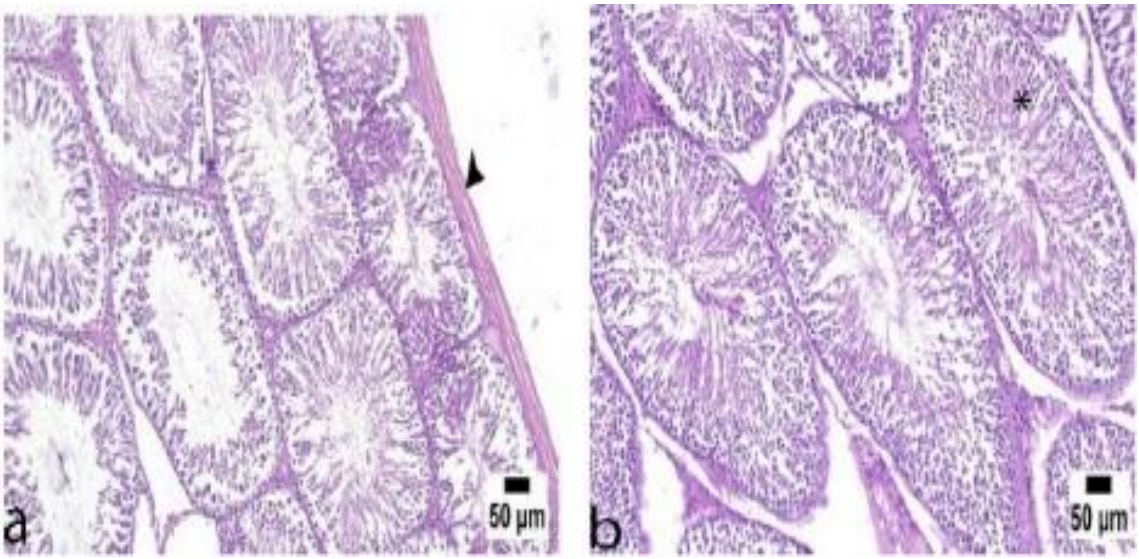

Figure 3 Photomicrographs of testis tissues of albino rats stained by (H&E) of the control group. (a, b) show a thin tunica albuginea layer (arrowheads) seminiferous tubules consisting of stratified germinal epithelium (asterisk)

Table 1 Effect of n-butanol extract (GII) and ethyl acetate extract (GIII) of *P. oxalicum* on body weight, relative reproductive organ weights, stages of spermatogenesis, and testicular morphometry of albino rats.

Treatment		Changes in body weight (%)	Relative reproductive organs weights (%)				Stages of spermatogenesis	Morphometric analysis (µm)		
			Testis	Epididymis	Vesicula seminalis	Prostate gland	Johnsen's score for spermatogenesis	Thickness of tunica albuginea	Diameter of seminiferous tubules	Epithelial height
GI	Control	24.12 ± 6.89	0.685 ± 0.04	0.305 ± 0.088	0.45 ± 0.06	0.307 ± 0.02	9.1 ± 0.27	17.67 ± 0.57	483.16 ± 25.1	107.5 ± 7.82
GII	24 h	-9.070 ± 0.78**	0.79 ± 0.08*	0.235 ± 0.008	0.255 ± 0.014*	0.205 ± 0.0086**	8.0 ± 0.25**	44.27 ± 0.77***	288.5 ± 5.67***	87 ± 1.52**
	96 h	1.28 ± 1.02*	0.735 ± 0.0086	0.265 ± 0.0086	0.265 ± 0.008*	0.145 ± 0.014***	5.6 ± 0.4***	36.60 ± 0.77***	303.5 ± 5.9***	85.5 ± 1.42**
	168h	13.85 ± 0.77	0.771 ± 0.023	0.175 ± 0.025	0.325 ±0.037	0.165 ± 0.002***	6.4 ± 0.22***	32.14 ± 0.57***	287.5 ± 4.78***	74.5 ± 2.5***
GIII	24 h	1.75 ± 1*	0.73 ± 0.115	0.185 ± 0.049	0.205 ± 0.002*	0.277 ± 0.0025	5.9 ± 0.48***	31.069 ± 0.54***	212 ± 11.74***	53 ± 2.09***
	96 h	3.31 ± 3.2*	0.725 ± 0.014	0.285 ± 0.020	0.41 ± 0.017	0.205 ± 0.008**	6.1 ± 0.50***	46.96 ± 1.08***	254.98 ± 8.5***	67.5 ± 1.55***
	168 h	11.1 ± 2.13	0.735 ± 0.095	0.51 ± 0.16	0.47 ± 0.05	0.25 ± 0.017	5.6 ± 0.16***	39.28 ± 0.57***	273.5 ± 8.63***	71.5 ± 1.58***

Values are expressed as means ± SEM; they were significantly different from those of the control group; *p≤0.05; **p≤0.01; ***p≤0.001.

Table 2 Effect of n-butanol extract (GII) and ethyl acetate extract (GIII) of *P. oxalicum* on sperm characteristics in albino rats

Treatments		Epididymal sperm count x 10^6/ml	Sperm motility (%)		Sperm morphology (%)							
			Motile sperm	Immotile sperm	Compact Head sperm	Amorphous head sperm	Headless sperm	Head with cytoplasmic droplet sperm	Tailless sperm	Bent tail sperm	Coiled tail sperm	Total sperm abnormalities (%)
GI	Control	58.5 ± 4.9	70 ± 3	30 ± 2	10 ± 0.7	1.5 ± 0.64	2.5 ±1.04	0	2.75 ± 0.47	0	0	16.75 ± 1.79
GII	24h	7.25 ± 1.10***	0 ± 0***	100***	11.5 ± 1.32	7 ± 1.08**	6 ± 1.08	0.75 ± 0.47	3.5 ± 0.64	0	1.25 ± 0.47*	30 ± 3.85*
	96h	5.5 ± 0.64***	0 ± 0***	100***	10.25 ± 1.30	7.25 ± 0.85**	6 ± 1.08	0.5 ± 0.288	4.25 ± 0.85	0	1 ± 0.40*	29.2 ± 3.47*
	168h	9.5 ± 0.64***	0 ± 0***	100***	10.25 ± 1.6	6.75 ± 0.62***	6.25 ± 1.03*	0.75 ± 0.47	5.25 ± 0.62*	0	1 ± 0.40*	30.2 ± 3.42*
GIII	24h	8 ± 1.4***	0 ± 0***	100***	8.2 ± 1.0	5.7 ± 1.4*	4.75 ± 0.85	0	6.5 ± 0.8**	2 ± 0.40**	0	27.2 ± 2.13**
	96h	7.75 ± 0.85***	4.5 ± 0.5***	95.5 ± 0.5***	11.2 ± 1.3	6.7 ± 0.75**	5.25 ± 1.03	0	7 ± 0.57***	2.25 ± 0.2***	0	32.5 ± 2.5**
	168h	8.75 ± 0.62***	7.5 ± 1.44***	92.5 ± 1.44***	9.5 ± 0.64	7 ± 1.22**	4.75 ± 0.8	0	7.5 ± 0.28***	1.75 ± 0.47**	0	30.5 ± 1.70***

Values are expressed as means ± SEM. They were significantly different from those of the control group. *p≤0.05; **p≤0.01; ***p≤0.001

3.5.2 n-butanol and ethyl acetate extract of *P. oxalicum* treated groups

During the injection and recovery periods, gross morphological examination of autopsied testes from rat groups demonstrated typical color and size compared with the control group. The testes of both fungal extract-treated groups (GII and GIII) showed highly significant ($p<0.001$) increases in the thickness of tunica albuginea and a decrease in tubule diameter in the injection and recovery periods in comparison to the control group (Table 1 and Figure 4 and 5). In GII, the testicular examination of the injected and recovery periods showed the presence of congested blood vessels, disrupted seminiferous tubules with vacuolated spermatogenic cells, mild interstitial edema, decreased spermatogenesis in the surrounding seminiferous tubules, and wide interstitial spaces (Figure 4). During the injection period of GIII, testicular examination indicated several histopathological alterations, including atrophied tubules characterized by an irregular basement membrane with degenerated seminiferous tubules, the absence of mature sperm, vacuolated spermatogenic cells, congested blood vessels, and wide interstitial spaces (Figure 5a and 5b). During the recovery periods (96 h and 168 h) of GIII, testicular examination revealed numerous vacuolations and exfoliation of spermatogenic cells in seminiferous tubules, wide interstitial spaces, mild interstitial edema, and congested blood vessels (Figure 5c, 5d, 5e, and 5f). In GII, the testicular biopsy (mean of Johnsen's score) was significantly lower ($p = 0.009$) than in the untreated rats during the injection period, while it demonstrated a more significant ($p<0.01$) decrease in testicular biopsy after 96 h and 168 h of exposure to n-butanol extracts (the recovery period). In GIII, the mean of Johnsen's score showed a more significant decrease ($p<0.01$) during the injection and recovery periods (Table 1). The results of Johnsen's scoring in both treatments indicated impaired spermatogenesis (Table 1).

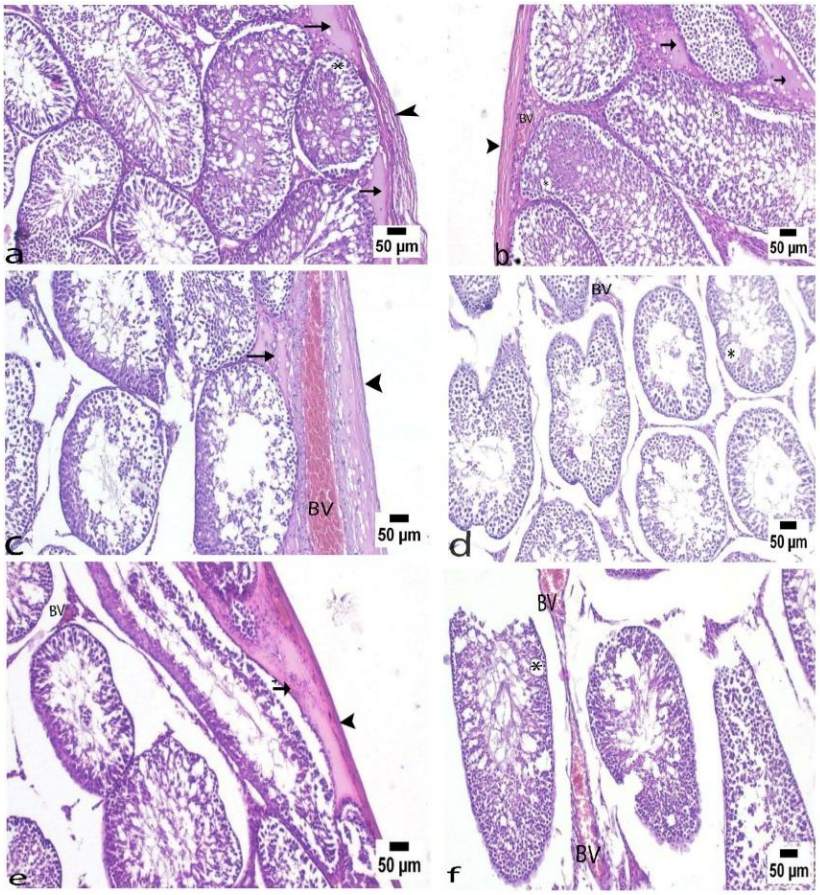

Figure 4 Photomicrographs of testes tissues of albino rats stained by (H&E) treated with n-butanol extract of *P. oxalicum*; (a, b) show thickened tunica albuginea (arrowheads), the presence of congested blood vessels (BV), disrupted seminiferous tubules with vacuolated spermatogenic cells (asterisk), and mild interstitial edema (arrows) after 24 h of treatment (the injection period); (c, d) The recovery period (after 96 h of cessation of treatment) shows thickening in the tunica albuginea (arrowhead), the presence of congested blood vessels (BV), and mild vacuolation of seminiferous tubules (asterisk), and mild interstitial edema (arrow); (e, f) The recovery period (after 168 h of treatment cessation) demonstrates thickening in the tunica albuginea (arrowhead) surrounded by peripheral edema (arrow), a mildly congested blood vessel (BV), and vacuolated spermatogenic cells (asterisk).

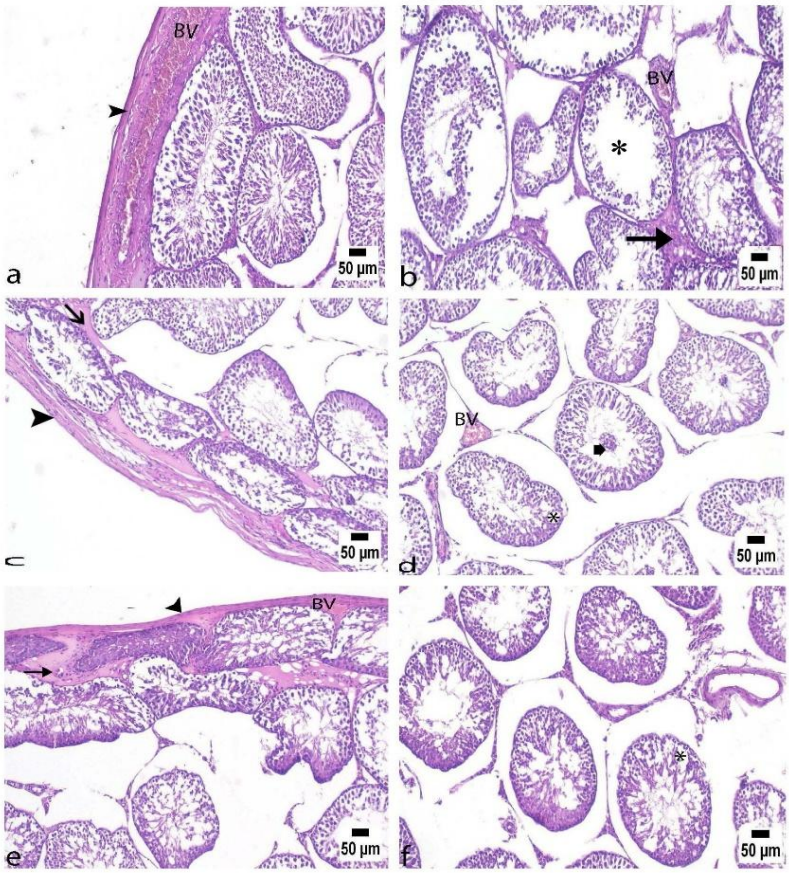

Figure 5 Photomicrographs of testes tissues of albino rats stained by (H&E) treated with ethyl acetate extract of *P. oxalicum*; (a, b) After 24 h of treatment (the injection period), there is a thickening in the tunica albuginea (arrowhead), a presence of congested blood vessels, disrupted seminiferous tubules with vacuolated spermatogenic cells, mild interstitial edema (arrow), empty seminiferous tubule (asterisk), and wide interstitial spaces; (c, d) The recovery period (after 96 h of ceased treatment) shows thickening in tunica albuginea (arrowhead), the presence of mild blood vessels congested (BV), disrupted seminiferous tubules with exfoliated spermatogenic cells (broad arrow), and vacuolated spermatogenic cells (asterisk), and mild interstitial edema (arrow); (e, f) The recovery period (after 168 h of cessation of treatment) shows thickened tunica albuginea (arrowhead), the presence of congested blood vessels (BV), disrupted seminiferous tubules with vacuolated spermatogenic cells (asterisk), and mild interstitial edema (arrow).

4 Discussion

Biological control based on fungi is one of the most promising techniques (Charnley and Collins 2007). The fungal secondary metabolites are significant due to their biological functions and efficiency (Greco et al. 2019; Shankar and Sharma, 2022; Weng et al. 2022). The present study assessed the possible antifertility effects of *P. oxalicum* QR20 extracts (n-butanol and ethyl acetate) on adult male albino rats to be used in integrated pest management programs. The present work relies on the genus *Penicillium*'s ability to produce many different arrays of bioactive metabolites (Zhang et al. 2020).

P. oxalicum is widespread in the soil (Currie and Thom 1915) and a natural source for producing several compounds such as organic acids, toxins, numerous enzymes, and alkaloids (Kubátová et al. 2019). These substances are used in several biocontrol methods, such as antibacterial, antifungal (Lucas et al. 2007; Yang et al. 2008), and insecticidal agents (Santamarina et al. 2002). Therefore, it is recommended as a biocontrol technique against various pests or plant pathogens (Kataria et al. 2018).

The obtained results from macro and microscopic characteristics of the isolated fungus (QR20) revealed that it was *P. oxalicum*, confirmed by the molecular test with 100% identity. The sequence was deposited at GenBank with accession number OM282858. In the molecular characterization, the PCR amplicon amplified with ITS1/ITS4 primers agreed with Umemoto et al. (2009), who revealed *Penicillium* species identification with 100% homology, using sequencing of ITS region, and with Stackebrandt and Goebel (1994) who confirmed that the higher value of identity percentage indicated the higher similarity of DNA sequences. Furthermore,

Gardens and Bruns (1993) reported that investigations of fungal phylogenetics through ITS regions of ribosomal DNA were widely used in genomes, which was attributed to the facilitated examination of these regions by PCR amplification.

The present study showed that n-butanol and ethyl acetate extracts of *P. oxalicum* were injected once intraperitoneally at 200 mg/kg b.wt. and strongly affected the male fertility of albino rats. No clinical signs appeared during the treatments, and all rats were active, healthy, and showed normal behavior. During the injection period, the body weight of both fungal extract-treated rats showed a significant reduction, while it did not indicate any significant changes during the recovery period of 168 h for both treatments compared with the non-treated group. The percentage of change in the body weight of treated rats could be recovered within a week, suggesting that the two fungal extracts had a slight effect on the body weight of treated rats, which was considered a negligible effect on the growth of animals. Body weight fluctuations are a highly sensitive indicator of overall health, and any significant changes in body weight indicate toxic materials (Muharni and Heni 2018; Taha 2022).

Spermatogenesis represents a significant biomarker of chemically-induced male reproductive toxicity (Reddy et al. 2011). The weight of accessory reproductive organs is an endpoint used to estimate the toxic effects of the tested chemical compounds on male fertility (Creasy 2003). In the current study, both fungal extracts generated considerable alterations regarding some reproductive organ weights and caused changes in both the structure and functions of these organs. These might be attributed to androgen insufficiency because androgens are essential for typical male reproductive organ growth (Mahmoud et al. 2018).

In both fungal extract treatments, the count and motility of sperms showed a highly significant ($p<0.001$) decrease during the injection and recovery periods compared to the untreated rats. These results indicated that both fungal extracts had highly destructive effects on the count and motility of epididymal sperm compared to the control rats. The results were consistent with those of other researchers, who reported that sperm count and motility were vital factors and that any changes in sperm count and motility could be used as an indicator for measuring normal fertility by measuring sperm fertilizing capacity in animals (Mahmoud et al. 2018; Taha and Soliman 2019). The reduction in the count and motility of epididymal sperm was attributed to the reduced supply of testosterone in the epididymis (Gong and Han 2006). Changes in sperm characteristics might be attributed to differences in the solvent polarity of n-butanol and ethyl acetate extracts. Different chemical compounds dissolve at different polarities; hence, there are differences in the quantity of both extracts obtained, which lead to different effects (Paini et al. 2014). The activity of the ATPase enzyme in the spermatozoa cell membrane could be affected by alkaloid compounds and the distributing balance of sodium and potassium ions (Hidayati et al. 2018). The morphology of sperm is a vital parameter that reflects their normality and maturity and is directly correlated with male fertility (Memon et al. 1986; Taha and Soliman 2019). Head and mid-piece sperm abnormalities were identified as mainly prime spermatogenesis defects (Schumacher and Moll 2011; Taha and Soliman 2019) and were valuable indicators of testicular degeneration (Bloom 1950; Taha and Soliman 2019). Alterations in sperm parameters were directly associated with the histopathological changes in testicular tissue that caused reproductive dysfunction (De Souza et al. 2010). In this study, both fungal extracts caused numerous testicular histological changes in treated rats during injection and recovery compared to non-treated rats. These results were similar to previous reproductive toxicity publications that showed gonad histology, testis biochemistry, and epididymis histology were used to characterize hazardous compounds that might induce reproduction difficulties in treated animals (Mathur et al. 2010). The increased tunica albuginea thickness resulted from decreasing testicular parenchyma volume (Arenas et al. 1997; Mahmoud et al. 2018; Taha and Soliman 2019). The appearance of numerous mild congestions in the blood vessels of the testes of injected rats was attributed to increasing the production of adenosine, which was consistent with hypoxia and caused vasodilation and expanded blood flow, restoring normal oxygen levels (Huether and McCance 2008; Mahmoud et al. 2018).

The tests on *P. oxalicum* extract-treated rats demonstrated shrinkage in tubular diameter. This was due to the disruption of cell junctions in the Sertoli germ (Mesbah et al. 2008; Taha and Soliman 2019). According to this study, both fungal extract treatments significantly lowered the epithelial height of seminiferous tubules during the injection and recovery periods. These results were accompanied by multiple changes in testicular histology, an increase in tunica albuginea thickness, a decrease in tubular diameter, decreases in the number and motility of epididymal sperm, and an increase in the percentage of sperm abnormalities. Mean score of the testicular biopsy (Johnsen's score) for both fungal extracts during the injection and recovery periods indicated a significant decrease compared to non-treated rats, revealing that both fungal extracts impaired spermatogenesis.

Conclusions

The current study concluded that n-butanol and ethyl acetate extracts of *P. oxalicum* have significant antifertility effects due to the inhibition of spermatogenesis. Further studies are required to clarify the exact mechanism of the antifertility effect and the separation and identification of the active ingredients responsible for the antifertility. To the best of our knowledge, this is the first research that evaluated the potential of *P. oxalicum* extracts as antifertility agents.

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Author contributions

All author conceived, designed the study, and contributed to data analysis and manuscript preparation. All authors read and approved the manuscript.

Conflict of interest

No conflicts of interest. Alone, authors are responsible about the content and writing of paper.

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The epidemiological and neurological risk factors of Japanese encephalitis virus in the population of Assam, Northeast India

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ABSTRACT

Japanese encephalitis is one of the world's most common public health issues, particularly it is prevalent in the north-eastern Indian states of Assam. This study aimed to find out the risk factors linked to clinical and epidemiological characteristics. A total of 245 cases were found as PCR-positive in Assam. The most common clinical symptoms were fever (87%), seizure (65%), altered sensorium (60%), cold with shivering (74%), vomiting (68%), throat irritation (31%), cough (67%), chest pain (10%), joint pain (18%), mouth ulcer (18%), diarrhea (29%), pain in the abdomen (42.9%), runny nose (64%), redness in eyes (78%), jaundice (25%), and blood in the sputum (25%). Further, the neurological symptoms included vision problems (66.5%), hearing difficulties (55 %), neck stiffness (62%), limb numbness (65%), dizziness (77%), headaches (75.5%), speaking difficulties (63%), hydrophobia (47%), and abnormal behavior (66%). The epidemiological risk factors included contact with pigs (57%), bats (21%), cattle (32%), and rates (66%). In addition, 24.5% of patients observed the death of animals/birds. The protection measure included window screening, sleeping under a mosquito net, and use of insect repellent while sleeping in open compounds (29%) and floods (63%) are considered important risk factors. JE-positive cases include daily habits like working in agriculture fields (28%), in standing water (16%), swimming in nearby lakes (24%), traveling outside their village (40%), and wearing shirts while working in the field (20%), storing water in open containers in or outside the house (62%). These were the epidemiological factors that affected the abundance of the potential mosquito vectors of the JE infection.

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1 Introduction

JE infection is caused by the JE virus which is a single standard RNA genome and the genome size is 11 kb (Habib et al. 2022). The JEV viral structure contains mainly three underlying structural and nonstructural proteins (NS) i.e. nucleocapsid or center protein (C), non-glycosylated film protein (M), and glycosylated envelope protein (E), as well as seven non-structural (NS) proteins i.e. NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS.7 (Ashraf et al. 2021). While structural proteins are always in charge of virus assembly, viral entry into host cells, and finally viral egress from those cells. The NS proteins play a key role in viral replication and immune response evasion by the host (Xiong et al. 2022).

Basically JE influences youngsters or adolescents as the immunization program is mainly developed for mature-aged people. The hereditary examinations recommend that JEV start from the Malay Archipelago area. The infection advanced, presumably a long time back, into various genotypes (I-IV) and spread across Asia (Solomon et al. 2003). JEV keeps on attacking other topographical regions and becoming a serious general medical issue. JEV, a mosquito-borne flavivirus (Furuya-Kanamori et al. 2022), belongs to the family Flaviviridae and causes single-stranded positive-sense RNA infection (Karabatsos 1985). Pigs and birds, for instance, significantly contribute to the development and proliferation of the infection (Ladreyt et al. 2019). One percent of human JEV causes develop into JE. However, CDC (2013) reported that more than half of survivors in these cases including 20-30% of fetal cases are alive with significant neurologic or mental sequelae or mental stress disorder (CDC 2013). JEV is one of the most serious general medical conditions and also causes severe neuropsychiatric sequelae (Solomon 2006). In agricultural nations like India, kids are primarily impacted by the infection and the rate is assessed as 0.30 to 1.5 per 100,000 population (Gajanana et al.1995; Parida et al. 2006). Climate change causes an increase in potential mosquito vectors, along with this, intensifying hosts, horticultural practices, individuals' socio-cultural activities, and some other factors can also contribute to the spread of JE. During the long summer months from June to August, the frequency of motion sickness increases (Simon et al.2022). Children are more likely to develop symptoms of infection in places where Japanese encephalitis is endemic. Among all the risk factors, travelers are also the most prevalent to infect with this JE infection. So, travel guidelines for travelers towards Asia should maintain as a strict rule (Mileno 2020).

Studying the risk factors related to JE infection is necessary to identify a clear concept of virulence and pathogenesis. Further, to understand the risk factors, it is very much necessary to track the vaccine profile. The viral encephalitis infection in the northeastern part of India has not been studied among human hosts. Therefore this study was carried out to study the important symptoms, the concept of virulence, pathogenesis, and vaccine status in Assam, NE state of India.

2 Materials and Methods

2.1 Subject and study area

Assam is a state in Northeast India with a population of around 3 crores, according to the 2012 census. More than 85% of the population resides in rural areas, and more than 52% of the labor force is employed in agriculture. This study checked for JEV infection in Assam from June 2020 to June 2022, among the two hundred and forty-five clinically suspected viral encephalitis patients. The serums of the JEV-positive samples were collected from Gauhati Medical College and Hospital (GMCH). The serum from 245 and serum with CSF from 118 patients was collected and stored at -80^{0}C till tested at Gauhati University. JEV IgM detection was done by using ELISA units at GMC Hospital, Assam. The examples showing units > 50 were deciphered as JE infection explicit IgM antibody positive; such sure cases were taken as JE patients with late infection disease.

2.2 Data collection for epidemiology

The data related to the clinical features of the last two weeks of infections, neurological symptoms, personal behaviors, and epidemiological risk factors along with the vaccine profiles were recorded.

2.3 Statistical Analysis

Results from serological and molecular tests were combined with demographic and epidemiological data (sex, age), and entered into a spreadsheet made with Microsoft Excel software. Measuring means and frequencies was part of a descriptive statistical analysis. Statistics were considered significant for P-values under 0.05.

3 Results

In Assam, from June 2020 to June 2022, a total of 293 individuals were clinically suspected of viral encephalitis cases and also found positive for JEV IgM in the Gauhati Medical College Hospital, Assam, India. From these patients, different information related to neurological and epidemiological characteristics was recorded in this study. Also, both serum and CSF were collected from the patients. Out of 293 patients with IgM JEV positive, 245 were also found positive in PCR testing. These patients were included in this study and among these selected patients, 127 and 118 were male and female patients respectively (Table 1). The age group of the selected patients was reported between 11 and 20 and found to have the highest levels among all the study groups. Further, JEV infection was found in 38.6% of males and 45% of females, and this rate was found to be the most frequent (Table 2).

Table 1 Demographic profile of the enrolled patient's sex

Sex	Clinically-suspected cases reported (n=293)	JE positive cases (n=245);83.6%	Frequency of positive cases
Male	148 (50.5%)	127 (51.9%)	85.8%
Female	145 (49.5%)	118 (48.1%)	81.3%

Table 2 Frequency polygon distribution of age based on sex

Age (years)	Sex		Midpoint interval	Frequency
	Male	Female		
1-10	19 (15%)	19 (16.1%)	19	3
11-20	49 (38.6%)	53 (45%)	51	1
21-30	27 (21.2%)	29 (24.5%)	28	3
31-40	11 (8.6%)	5 (4.2%)	8	2
41-50	11 (8.6%)	7 (6%)	9	2
51-60	5 (4%)	3 (2.5%)	4	0
61-70	5 (4%)	2 (1.7%)	3.5	0
Total	127 (100%)	118 (100%)	-	-

Clinical features were presented in Table 3, where the data is represented by comparing the male and female populations. It was found that almost 87% of the JEV-positive patients had a history of fever and found it statistically significant ($p<0.5$). Seizures were found as an important feature of JE infection and 65% of patients had shown positive rates, which is statistically significant ($p <0.05$). Also, it was found that 76% had generalized and 24% had focal seizures. All the patients were carefully asked about their altered sensorium state during hospitalization, and 60% were found to be active with sensorium.

Table 3 Clinical features of the patients during the current illness

Factors	JEV PCR Positive Male (N=127) Frequency (%)	JEV PCR Positive female (N=118) Frequency (%)	P Value
Fever			
Present (N=213) 87%	116 (91%)	97 (83%)	<0.05
Absent (N=32) 13%	11 (9%)	21 (17%)	
Unknown (N=0) 0%	0 (0%)	0 (0%)	
SEIZURE			
Present (N=160) 65%	90 (70%)	70 (60%)	<0.05
Absent (N=80) 33%	36 (28%)	44 (38%)	
Unknown (N=5) 2%	1 (2%)	4 (2%)	
SEIZURE type			
Focal (N=58) 24%	36 (28%)	22 (19%)	0.3
General (N=187) 76%	91 (72%)	96 (81%)	
Unknown (N=0) 0%	0 (0%)	0 (0%)	
ALTERED SENSORIUM			
Present (N=146) 60%	78 (61%)	68 (58%)	0.19
Absent (N=99) 40%	49 (39%)	50 (42%)	
Unknown (N=0) 0%	0 (0%)	0 (0%)	
Cold/cold with shivering			
Present (N=182) 74%	102 (80%)	80 (68%)	<0.05
Absent (N=42) 17%	24 (19%)	18 (15%)	
Unknown (N=21) 9%	1 (1%)	20 (17%)	
VOMITING			
Present (N=166) 68%	95 (75%)	71 (60%)	<0.05
Absent (N=71) 29%	31 (24%)	40 (34%)	
Unknown (N=8) 3%	1 (1%)	7 (6%)	

Factors			JEV PCR Positive Male (N=127) Frequency (%)		JEV PCR Positive female (N=118) Frequency (%)		P Value
Irritation/Pain in the throat							
Present	(N=76)	31%	31	(24%)	45	(38%)	<0.05
Absent	(N=166)	67%	96	(76%)	70	(59.5%)	
Unknown	(N=3)	2%	0	(0%)	3	(2.5%)	
Cough							
Present	(N=164)	67%	93	(73%)	71	(60%)	<0.05
Absent	(N=77)	31%	34	(27%)	43	(36%)	
Unknown	(N= 4)	2%	0	(0%)	4	(4%)	
Chest Pain							
Present	(N=25)	10%	7	(5%)	18	(15%)	<0.05
Absent	(N= 219)	89%	119	(94%)	100	(85%)	
Unknown	(N=1)	1%	1	(1%)	0	(0%)	
Joint Pain							
Present	(N=44)	18%	17	(13%)	27	(23%)	<0.05
Absent	(N=192)	78%	109	(86%)	83	(70%)	
Unknown	(N=9)	4%	1	(1%)	8	(7%)	
Mouth Ulcers							
Present	(N=45)	18%	15	(12%)	30	(25%)	<0.05
Absent	(N=198)	81%	112	(78%)	86	(73%)	
Unknown	(N=2)	1%	0	(0%)	2	(2%)	
Diarrhea							
Present	(N=71)	29%	39	(31%)	32	(27%)	0.6
Absent	(N=174)	71%	89	(69%)	85	(73%)	
Unknown	(N=0)	0%	0	(0%)	0	(0%)	
Pain in the abdomen							
Present	(N=105)	42.9%	51	(40%)	54	(46%)	<0.05
Absent	(N=138)	56.3%	77	(60%)	61	(52%)	
Unknown	(N= 2)	0.8%	0	(0%)	2	2%)	
Runny nose							
Present	(N=156)	64%	83	(65%)	73	(62%)	0.1
Absent	(N= 89)	36%	44	(35%)	45	(38%)	
Unknown	(N=0)	0%	0	(0%)	0	(0%)	
Redness of eyes							
Present	(N=151)	78%	82	(65%)	69	(59%)	<0.05
Absent	(N= 47)	19%	43	(33.4%)	44	(37%)	
Unknown	(N=7)	3%	2	(1.6%)	5	(4%)	
Jaundice							
Present	(N= 62)	25%	36	(27%)	26	(23%)	0.4
Absent	(N= 173)	71%	86	(68%)	87	(74%)	
Unknown	(N=10)	4%	6	(5%)	4	(3%)	
notice any blood in sputum/vomiting/urine/faces							
Present	(N= 61)	25%	34	(27%)	27	(23%)	0.8
Absent	(N=184)	75%	93	(73%)	91	(77%)	
Unknown	(N= 0)	0%	0	(0%)	0	(0%)	

*p<0.05=Significant data

Further, 74% of JE patients show a history of cold or cold with shivering. Clinical findings also showed that 68% of patients were positive for vomiting. But a few patients (31%) had pain in the throat and most of the patients (67%) did not have any irritation in the throat. About 67% of JE-positive patients had a cough while 31% of patients did not have a cough. In this study, chest pain among the JE patients was not a major risk factor because 89% did not exhibit these symptoms and only a few (10%) have this type of symptom. The majority of the patients did not have joint pain, and only 18% of JE patients showed this symptom. Mouth ulcers were absent in 81% of patients. Most of the JE-positive patients had no issues with diarrhea (71%). Also, pain in the abdomen was reported in 56.3%, and 42.9% of respondents. Further, 64% of patients had runny noses, and 78% showed redness in the eyes. Jaundice was found positive only in 25% of JEV patients and blood in the sputum, vomiting, urine, or faces was observed only in 25% of

patients. JE patients with various neurological symptoms were also recorded when they were ill (table 4). Among the selected patients, 66.5% had problems with vision, 18% of patients had problems with photophobia, 10% of patients had double vision problems, and 49% showed blurred vision. Most of the patients had problems with hearing (55%). Further, 62% of positive JE patients had a major issue with their necks because they could not move them or neck stiffness occurred. A factor known as numbness in the limbs

Table 4 Neurological Symptoms of JEV patients during the illness of JEV

Factors	JEV PCR Positive Male (N=127); Frequency (%)	JEV PCR Positive female (N=118); Frequency (%)	P Value
Problems in Vision			
Present (N=163); 66.5%	75 (N=59%)	88 (N=75%)	<0.05
Absent (N=69) ; 28.2%	46 (N=32%)	23 (N=19.5%)	
Unknown(N=13) ;5.3%	6 (N=9%)	7 (N=5.5%)	
Bothered-by light/Photophobia			
Present (N=45); 18%	32 (N=25%)	13 (N=11%)	<0.05
Absent (N=186);76%	88 (N=69%)	98 (N=83%)	
Unknown(N=14); 6%	7 (N=6%)	7 (N=6%)	
Double vision/Diplopia			
Present (N=24);10%	18 (N=14%)	6 (N=5%)	<0.05
Absent (N=212);87%	101 (N=80%)	111 (N=94.2%)	
Unknown(N=9);3%	8 (N=6%)	1 (N=0.8%)	
Blurred vision			
Present (N=121);49%	73 (N=57%)	48 (N=41%)	<0.05
Absent (N=81); 33%	47 (N=37%)	34 (N=29%)	
Unknown(N=34) ;18%	7 (N=6%)	36 (N=30%)	
Any difficulties in hearing			
Present (N=136);55%	74 (N=57%)	62 (N=53%)	<0.05
Absent (N=97);40%	49 (N=38%)	48 (N=41%)	
Unknown(N=12);5%	4 (N=5%)	8 (N=6%)	
Unable to move neck/Neck stiffness			
Present (N=152);62%	87 (N=69%)	65 (N=55%)	<0.05
Absent (N=92); 37.5%	40 (N=31%)	52 (N=44%)	
Unknown (N=1); 0.5%	0 (N=0%)	1 (N=1%)	
Experience numbness in limbs			
Present (N=159);65%	93 (N=73%)	66 (N=56%)	<0.05
Absent (N=85); 34.5%	33 (N=26%)	52 (N=44%)	
Unknown(N=1);0.5%	1 (N=1%)	0 (N=0%)	
Dizziness			
Present (N=188);77%	106 (N=83%)	82 (N=69%)	<0.05
Absent (N=57);23%	21 (N=17%)	36 (N=31%)	
Unknown(N=0);0%	0 (N=0%)	0 (N=0%)	
Headaches			
Present (N=185);75.5%	103 (N=81%)	82 (N=70%)	<0.05
Absent (N=55);22.5%	23 (N=18%)	32 (N=27%)	
Unknown(N=5);2%	1 (N=1%)	4 (N=3%)	
Difficulties in speaking			
Present (N=155);63%	91 (N=72%)	64 (N=54%)	<0.05
Absent (N=90);37%	36 (N=28%)	54 (N=46%)	
Unknown(N=0);0%	0 (N=0%)	0 (N=0%)	
Any repulsion to drinking water/Hydrophobia			
Present (N=115);47%	65 (N=51%)	50 (N=42%)	0.18
Absent (N=124);51%	56 (N=44%)	68 (N=58%)	
Unknown(N=6);2%	6 (N=5%)	0 (N=0%)	
Any abnormal behavior			
Present (N=162);66%	86 (N=68%)	76 (N=64%)	0.14
Absent (N=77);31%	38 (N=30%)	39 (N=33%)	
Unknown(N=6);3%	3 (N=2%)	3 (N=3%)	

* p<0.05=Significant data

was also observed in 65% of the JE patients. In the case of dizziness, a total of 77% of patients have positive responses towards dizziness. Further, 75.5% of patients had headaches while admitted to the hospital. Among 63% of positive JEVs, there was a problem with speaking. Hydrophobia was recorded in 47% of positive patients. As JE is mainly a neurological disorder, 66% of patients showed abnormal behaviors.

When the study was done along with the clinical factors, a few important factors were noticed as presumptive risk factors (Table 5). It was found that 57% had contact with pigs in the last four weeks. There are also many people related to pig farming, so there is a chance of transmission of JE from pigs. Only 21% of patients had contact with bats and 32% had contact with cattle. Also, it was found that 66% of positive cases were in contact with the rates while 24.5% of JE-positive cases were in contact with dead animals. Further, the majority of patients (85%) did not have window screening in their homes, and 81% did not have insect repellents to protect themselves from vectors. Most of the patients used mosquito nets but 29% of patients slept outside their houses in the open compound in the last month of their infections. Further, 63% of patients had faced flood problems during the time of infection or recently occurred. Earlier studies from various parts of the world discovered that during flood times, incident rates of JEV

Table 5 Epidemiological Risk Factors of the JEV Patients among male and female groups

Factors	JEV PCR Positive Male (N=127)	JEV PCR Positive Female (N=118)	P Value
In the last four weeks have you been in contact with Pig			
YES (N=140); 57%	90	50	<0.05
NO (N=101); 41%	33	68	
UNKNOWN (N=4); 2%	4	0	
In the last four weeks have you been in contact with Bats			
YES (N=52); 21%	36	16	<0.05
NO N=182); 74%	83	99	
UNKNOWN (N=11); 5%	8	3	
In the last 4 weeks have you been in contact with Cattle			
YES (N=79); 32%	53	26	<0.05
NO (N=153); 62%	67	86	
UNKNOWN (N=13); 6%	7	6	
In the last four weeks have you been in contact with Rats			
YES (N=161);66%	92	69	<0.05
NO (N=68); 28%	28	40	
UNKNOWN (N=16); 6%	7	9	
Was there any animals/bird death			
YES (N=60); 24.5%	45	15	<0.05
NO (N=146); 59.5%	61	85	
UNKNOWN (N=39); 16%	21	18	
Do you have window screening in your house?			
YES (N=32); 13%	23	9	<0.5
NO (N=207); 85%	98	109	
UNKNOWN (N=6); 2%	6	0	
Do you sleep under the mosquito Net?			
YES (N=194);79%	108	86	<0.5
NO (N=51);21%	19	32	
UNKNOWN(N=0); 0%	0	0	
Use insect repellents?			
YES (N=44); 18%	34	10	<0.5
NO (N=199); 81%	92	107	
UNKNOWN (N=2); 1%	1	1	
Sleep outside your house in the open compound anytime in the last month?			
YES (N=72); 29%	51	21	<0.5
NO (N=173);71%	76	97	
UNKNOWN (N=0); 0%	0	0	
Recently flood occurred in the area?			
YES (N=155); 63%	90	65	<0.5
NO (N=90); 37%	37	53	
UNKNOWN (N=0); 0%	0	0	

Table 6 Personal behaviors of the patients during infection

Factors	JEV PCR Positive Male (N=127); Frequency (%)	JEV PCR Positive Female (N=118); Frequency (%)	P Value
Work in agricultural land			
YES (N=69); 28%	46	23	<0.5
NO (N=171); 71%	78	93	
UNKNOWN (N=3); 1%	3	0	
Work in standing water			
YES (N=39);16%	30	9	<0.5
NO (N=206); 84%	97	109	
UNKNOWN	0	0	
Swim in a nearby lake/pond			
YES (N=59); 24%	26	33	<0.5
NO (N=186);76%	101	85	
UNKNOWN	0	0	
Travel outside your village			
YES (N=97); 40%	72	25	<0.5
NO (N=148);60%	55	93	
UNKNOWN	0	0	
Usually, wear a shirt while working in the field			
YES (N=50);20%	34	16	<0.5
NO (N=45);18%	45	0	
Not applicable (N=150);62%	48	102	
Store water in open containers in or outside your house			
YES (N=153);62%	92	61	<0.5
NO (N=92);38%	35	57	
UNKNOWN	0	0	
Usually, take Bath			
River /Stream (N=14);5.7%	5	9	<0.5
Pond (N=13);5.3%	5	8	
House (N=218);89%	117	101	

*p<0.05=Significant data

infection rise because the conditions become more favorable for mosquitoes. In table 6, it was found that personal behaviors also have a significant role in JEV infections. In the case of working conditions, 28% of patients worked on agricultural land and 16% of patients worked on standing water. Also, 24% of patients swam near lakes or ponds while 40% of JEV patients used to travel outside their villages. Only 20% of patients usually wore shirts while working in the fields. Further, 62% of patients used to store water in open containers in or outside their houses while 89% of the patients used to take baths in houses, 5.3% used to take baths in ponds, and 5.7% used to take baths in rivers or streams.

4 Discussion

The single-stranded, enclosed RNA virus known as Japanese encephalitis virus (JEV), has been linked to severe neurological problems, particularly in young infants, and is a health concern throughout Asia (Robert and Gandhi 2020). The main natural hosts for JEV are pigs, bats, and birds, though humans can also contract the disease. JEV needs a few specific host proteins for their multiplication inside the host cells and these are available in mammals (Kumar et al. 2022). The disease transmission rate and study of the research center affirmed JE differed notably by prefecture, with transcendence in young generations in Assam. The JEV vaccinations helped adults to eradicate the infection. Results of this study showed 83.6% of serologically confirmed JE cases in 2 consecutive years (2020–2022), with reports of 293 clinically-suspected viral encephalitis patients in Assam. A viral-specific JEV IgM antibody test showed a positive confirmation of JEV. Both the CSF and serum were used to study the positive rates of admission to hospitals. The proof of ongoing JE infection in patients with the clinical elements of meaning encephalopathy and an epidemiological foundation of the illness upheld the affirmation of JE cases without CSF testing.

Previous studies reported that JE is more prevalent among the young age groups, and it is necessary to find age-wise infection information (Li et al. 2016). This may be because of their high openness to tainted mosquito nibbles with lower insusceptibility. The event was more noteworthy in the long-term age group, which may be because of the age-related hyperactivity of the youngsters.

This study showed higher involvement of the adult population aged between 11 and 20. Similarly, the male population (51.9%) is more susceptible as compared to the female population (48.1%). This might be due to the higher exposure of male patients to

mosquito vectors or the use of less protective measures being used by male populations. A new set of treatments to stop JE may be developed as a result of our better understanding of the JE pathophysiology (Ashraf et al. 2021). The clinical features observed in the study were found to be significant. But variations can be observed among the male and female populations. Although the vast majority have, for all intents and purposes, no side effects, others can endure mind contamination that can cause cerebral pains, spasms, retching, confusion, and seizures. In the current study, the most widely recognized factor was fever and these results are in agreement with the previous studies of Sen et al. (1976) and Patgiri et al. (2014).

In this study, symptoms like fever, seizure, cold, vomiting, pain in the throat, cough, chest pain, joint pain, redness in the eyes, mouth ulcer, and pain in the abdomen are reported and these findings corroborated by the findings of Xu et al. (2022). However, abdominal pain might be associated with dietary habits or any other bacterial infections that might be given importance while the patients are admitted to the hospital. Among the studied vectors, the predominant vector in South India is *Culex tritaeniorhynchus*, (Arunachalam et al. 2004) and in a few other JE-impacted regions in India (Kanojia et al. 2003). Overall in all parts of India JE reported about every year. From that point forward, the infection was found to be dynamic in practically all aspects of India, and episodes have been accounted for consistently. Stop inoculation remains the best preventive procedure for JEV control because of its complicated eco-the study of disease transmission. The Disease's expansion to the gullible non-endemic region of the Country and the conditions in India's Northern and north-eastern regions were both constantly attributed to the infection's travel. As of late, India saw one more huge flare-up in Malkangiri in 2012 and Manipur in July 2016 (Dwibedi et al. 2015).

As JE is also a neurologic disorder (Ghosh and Basu 2009), various neurological features have also been studied among the patients. However, it was observed that patients are at high-risk with problems ($p<0.05$) like vision, being bothered by light or photophobia; double vision; blurred vision; difficulties in the hearing; unable to move neck/neck stiffness; experiencing numbness in limbs; dizziness, headaches, and difficulties in speaking. At the end of four weeks, patients had been in contact with pigs, bats, cattle, and rats, which are considered to have a higher risk of causing JEV ($P<0.05$). Also, 24.5% of patients had seen animals and birds in dead conditions. Window screening, mosquito nets, and insect repellents can also be considered protective measures against JE infection. As we know that mosquitoes lay eggs in open water storage containers, it was also found that patients who slept in open compounds before the infection occurred are more prone to JE infections ($p<0.05$). Further, severe and outrageous flooding during the summer of May has caused broad harm in Assam consistently for many years. Floods enhance JE infection in two ways, first, it gives more than adequate rearing locales to equipped vectors of the infection, for example, *Culex annulirostris*. Second, it gives the territory to water birds, which can move Japanese encephalitis over significant distances. As a result, flooding is identified as a major source of concern for JE infection ($p<0.05$).

The illness is generally regarded as rustic, and proximity to rice fields and pigs is associated with an increased risk of transmission. Significant mosquito vectors prefer to breed in rice fields, and pigs are thought to be important in enhancing infection and leading to death (Henriksson et al. 2021). This study found that working on agricultural land, working in standing water, swimming in nearby lakes/ponds, and wearing shirts or fully covered clothes while working in the field have a significant effect on this JE infection ($p<0.05$). As we know, standing water is the main breeding spot for mosquitoes. Mosquitoes breed by laying eggs in stale water. So in this study, it's also found that storing water in open containers in or outside your house is a major risk factor for an increase in JE infection ($p<0.05$).

While past evaluations propose that the gamble for an explorer to Asia of contracting JE was one in a million (Hills et al. 2010), today there is a gamble of openness to JE infection and suggestive sickness. The risk of getting JE increases during the active transmission season. Thus, travel medicine specialists give risk avoidance and mindfulness counseling to those voyagers at their most serious risk. In this study, we observed that patients who travel outside their villages have a high risk of getting a JEV infection. The results of our study also showed that taking a bath in a river, or pond, can be considered a risk factor for JEV infection. This epidemiological study reflects that the JEV is a burden on Assam and it's a severe public health issue. The present study touched on many important aspects of JEV that developed in the past few years. The burden of the viral JE infection in the North Eastern part of India is not well characterized to date. So, the results of this study will significantly contribute to the development of policies and immunization programs to control JE in NE states Assam which helps in reducing the mortality rates.

Conclusion

The data from the NE region of India is used to understand neurological problems and underlying infections associated with the JE infection. It is necessary for those who reside in JE endemic areas, to have the necessary immunizations at the appropriate times because this infection poses a high risk to any group of people. It is also important to research the vaccine history to comprehend infection eradication. Additionally, this disease is transmitted via vector so proper management of important vectors is also necessary which seems difficult because pig and rice farming are

crucial for the Nation's economic prosperity, so some future research should be planned for the proper management of vectors.

Declaration of interest statement

None

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Ethical approval

The ethical clearance has been taken from Gauhati Medical College Hospital, Guwahati.

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Validation of a method to elute viruses from different types of face masks

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ABSTRACT

Due to the SARS-CoV-2 pandemic, it is crucial to study the efficiency of face masks in retaining viruses for the upcoming years. The first objective of this study was to validate a method to elute viruses from polyester and cotton face masks. We observed that deionized water followed by 3% beef glycine (pH 9.5 or pH 7.2) was significantly more efficient ($p < 0.05$) in eluting the bacteriophage phiX174 virus from polyester (4.73% ± 0.25% to 28.67% ± 1.89%), polyester/cotton (3% ± 0.33%), and cotton (1.7% ± 0.21%) face masks than 3% beef glycine only (pH 9.5 or pH 7.2) as a single eluent (3.4% ± 0.16% to 21.33% ± 0.94% for polyester, 1.91% ± 0.08% for polyester/cotton, and 1.47% ± 0.12% for cotton face masks). Also, deionized water was significantly less efficient as a single eluent for eluting bacteriophage phiX174 from all the studied face mask types. The polyethylene glycol (PEG) precipitation method was substantially more efficient ($p < 0.05$) as a second step concentration method for the viruses in the eluates than the organic flocculation (OF) method. Higher viral loads were eluted from polyester face masks than cotton ones. We also found varying viral loads in the eluate solutions from different commercial polyester face masks, with the highest percentage seen for the N95 face mask. The second objective was to apply the validated method to study the effect of autoclaving on the different face mask materials. Results of the study did not show any significant differences in the viral loads eluted from the

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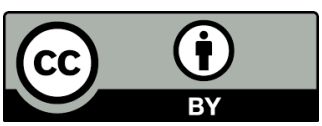


studied face masks before and after one and five autoclaving cycles. Moreover, a scanning electron microscope (SEM) analysis revealed no changes in the yarns, elongation, tensile strength, and contact angle measurements of the polyester or cotton materials after one or five autoclaving cycles.

1 Introduction

Coughing, talking, and breathing are different transmission routes for respiratory viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which spreads through microdroplets expelled from the human respiratory tract into the air. The airborne transmission of these microdroplets depends on their sizes, which usually are in the µm to mm range, while some larger ones can settle on surfaces. Several viral diseases can be transmitted through airborne routes, such as influenza, SARS, respiratory syncytial virus, adenoviruses types 4 and 7, porcine coronavirus, and foot and mouth disease virus. However, they can also be transmitted *via* direct contact with infected persons (Yu et al. 2004; Atkinson and Wein 2008; Kuo et al. 2009; Gloster et al. 2010; Lindsley et al. 2010; Verreault et al. 2010).

Bacteriophages are believed to represent good surrogates for studying airborne viruses as they are safe for laboratory workers, relatively easy to produce on a large scale, and can be purified using several available techniques (Gill and Hyman 2010). Due to their high genetic and morphological diversity, they can be easily distinguished from a large pool of viruses (Ackermann and Prangishvili 2012). Some phages display structural features similar to eukaryotic viruses (Krupovic and Bamford 2008). Double-stranded DNA-tailed phages (Caudovirales order) were the most studied among bacterial viruses and were used in a wide range of fields, including aerosol studies (Verreault et al. 2008). However, since eukaryotic viruses are tail-less, members of tailed phages (Caudovirales order) such as coliphages T4 and T7 might not be the most suitable models. So, tail-less phages like MS2, Φ6, and ΦX174 may be better to be used as viral aerosol models (Verreault et al. 2008).

The Center for Disease Control (CDC) guidelines regarding COVID-19 recommend that even fully vaccinated individuals should wear masks in public (https://www.news4jax.com/news/2021/07/25/fauci-cdc-may-back-wearing-face-masks-more/). The detection of new SARS-CoV 2 variants, including the Delta (https://www.sciencemediacentre.org/expert-reaction-to-cases-of-variant-b-1-617-the-indian-variant-being-investigated-in-the-uk) and Omicron (https://www.who.int/news/item/26-11-2021-classification-of-omicron-(b.1.1.529)-sars-cov-2-variant-of-concern) variants might warrant the usage of face masks for the next months or years. Wearing face masks might be strongly recommended until most of the global population is vaccinated against SARS-CoV-2. Moreover, frequent usage of face masks worldwide for the following years might protect people from respiratory infections. Masks are physical coverings that prevent the spread of respiratory droplets and, therefore, protect the wearer (https://www.cnbc.com/2021/01/25/dr-fauci-double-mask-during-covid-makes-common-sense-more effective.html). Although the CDC and World Health Organization (WHO 2021) guidelines strongly recommend the usage of face masks to prevent the spread of SARS-CoV 2, the information about their protective efficiency against the airborne transmission of infectious SARS-CoV-2 and other respiratory viruses through droplets/aerosols is insufficient (Ueki et al. 2020). The ability of masks to filter particles depends on the particle size and trajectory, as smaller floating aerosols are more challenging to filter than larger particles with momentum. Speech produces more SARS-CoV-2 virus particles and the asymptomatic transmission of SARS-CoV-2 is associated with upper respiratory tract shedding, where the virus-laden particles are formed during vocalization (Howard et al. 2021). Ueki et al. (2020) developed an airborne transmission simulator for infectious SARS-CoV-2-containing droplets/aerosols produced by human respiration and cough. They also assessed the transmissibility of the infectious droplets/aerosols and the ability of various types of face masks to block the transmission. They found that cotton, surgical, and N95 masks can prevent the transmission of infective SARS-CoV-2 droplets/aerosol and this protective efficiency was higher when masks were worn by infected individuals. However, the masks could not completely block viral transmission through droplets or aerosols even when sealed. Also, Ueki et al. (2020) did not estimate the efficiency of different face mask types by quantifying the viruses collected in the solution passed through these face masks. Therefore, taking into account the viruses that are already adsorbed and/or absorbed on face masks is important. It is also necessary to evaluate various parameters about the efficiency of the different viral elution methods, such as comparing different types of face masks and the effect of autoclaving on the efficiency of face masks. Moreover, to estimate the efficiency of the face masks in retaining viruses for several applications, the methods for eluting viruses from different textile types need to be validated. This will help in distinguishing between the elution efficiency for viruses from each type and the capability of the same material to retain viruses. It is also essential to validate the viral concentration method for eluting viruses from different face mask types and then re-concentrating the viruses from the eluate.

The first objective of this work was to validate a method to elute viruses from polyester and cotton face masks. Secondly, we applied this method to study the effect of autoclaving on different

face mask materials by testing the viral loads eluted from these masks. This indicates the effect of autoclaving on the efficiency of the face masks to adsorb or absorb viruses.

2 Materials and Methods

2.1 Description of examined face masks

Eight different commercial face masks were purchased from markets (A, B, C, D, E, F, G, and H). One of them (F) is the N95 face mask, while face masks A, B, C, D, and E are surgical face masks, and masks G and H are made of cloth. They were analyzed in our labs for their internal and external composition, tensile strength, elongation, air permeability assessment, and contact angle measurements.

2.2 Validation of a method to elute phiX174 bacteriophage virus from the studied face masks

Different types of face masks were put on the mannequin face which simulates the human face. One milliliter (ml) of three different doses of bacteriophage phiX174 virus ($5x10^8$, $5x10^6$, and $5x10^5$ PFU/ml) were dispersed using a plastic aerosol sprayer perpendicularly from 20 cm distance on the different types of face masks that the mannequin wears on his face. The diameter of the droplets dispersed on the face masks was measured using the immersion sampling method as will be described later. A plastic container was put below for the dispersing process to receive the rejected droplets from the face mask and re-disperse them on the face mask. One mask from each type was used for each experiment and the experiment of each type of face mask was performed separately with different concentrations of bacteriophage phiX174 virus. The experiments were performed for different types of unautoclaved masks, masks autoclaved for one cycle, and masks autoclaved for five cycles in an experimental cabin with sterile air. After 15 minutes of virus dispersion on face masks, the masks were put separately in nylon bags filled with 100 ml of the different eluents [deionized water in 2 bags and 3% beef in 0.05M glycine (at either pH 9.5 or pH 7.2) in one bag]. For bags of deionized water and after 24 hours at 4°C, rubbing of masks with hands for 2 minutes and then squeezing of the masks was performed. Then bacteriophage phiX174 in one of the two bags of deionized water was quantified. The masks in the other bag which contains deionized water were exposed to re-elution using 100 ml [3% beef-glycine (at either pH 9.5 or pH 7.2)] for 30 minutes contact time and then rubbing with hands for 2 minutes and then squeezing the masks performed. Virus in the beef extract was quantified directly (neutralization of beef-glycine 3% at either pH 9.5 or 7.2 was performed by adjusting the pH to 7 before inoculation) or after the re-concentration processes using organic flocculation according to Katzenelson et al. (1976) and PEG precipitation method according to Lewis and Metcalf (1988). For PEG concentration, 0.25 volumes of a 5x PEG 8000/1.5 M NaCl solution were added to the eluates and incubated with rocking at 60 rpm at 4°C for 60 min. After centrifugation at 10,000 ×*g* for 30 min at 4°C, the pellets were suspended in 500 µl of PBS and stored at −70°C. For OF reconcentration method, the eluates were adjusted to pH 3.5 and kept with rocking at 60 rpm at 4°C for 30 min. After centrifugation at 3000 rpm for 15 min at 4°C, the pellets were suspended in 500 µl of PBS and stored at −70°C. Each experiment was repeated three times and calculations of mean and standard deviation were done.

2.3 The concentration of bacteriophage phiX174 from aerosols

The area of the experimental cabin was 1.8 m^3 and viruses in aerosols were concentrated in parallel to each experiment according to Harstad (1965). Briefly, glass impingers were used with a flow rate of 12 liters per minute with 25 ml of 0.1% tryptone nutrient broth (Bio-Basic Canada). The glass impingers were put inside the experimental cabin during the dispersion of the different doses of bacteriophage phiX174 virus on the different types of face masks. The same experiment which was explained above was repeated with the addition of using the glass impingers containing 25 ml of 0.1% tryptone nutrient broth and with continuous suction using a suction pump with a flow rate of 12 liters per minute. Briefly, different types of face masks were put on the mannequin face which simulates the human face. One milliliter (ml) of three different doses of bacteriophage phiX174 virus ($5x10^8$, $5x10^6$, and $5x10^5$ PFU/ml) were dispersed using a plastic aerosol sprayer perpendicularly from 20 cm distance on the different types of face masks that the mannequin wears on his face. The diameter of the droplets dispersed on the face masks was measured using the immersion sampling method as will be explained later. A plastic container was put below for the dispersing process to receive the rejected droplets from the face mask and re-disperse them on the face mask. After 15 minutes of virus dispersion on face masks, suction was stopped and the 0.1% tryptone nutrient broth was collected to quantify the bacteriophage phiX174 virus which was collected in it.

2.4 Quantification of infectious bacteriophage phiX174 virus either eluted from face masks or concentrated from aerosols

Quantification of infectious bacteriophage phiX174 was performed according to the standard methods for the examination of water and wastewater, 23rd edition (APHA 2017). Bacteriophage phiX174 strain (ATCC 13706B1) and *Escherichia coli* strain C (ATCC 13706) ATCC as a viral host were used in this study. Briefly, Three mL of melted tryptone top agar were held at 44.5 °C in each of ten 16x150 mm test tubes for sample assay and in each of two additional test tubes to serve as negative and positive controls. Test tubes were held in a water bath to avoid premature solidifying of agar. Then, 0.1 mL of host culture was

added to each of the 12 test tubes. One ml tryptone broth was added to the test tube serving as the negative control and one ml phiX174 preparation (30 to 80 PFU/ml) was added to the test tube serving as the positive control. To each of the remaining 10 tubes, one ml sample (direct and after serial ten-fold dilutions) was added. For each tube, mixing and immediately pouring contents over the bottom agar of a Petri dish that has been suitably labeled was performed. Tilting and rotating the dish to spread suspension evenly and placing it on a level surface to let agar solidify were performed. Then, incubation at 36.5 °C overnight and examination for plaques the following day, and counting of the total number of plaques on the ten dishes receiving the sample (for each direct or diluted sample) were performed. Calculation of the somatic coliphage concentration according to the formula: Ca = (P÷10) X D was done, where: Ca: is the somatic coliphage concentration, PFU/ml, P: is the total number of plaques from the 10 dishes, D: is the reciprocal of dilution made on the inoculum before plating (D =1 for undiluted samples).

2.5 Sterilization of different types of face masks

Different types of examined face masks were sterilized using an autoclave at 126°C for 30 minutes and at 0.15 Mpa to examine the effect of autoclaving on the viral loads that could be eluted from different types of face masks. All types of face masks were put inside thermal bags before being put inside the autoclave to maintain the uniformity of the face masks after the autoclaving process. Normal bags which are not suitable for high temperatures and pressure will affect the form of face masks after the autoclaving process. Elution of bacteriophage phiX174 virus from face masks exposed to one or five autoclaving cycles was performed using the same methods explained above and each cycle was performed at 126°C for 30 minutes and 0.15 Mpa.

2.6 Electron microscope examination

SEM was used according to the manufacturer's instructions to examine if there are changes in the yarns of the studied face masks before autoclaving and after five autoclaving cycles. Briefly, a piece of the mask (1 cm X 1 cm) was cut, placed on copper tape, stuck with double-face carbon tape, coated with a thin gold layer using a sputter coater (S 150A, Edwards, England), and examined using SEM (Quanta 250 FEG, Holland).

2.7 Mechanical properties of studied face masks

2.7.1 Tensile strength and elongation

Tensile strength and elongation at a break were reported by (ASTM D1388-14e1 1994) using a cantilever bending test instrument. All reported values were the average of three readings.

2.7.2 Air-permeability assessment

The air permeability of both treated and pristine cotton samples was recorded according to (ASTM D737 1996) standard method employing TEXTEST FX-3300 at a pressure gradient of 100 Pa. For each sample, an average of five measurements recorded at five different locations was reported.

2.7.3 Contact angle measurements

The contact angle is defined as the angle between the drop's outline tangent at the three-phase contact point, and the substrate. Contact angle measurements were performed using Theta Optical Tensiometer (Dataphysics, Model OAC 13EC, Dataphysics Instrument GmbH, Germany) and according to the manufacturer's instructions. An automatic single-liquid dispenser was used to automatically dispense a precise volume of 1µl liquid drop and then descended until the drop was contacted with the paper surface. It was raised again until the water drop stayed at the sample surface. The water drop image was taken by the camera and analyzed by Dataphysics software using Young fitting mode to obtain the contact angles.

2.8 Immersion sampling method for measuring the diameter of the droplets containing viruses

Droplets are collected on a glass plate coated with silicone oil and they are immediately photographed at high magnification for subsequent scanning. In this method, the collected droplets quickly settle in the silicone oil and do not evaporate even underneath the strong light while being photographed. Stuck in silicone oil, they are measured as perfect spheres (Hurlburt and Hanratty 2002).

2.9 Statistics

A paired Student's t-test was applied to ascertain the significance at $p < 0.05$ of differences in the mean of the virus recovery after direct beef-glycine quantification, organic flocculation, and PEG precipitation methods. To ascertain the significance at $p < 0.05$ between elongation, tensile strength, air permeability, and contact angles before and after autoclaving, the measurement results obtained from the tests were analyzed and evaluated with the help of the SPSS 24 Statistical Analysis Package Program. In this context, after determining the normal distribution of the data, analysis of variance (ANOVA) was performed to determine the relationship between the groups, and correlation analysis was performed to determine the strength and direction of the relationship. The one-way analysis of variance (ANOVA) test was used to determine whether there was a significant difference ($p < 0.05$) in the virus recovery by comparing the mean values of the viral loads eluted from the different types of face masks before and after the autoclaving process. On the other hand, each

experiment was repeated three times and both mean and standard deviation (SD) were calculated.

3 Results and Discussion

We obtained six types of commercial face masks (A, B, C, D, E, and F) with 100% polyester in both internal and external parts, including N95 masks (F). Face mask H was composed of 100% cotton, both internally and externally. No changes were observed in the internal and external compositions of these face masks after one or five autoclaving cycles. Face mask G was a polyester and cotton blend composed of 91.75% polyester + 8.25% cotton externally and 67.95% polyester + 32.05% cotton internally (before autoclaving). The external and internal composition changed to 93.95% polyester + 6.05% cotton and 68% polyester + 32% cotton, respectively, after one autoclaving cycle, and to 94.77% polyester + 5.23% cotton and 68% polyester and 32% cotton after five autoclaving cycles, respectively.

The bacteriophage phiX174 was eluted from all the studied face masks using deionized water, 3% beef glycine (pH 9.5 or pH 7.2), and deionized water followed by 3% beef glycine (pH 9.5 or pH 7.2) (Figure 1). No significant differences were observed between the viral loads in the eluate solutions when either pH 9.5 or pH 7.2 was used. The highest elution efficiency was observed using deionized water followed by 3% beef glycine (pH 9.5 or pH 7.2) with the studied face masks. Direct elution with only 3% beef glycine (pH 9.5 or pH 7.2) was less efficient than deionized water, followed by 3% beef glycine (pH 9.5 or pH 7.2), but more efficient than deionized water as a single eluent. Deionized water followed by 3% beef glycine (pH 9.5 or pH 7.2) was significantly more efficient ($p < 0.05$) in eluting the bacteriophage phiX174 from polyester (from 4.73% ± 0.25% to 28.67% ± 1.89%), polyester/cotton (3% ± 0.33%) and cotton (1.7% ± 0.21%) face masks than 3% beef glycine (pH 9.5 or pH 7.2) as a single eluent (3.4% ± 0.16% to 21.33% ± 0.94% for polyester, 1.91% ± 0.08% for polyester/cotton, and 1.47% ± 0.12% for cotton face masks). Deionized water also was significantly less efficient as a single eluent for the bacteriophage phiX174 from all the face masks (2.2% ± 0.16% to 17.87% ± 0.41% for polyester, 1.62% ± 0.24% for polyester/cotton, and 1.41% ± 0.17% for cotton face masks). However, the elution was significantly better when N95 face masks were used, followed by other commercial polyester face masks. Lower elution percentages were observed with the polyester/cotton face masks, with the lowest being for cotton face masks. No significant differences in the viral elution percentages were observed with all the viral concentrations examined ($5x10^8$, $5x10^6$, and $5x10^5$ PFU/ml).

No significant differences ($p > 0.05$) were observed in the viral load eluted from face masks that were non-autoclaved and autoclaved for one and five cycles with different eluents. After a single autoclaving cycle, 2.16% ± 0.12% to 17.81% ± 0.63% of sprayed bacteriophage phiX174 could be eluted from polyester,

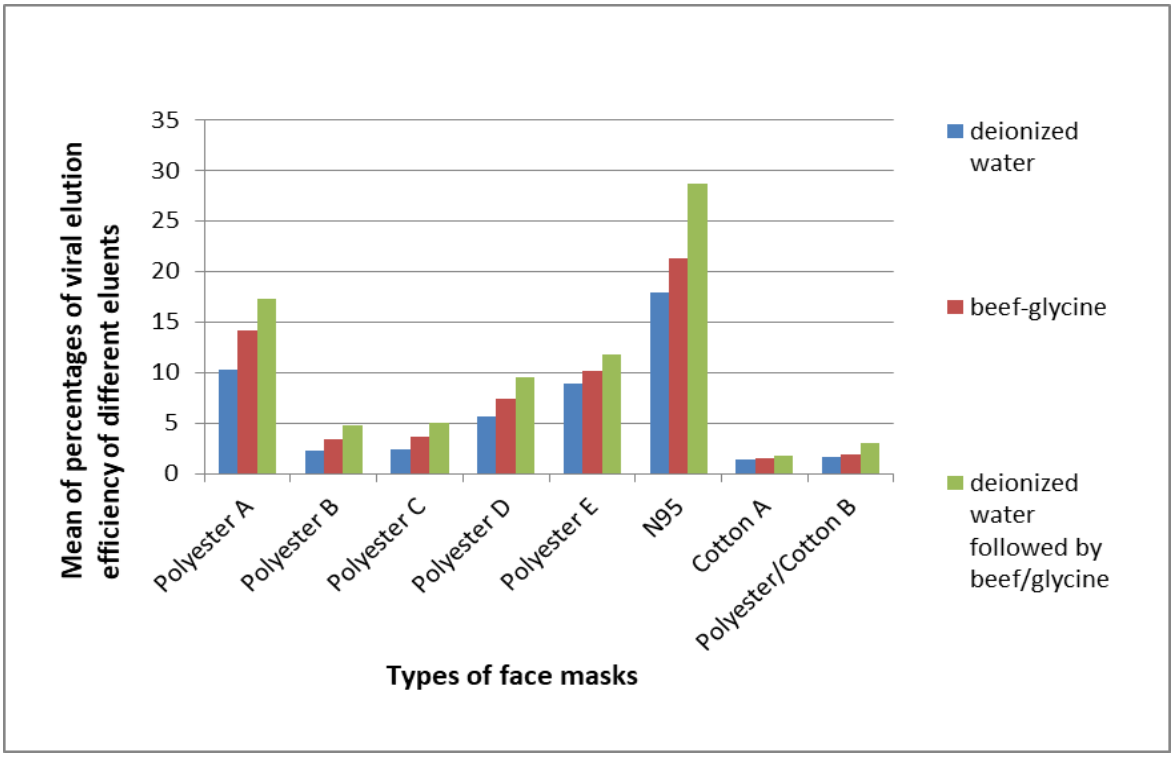
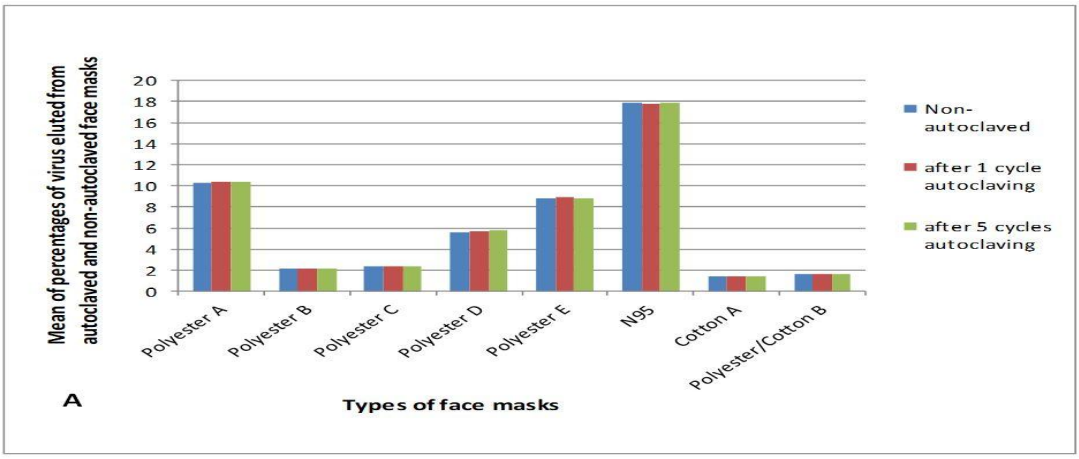
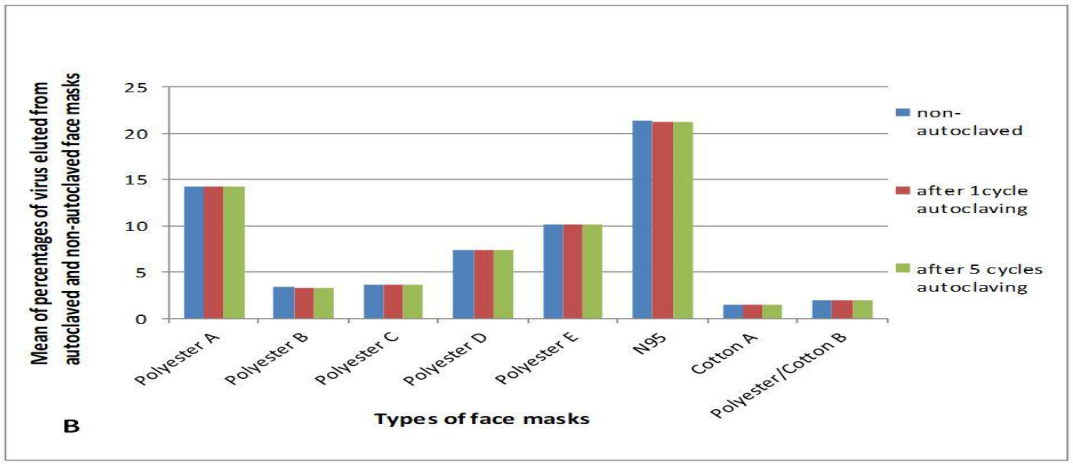


Figure 1 Validation of the elution of bacteriophage phiX174 from non-autoclaved polyester and cotton face masks using different eluents.

1.64% ± 0.11% from polyester/cotton, and 1.4% ± 0.15% from cotton face masks using deionized water as eluent. When 3% beef glycine was used as eluent, 3.33% ± 0.24% to 21.29% ± 0.82% of sprayed bacteriophage phiX174 virus could be eluted from polyester, 1.94% ± 0.14% from polyester/cotton, and 1.49% ± 0.09% from cotton face masks. Further, 4.58% ± 0.27% to 28.81% ± 0.71% of sprayed bacteriophage phiX174 virus could be eluted from polyester, 2.91% ± 0.11% from polyester/cotton, and 1.73%





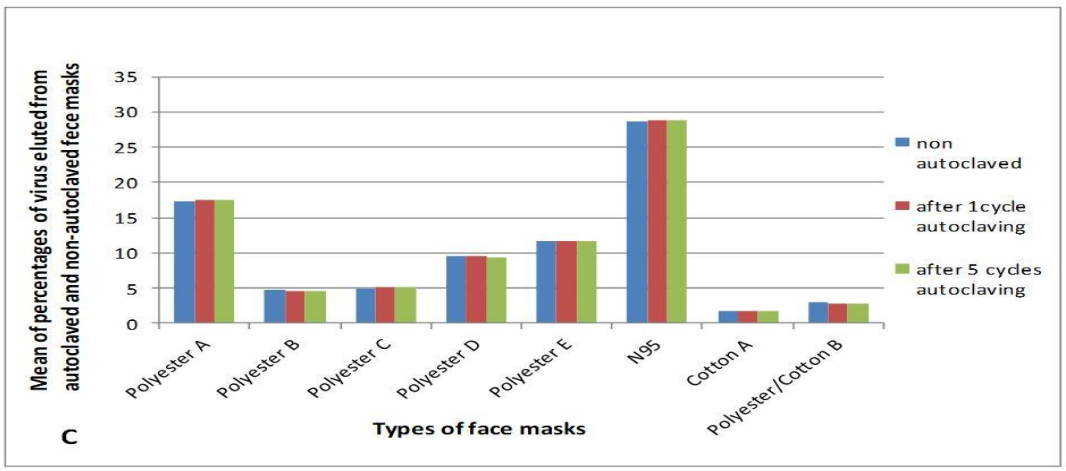


Figure 2 (A, B, and C) Validation of elution of bacteriophage phiX174 from non-autoclaved and autoclaved polyester and cotton face masks using different eluents. A: deionized water. B: Beef glycine. C: deionized water followed by beef glycine

± 0.14% from cotton face masks using deionized water followed by 3% beef glycine as eluent. After five autoclaving cycles, 2.13% ± 0.16% to 17.9% ± 0.36% of sprayed bacteriophage phiX174 virus could be eluted from polyester, 1.6% ± 0.12% from polyester/cotton, and 1.44% ± 0.08% from cotton face masks using deionized water as eluent. Similarly, 3.3% ± 0.23% to 21.315 ± 0.93% of sprayed bacteriophage phiX174 virus could be eluted from polyester, 1.91% ± 0.18% from polyester/cotton, and 1.5% ± 0.12% from cotton face masks using 3% beef glycine as eluent. Then, 4.62% ± 0.32% to 28.84% ± 0.94% of sprayed bacteriophage phiX174 virus could be eluted from polyester, 2.89% ± 0.18% from polyester/cotton, and 1.77% ± 0.13% from cotton face masks using deionized water followed by 3% beef glycine as eluent (Figure 2).

No significant differences ($p > 0.05$) were observed between the results of the viral loads in the 3% beef-glycine eluate or after using organic flocculation as a secondary concentration method. However, significantly higher efficiency ($p < 0.05$) was observed using PEG compared to organic flocculation as a secondary concentration method. The efficiencies for polyester, polyester/cotton, and cotton face masks were 7.8% ± 2.16% to 39.33% ± 2.49%, 5.53% ± 0.5%, and 4.2% ± 0.43%, respectively when PEG was used in the second concentration step. While we observed that the efficiencies for polyester, polyester/cotton, and cotton face masks were 3.2% ± 0.59% to 22.47% ± 2.17%, 1.97% ± 0.16%, and 1.46% ± 0.11%, respectively when organic flocculation was used as a second viral concentration step (Figure 3).

We simultaneously measured the viral loads in the aerosols in the experimental cabin along with the dispersion of viruses on the different face masks and during the 15 mins contact period. We found lower viral loads in aerosols with higher viral elution rates from different face masks. The viral load was highest in the aerosols when cotton face masks were used, which also showed the lowest elution efficiency from their surface and threads (16.53% ± 0.09%, 16.73% ± 0.09%, and 16.8% ± 0.28% when using deionized water followed by beef/glycine, beef/glycine, and deionized water as different eluents respectively). However, the bacteriophage phiX174 load was lowest in the aerosols when N95 face masks were used, which also demonstrated the highest virus elution efficiency from their surface and threads (0.64% ± 0.03%, 0.97% ± 0.02%, and 1.85% ± 0.11% when using deionized water followed by beef/glycine, beef/glycine, and deionized water as different eluents respectively). No significant differences ($p > 0.05$) were observed in the percentages of viruses in the aerosols when the face masks were autoclaved for one or five cycles. After one cycle of autoclaving, the bacteriophage phiX174 was highest in the aerosols with cotton face masks (16.56% ± 0.1%, 16.7% ± 0.18%, and 16.72% ± 0.24% when using deionized water followed by beef/glycine, beef/glycine, and deionized water as different eluents respectively) and was lowest when N95 face masks were used (0.61% ± 0.05%, 0.94% ± 0.03%, and 1.89% ± 0.08% when using deionized water, followed by beef/glycine, beef/glycine, and deionized water as different eluents respectively). After five cycles of autoclaving, the viral load was highest in the aerosols when cotton face masks were used (16.49% ± 0.07%, 16.7% ± 0.05%,

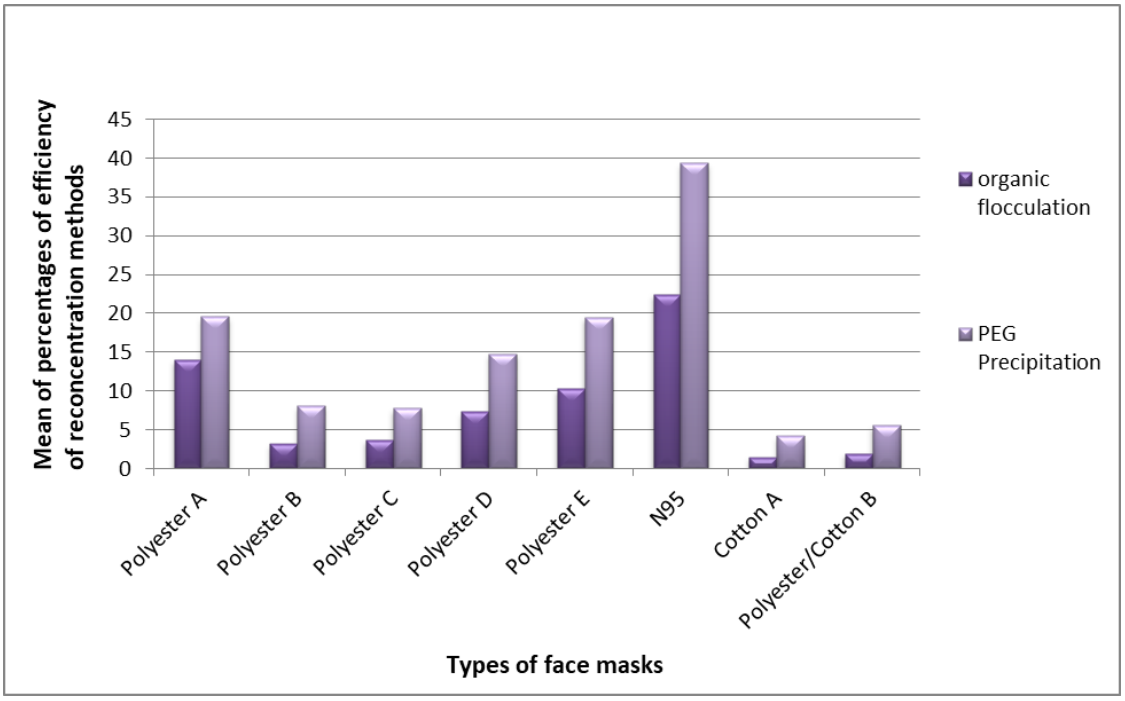
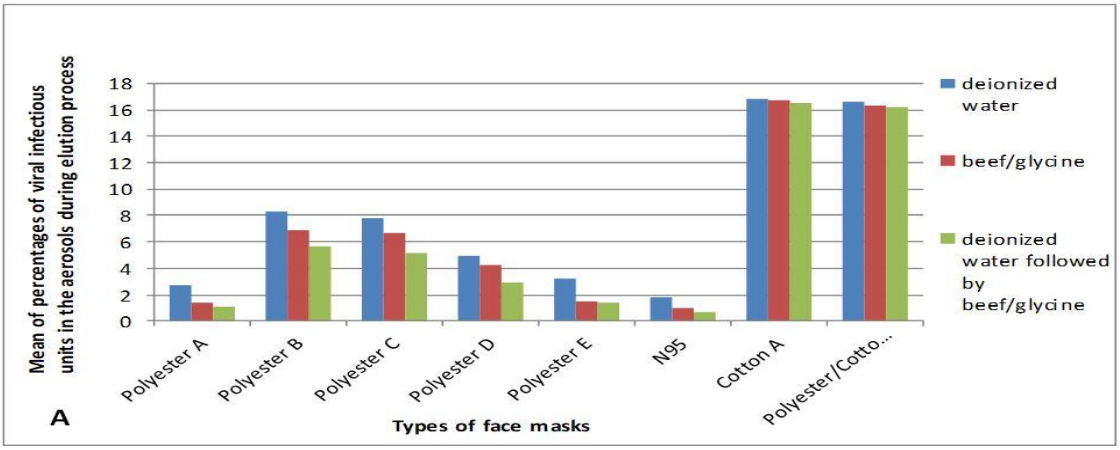
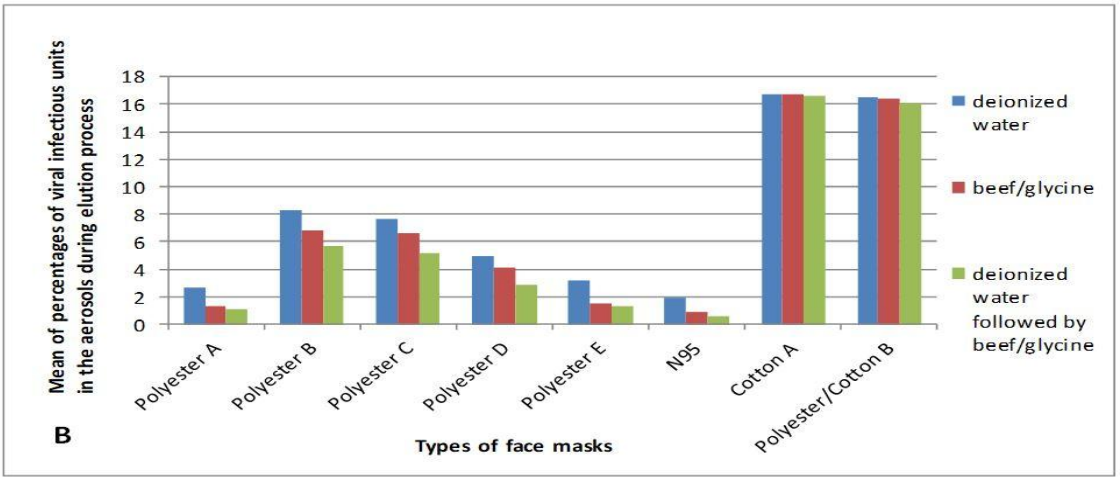


Figure 3 Percentages of bacteriophage phiX174 virus re-concentrated using either organic flocculation and/or PEG precipitation methods.

and 16.74% ± 0.09% when using deionized water, followed by beef/glycine, beef/glycine, and deionized water as different eluents respectively) and lowest when using N95 face masks (0.58% ± 0.04%, 0.95% ± 0.06%, and 1.88% ± 0.03% when using deionized water, followed by beef/glycine, beef/glycine, and deionized water as different eluents respectively) (Figure 4).





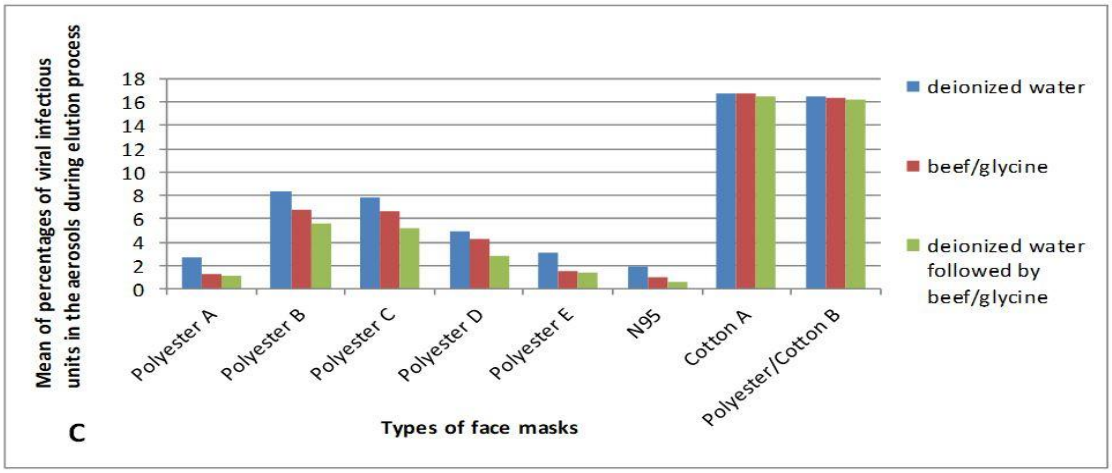


Figure 4 (A, B, and C) Number of bacteriophage phiX174 infectious units in the aerosols in the experimental cabin after using non-autoclaved and autoclaved face masks with different eluents. A) non-autoclaved and autoclaved for B) one cycle and C) five cycles

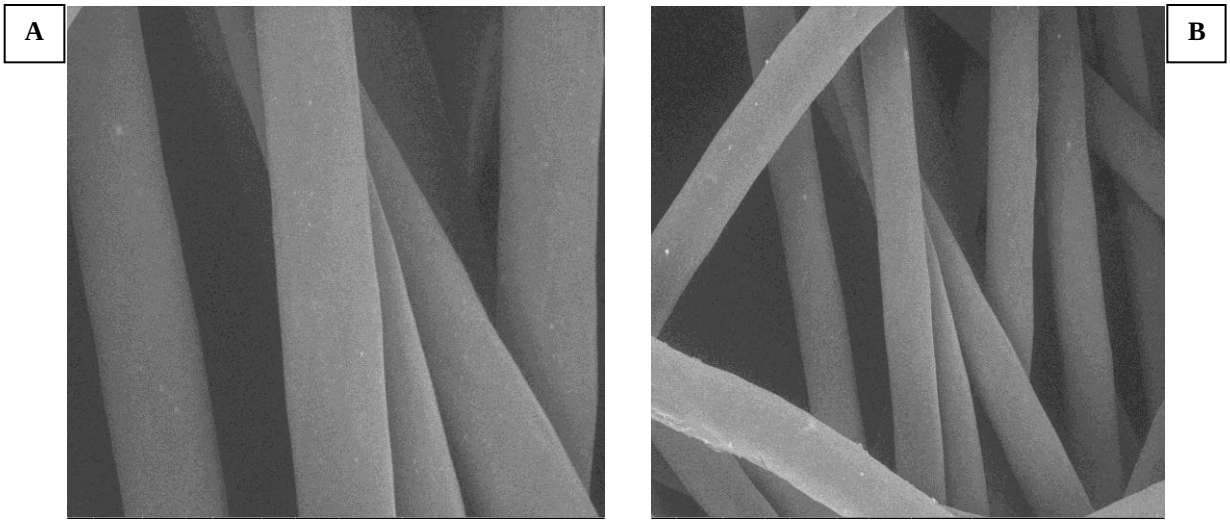


Figure 5 The threads (yarn) of a polyester face mask. A) Before and B) after autoclaving for five cycles

Table 1 Tensile strength, elongation, and air permeability of different sterile and non-sterile face masks

Types of face masks	before sterilization			After sterilization once			After sterilization five times		
	tensile strength (Kgm)	elongation	air permeability ($Cm^3/Cm^2/S$)*	tensile strength (Kgm)	elongation	air permeability ($Cm^3/Cm^2/S$)*	tensile strength (Kgm)	elongation	air permeability ($Cm^3/Cm^2/S$)*
Polyester A	17	15	333	12	13	ND	10	9	339.5
Polyester B	40	25	238	39.79	24.86	ND	39.65	24.43	239.5
Polyester C	27	30	124	27	29.89	ND	26.88	29.05	137.6
Polyester D	32	60	306	31.96	59.89	ND	31.99	59.81	357
Polyester E	25	35	70.4	24.89	34.79	ND	24.97	34.77	80.8
N95	37	20	ND	36.56	19.91	ND	35.99	19.95	ND
Cotton A	107	110	ND	106.02	108.54	ND	105.98	108.34	ND
Polyester/ Cotton B	45	80	ND	44.74	79.82	ND	44.03	79.05	ND

* Cubic centimeter of air per square centimeter of face mask per second; ND: Not done

Figure 5 shows the effect of five autoclaving cycles on polyester face masks evaluated using SEM, indicating no difference in the textile threads (yarns) before and after autoclaving. There were no changes in the yarn, such as cutting, grooves, breaks, and defects. Similar results were observed for all the studied face masks.

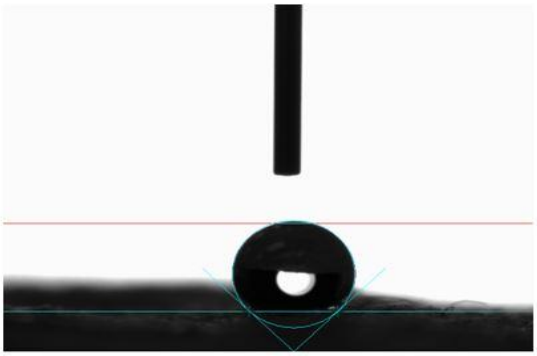
The diameter of the droplets containing bacteriophage phiX174 dispersed on the studied face masks ranged from 1 to 10 µm. Except for the polyester face mask A, there were no significant differences in both the tensile strength and elongation before and after autoclaving (for one or five cycles) in all the studied face masks (Table 1). The results of air permeability for the five polyester face masks tested before and after five autoclaving cycles indicate a slight increase in the air permeability of the masks autoclaved for five cycles compared to the non-autoclaved ones (Table 1).

Figures 6 A and B illustrate the right and left contact angles between 1 µl water droplets and the surface of different face masks, which were 0 values in the case of the cotton masks. The right and left contact angle values of the polyester/cotton masks were lower than those with polyester masks. The highest values were observed with the N95 mask.

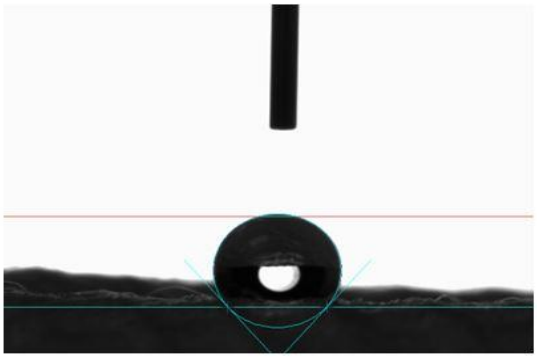
No significant differences were observed in both right and left angle values before and after five cycles of autoclaving for all the studied face masks. Previous studies have shown that using masks and social distancing can potentially reduce SARS-CoV-2 transmission and the number of associated cases (Choi and Ki 2020; Chu et al. 2020). Ma et al. (2020) reported that the virus-blocking rates of surgical and homemade masks were approximately 97% and 95%, respectively, by using an automated system that mimicked human breathing. Morais et al. (2021) reported that similar

Fig. 6 A

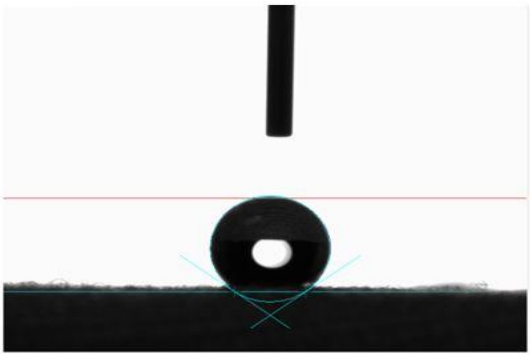
6 AA



6 AB



6 AC



6 AD

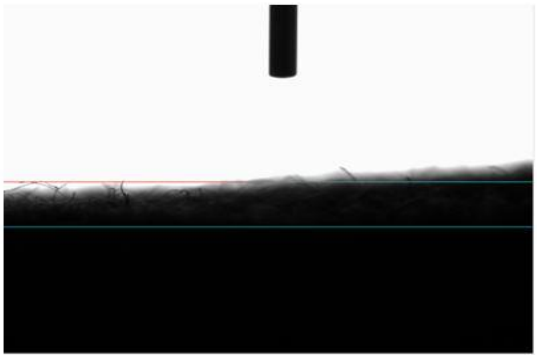


Fig. 6 B

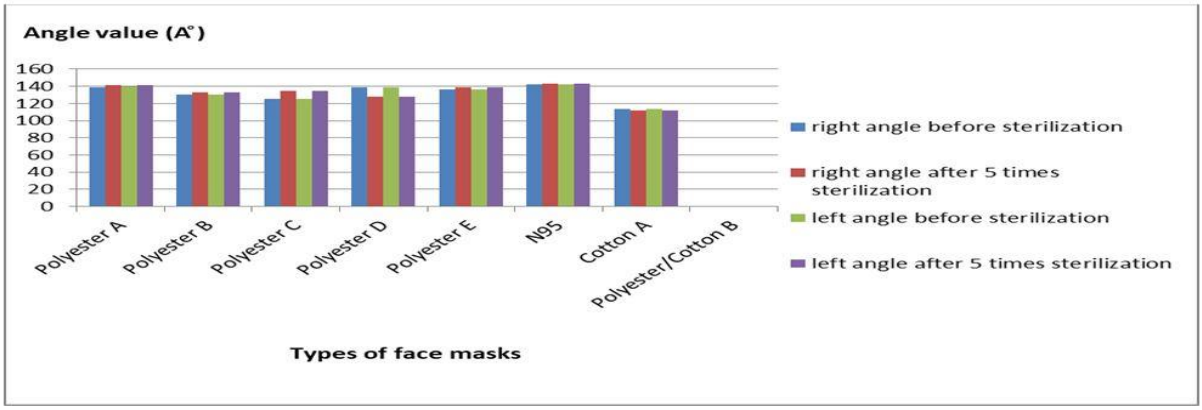


Figure 6 (A and B) Contact angle measurements of different face masks. The values for 6AA) polyester; 6 AB) N95; 6 AC) polyester/cotton mix, and 6 AD) cotton face masks

results were obtained when using a similar methodology for evaluating different types of masks, where surgical masks showed an 89% filtration rate while those for homemade masks ranged from 40% to 83%, depending on the fabric type. The first objective of this study is to validate a method to elute and re-concentrate bacteriophage phiX174 from polyester, polyester/cotton, and cotton face masks. Our results showed that deionized water followed by 3% beef glycine (pH 9.5 or pH 7.2) was the best eluent for bacteriophage phiX174 from all the studied face masks as it showed the highest elution efficiency. This was followed by

the single eluents, 3% beef glycine (at either pH 9.5 or pH 7.2), and finally, deionized water (lowest elution efficiency). This indicates that two consecutive eluents might increase the chance of eluting more viruses that are still adsorbed and/or absorbed on the yarns of the face masks as the viral particles remaining on the yarns of the face masks after elution using deionized water had to be re-eluted using another eluent (3% beef glycine) in our study. Our results also showed that 3% beef glycine (pH 9.5 or 7.2) is better than deionized water as a single eluent from different face masks. The beef glycine solution was previously used to elute enteric and non-enteric viruses from cotton gauze pad fibers used in the concentration of sewage and water samples (Coin 1967; Grabow 1968; Rao and Labzoffsky 1969; Hill et al. 1971; Liu et al. 1971; Ikner et al. 2012), with low viral adsorption efficiency. The higher recovery percentages in our study seen when beef glycine was used as an eluent either directly or after using deionized water compared to the previous studies might be due to the difference in the composition of the materials used in our study. It might be easier to elute viruses from the surface or yarns (threads) of polyester than cotton, as evidenced by the low recovery percentages of cotton masks. This might be attributed to the hydrophobic nature of polyester compared to the hydrophilic nature of cotton. Hence, polyester masks might prevent complete absorption of dispersed droplets (1 to 10 µm) carrying the viral particles, increasing the viral load on the mask itself, unlike that seen in the case of cotton masks. The contact angle of a liquid droplet applied to the substrate surface is affected by both fluid and substrate (Sarah and Ulrich 2018). There is a lack of equilibrium between drop and surface on absorbing substrates. Thus, a dynamic contact angle is measured, indicating the samples' hydrophobicity. The results of the right and left contact angles confirmed the hydrophobic nature of the polyester face masks, as higher contact angles were recorded for these than for polyester/cotton face masks. The contact angles were 0 for the cotton face masks, in which the droplets were completely absorbed. Liquid behavior on porous substrates is determined simultaneously by the spread of the substrate's surface and penetration into the bulk (Holman et al. 2002; Wijshoff 2018). Differences in yarn mass, density, and manufacturing methods within the different face mask types with the same composition might influence the efficiency of the elution process. This might explain the higher elution efficiency of bacteriophage phiX174 from the N95 face mask (100% polyester internally and externally) compared to the other commercial polyester face masks. At different pH values of beef glycine solution (pH 9.5 or pH 7.2), there were no significant differences in the viral loads in the eluate solutions from all the studied face masks. As hydrophobic interactions are considered to be the dominant forces stabilizing viral attachment to the membrane filters at high pH (Farrah et al. 1981), we choose to use beef glycine at pH values ranging from moderate alkaline (pH 9.5) to approximately neutral pH (7.2) as an eluent in this study.

The quantification process (plaque assay) for bacteriophage phiX174 depends on the quantification of the infectious viral particles. There was no significant difference between the results of the viral loads in the beef-glycine eluate or after using organic flocculation as a secondary concentration method. Previous reports indicated that beef extract might inhibit the molecular techniques and/or plaque assay of some enteric viruses when concentrated in water and wastewater. Plaque assays and molecular techniques are sensitive to organic inhibitors, such as humic and fulvic acids, which are naturally present in waste-, surface-, and tap water (Farrah et al. 1976; Sobsey and Glass 1984; Sobsey and Hickey 1985; Ikner et al. 2012). Richards and Weiheimer (1985) reported that 3% beef extract significantly reduced plaque counts and sizes. The heterogeneous organic composition of beef extract further contributes to this inhibition. This might be explained by the nature of some viruses, such as bacteriophage phiX174 which might not be affected by the inhibitory effect of beef extract during the plaque assay or by the lack of water or wastewater matrices containing organic inhibitors. Another possibility is the lower efficiency of organic flocculation compared to other methods, such as PEG precipitation as a secondary concentration process (Le Guyader et al. 2009; Pérez-Sautu et al. 2012; El-Senousy et al. 2013). This is consistent with our results showing significantly higher efficiency of the PEG precipitation method than the organic flocculation method as a re-concentration method.

Our results also showed an inversely proportional relationship between the viral loads in the aerosols in the experimental cabin with that eluted from the different face mask types. This might result in a higher presence of viruses in the aerosols, especially those un-adsorbed and/or unabsorbed in the face masks. Therefore, the higher elution rates, which might indicate higher adsorption and/or absorption of viruses on the face masks, might decrease the viral load in the aerosols. Herein, we used glass impingers with a flow rate of 12 L per min with 25 mL of 0.1% tryptone nutrient broth according to Harstad (1965) to concentrate viruses from the aerosols. This has been frequently used previously to concentrate viruses from aerosols (Tseng and Li 2005; Zhao et al. 2014; Bekking et al. 2019; Chen et al. 2021). However, till now, there is no standard method to concentrate viruses from aerosols. Recently, Raynor et al. (2021) reported that high-flow rate samplers recovered higher quantities of the virus than low-flow samplers. However, lower flow rate samplers were able to better measure the air concentrations of infectious viruses and viral RNA. To detect and accurately assess airborne viruses in animal agriculture and other settings, a two-sampler approach may be warranted. A high-flow sampler is likely to provide low limits of detection to determine if the virus is present in the air. If a virus is detected, a lower flow sampler might then be used to accurately measure airborne virus concentrations. In addition to viruses in aerosols, other viral numbers might still be adsorbed and/or absorbed into

the face masks, depending on the efficiency of the elution process. Another important factor is the efficiency of the concentration method for viruses from aerosols. These factors might explain the differences between the inoculated and quantified viruses either in the eluate solutions or in the aerosols.

Our second objective was to study the effect of sterilization of face masks at 126°C for 30 mins and at 0.15 Mpa on the viral elution efficiency from these masks as an application of the validated method. Our results indicated no significant differences in the viral elution efficiency from all studied face masks before, after one cycle, or five autoclaving cycles. This was evident when the elution efficiency of bacteriophage phiX174 from autoclaved and non-autoclaved face masks (after one cycle or five cycles of autoclaving) or the number of viruses in the aerosols were compared in parallel to the dispersion/elution process using non-autoclaved and autoclaved face masks (after one cycle or five cycles). This might indicate that autoclaving does not affect the efficiency of the face mask yarns to adsorb and/or absorb virus and that probably no changes occur in the yarns, such as cutting, grooves, breaks, and any other defects. This was confirmed in our study when the face masks were examined using SEM before and after five autoclaving cycles. This can also be validated by the results of textile composition before autoclaving and after one and five autoclaving cycles, in which no significant differences were observed almost in all face masks (polyester, polyester/cotton, and cotton). Also, no significant differences were observed in the tensile strength and elongation of all studied face masks before and after one and five autoclaving cycles. We also confirmed the lack of significant differences between the measured right and left contact angles for all the studied face masks before and after five autoclaving cycles. Moreover, there were no significant differences between the air permeability of the five studied polyester face masks before and after five autoclaving cycles. de Man et al. (2020) reported that multiple heat sterilization procedures did not change the permeability of face masks for small particles. Also, face masks can be reprocessed with minimal reduction of particle filtration efficiency by exposing them to 121°C steam for 15 mins or H_2O_2 plasma sterilization (van Straten et al. 2021), which enables the reuse of face masks. Moreover, families can easily do this at home using a standard pressure cooker. It might be difficult for some families, especially in poor countries, to follow the WHO guidelines for wearing and appropriately changing face masks because of the high cost of daily face masks for all family members.

Therefore, autoclaving the face masks at home using a pressure cooker might solve this economic problem. However, the efficiency and the filter breathability might be compromised by sterilization in an autoclave and ethanol treatment, especially for the filtered N95 face mask. Physical damages were observed in N95 respirators after autoclaving. The effect depends on several factors, such as particle size, breathing flow rate, protective device, and type of treatment (Grinshpun et al. 2020). This result contradicts ours, indicating no changes in air permeability for the five studied polyester face masks. However, we could not successfully test the air permeability test for N95 face masks. Riepe et al. (1999) showed that re-sterilization of polyester vascular grafts at 134°C with 2.4 bar steam pressure for 6 mins did not change the textile strength, single-filament strength, weight, infra-red spectroscopy, and electron microscopy of the surface. Therefore, it was concluded that it is safe to use once-autoclave-re-sterilized surplus un-coated polyester grafts, provided sterility is guaranteed. Moreover, Yen et al. (2022) also showed that 25 cycles of vaporized hydrogen peroxide reprocessing of 3M 1860/1860S N95 respirators did not compromise filtration efficiency, seal check, or qualitative and quantitative fit.

Conclusions

Our conclusions may be summarized as first, deionized water followed by beef-glycine 3% (at either pH 9.5 or pH 7.2) is a better eluent of bacteriophage phiX174 from polyester, polyester/cotton, and cotton face masks than beef-glycine 3% (at either pH 9.5 or pH 7.2) as a single eluent. Also, deionized water has a lower efficiency as a single eluent for the bacteriophage phiX174 virus for all the types of studied face masks. Second, PEG precipitation has higher efficiency than organic flocculation as a secondary concentration process for bacteriophage phiX174 eluted from different types of face masks (polyester, polyester/cotton, and cotton) using beef-glycine 3% (at either pH 9.5 or pH 7.2). So, elution of bacteriophage phiX174 from different types of face masks using beef-glycine 3% (at either pH 9.5 or pH 7.2) followed by PEG as a secondary concentration method achieved the highest percentage of the viral recovery. Finally, autoclaving face masks for up to five cycles (at 126°C for 30 minutes and 0.15 Mpa for each cycle) does not significantly affect their characteristics such as composition, tensile strength, elongation, air permeability, contact angles, and no changes between viruses in the eluate solutions when using autoclaved and non-autoclaved face masks even after five cycles of autoclaving.

Acknowledgment

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Author Contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Waled Morsy El-Senousy, Faten Hassan Hassan Abdellatif,

Hend Mohamed Ahmed, and Sherif Abd-Elmaksoud. The first draft of the manuscript was written by Waled Morsy El-Senousy and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Phytoremediation study of mining soils: case of the Mibladen and Zaida mine (High Moulouya, Morocco)

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ABSTRACT

The Upper Moulouya region, including the Mibladen (M) and Zaïda (Z) mines, is one of the highest lead (Pb) deposit areas in Morocco. These mines, abandoned without any measure of rehabilitation, constitute the main source of soil pollution by Metallic Trace Elements (MTEs) accumulation in the region. In this study, two greenhouse phytoremediation experiments (for the Mibladen and Zaida sites) were set up using Italian ryegrass (*Lolium multiflorum*) specie to assess its capacity and ability to remediate soils contaminated by zinc (Zn), cadmium (Cd), copper (Cu), and Pb. For both experiments, various factors including (i) three substrates (waste treatment [Wt]; clay uncovering [Cun]; and unpolluted control soil [Ucs]) and (ii) three treatments (no treatment, treatment with organic matter, and treatment with chemical fertilizers) were studied. The results before planting indicated that Wt substrates had poorer physicochemical properties than those of Cun, thus they are the most exposed to the degradation phenomena. This is confirmed by pollution index (PI) results that revealed the trend of PI (Z_{Wt})> PI (M_{wt})> PI (M_{Cun})> PI (Z_{Cun})> PI (Z_{Ucs})> PI (M_{Ucs}). The results of experiments indicated that ryegrass crops can grow on substrates contaminated with MTEs. Depending on the applied fertilizers, available metals, and the type of soil, the phytoremediation results showed that *L. multiflorum* can tolerate, hyperaccumulate, and translocate MTEs from polluted substrates. Our findings suggest that this plant can be a solution for remediating alkaline soils polluted by Cd, Pb, Zn, and Cu in Mediterranean conditions.

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1 Introduction

Soil is one of the most important sources for the continuity of life. However, this non-renewable natural resource, once degraded, could not be recovered (Rădoi 2021). Soils are subject to a range of threats such as erosion, loss of organic matter, compaction, acidification, salinization, and contamination (Khan and Al Shoumik 2022). Recent studies have shown that soil pollution is caused by many anthropogenic activities including mining, industrial activities, agriculture, smelting, fossil fuel combustion, and waste disposal (El Aafi 2016; Samsuri et al. 2019). These activities seriously threaten ecosystems and human health. Metallic Trace Elements (MTEs) from mining activity are one cause of soil pollution. Contrary to the organic elements, the MTEs are not biodegradable and accumulate for a long time in the soil. MTEs can also be transferred to human bodies causing serious health issues such as disabilities associated with malnutrition and Alzheimer's disease (Singh et al. 2023).

In Morocco, abandoned mine sites are a source of MTEs pollution (Laghlimi et al. 2022), particularly where no environmental management program has been put in place to reduce the extent of the impact. Located in Midelt city, the Mibladen and Zaïda mines, were the main source of lead mining during the 20th century. These mines were abandoned in 1985 without rehabilitation. Various studies have highlighted the potential risks posed by these mine tailings and the level of soil pollution by MTEs around abandoned sites. However, few research works have focused on the treatment of toxic and hazardous MTEs contamination from these mines using sustainable approaches based on natural processes such as phytoremediation. This last method directly uses green plants based on their ability to intercept, absorb, accumulate, sequester, stabilize, or transfer contaminants. Plants are particularly useful in the bioremediation process by preventing the spread of contaminants through climatic phenomena into nearby areas.

The main aim of this study was to highlight the capacity of Italian ray grass (*Lolium multiflorum*) to depollute the contaminated soils of the Mibladen and Zaïda mines with a focus on Cd, Pb, Zn, and Cu MTEs. The species chosen are grasses, which are generally used to be pioneers and adapted plants for covering polluted substrates.

2 Materials and Methods

2.1 Study areas

The sites chosen for this study are in the Upper Moulouya region, which is a lead mining district and includes the mines of Aouli, Mibladen, and Zaïda. This region contains the largest lead deposit in the country and covers an area of over 300 km^2 (figure 1).

The Zaida and Mibladen mine sites were selected for this study. The Zaïda mine (1490 m altitude; active exploitation was from 1972 to 1985) is located about 30 Km northwest of Midelt city. The temperature ranges from 6 to 36 °C and the mean annual precipitation is about 300 mm.

The mining area of Mibladen (1130 m altitude; exploitation was from 1936 to 1985) is situated 15 km Northeast of Midelt city and covers an area of 60 km^2. There is a foundry at this site, and the vegetation has been completely destroyed (El Aafi 2016). The climates of both sites are arid in nature.

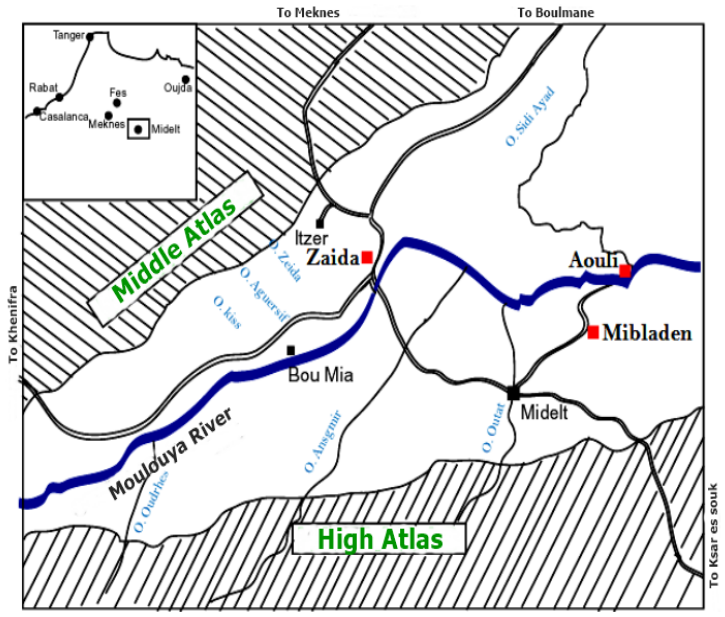

Figure 1 Geographic location of two studied sites Zaïda and Mibladen (El Aafi 2016)

2.2 Soil preparation and analysis

The metal contaminated substrates and unpolluted control soil (Ucs) samples were collected from a depth of 0-20 cm. Waste treatment (Wt) and clay uncovering (Cun) substrates were taken from the sites of Mibladen and Zaïda, while the Ucs was obtained from agricultural soil (uncultivated) of the National Institute of Agricultural Research (INRA) in Rabat, Morocco. Collected samples were taken to the soil laboratory and each one was air-dried, crushed, and sieved through a 2 mm sieve.

All samples were analyzed before planting the Italian ryegrass seeds to determine physicochemical proprieties and quantify MTEs. The physicochemical analysis of soils included the following: granulometry was measured by the Mériaux method (Mériaux 1954); organic matter (OM) was measured using the Walkley and Black method (Walkley and Black 1934); pH was measured using the potentiometric technique (in a soil/water ratios of $\frac{1}{2.5}$); electrical conductivity (EC) was determined with a conductivity meter in a saturated paste of soil $\frac{1}{5}$ (Montoroi 1997); total nitrogen (N) was obtained according to the Kjeldahl method (McGill and Figueiredo 1993); and the assimilable phosphorus (P) and the exchangeable potassium (K) were measured by Olsen et al. (1954) and Quemener (1979) procedures respectively. For MTEs quantification (Cd, Pb, Cu, and Zn), the Atomic Absorption Spectrometer (AAS Varian) method was used (Pinta 1976).

2.3 Greenhouse trials

For the greenhouse study, two experiments (one for the Zaïda site and one for the Mibladen site) were set up at INRA of Rabat. Various factors including (i) three substrates (Wt, Cun, and Ucs) and (ii) three treatments (no treatment, treatment with OM, and treatment with chemical fertilizers) were studied for each experiment. The number of fertilizers applied to the substrates used to sow Italian ryegrass seeds was calculated based on the physicochemical analysis parameters. In this study, four replicates were performed per pot. Fifteen grams per pot of OM along with peat was used for the Mibladen experiment and ovine manure for the Zaïda experiment. In the case of chemical fertilizers, we used 0.16 g/pot of ammonium phosphate and 0.50 g/pot of potassium sulfate for the substrates of Mibladen. For the Zaïda soils, we used 0.11g/pot of ammonium nitrate, 0.12 g/pot of potassium sulfate, and 0.08 g/pot of triple super phosphate. Five and seven seeds were grown in all substrates placed into plastic pots (15 cm × 20 cm, and 20 cm height) for Mibladen and Zaïda experiments respectively. The pots were irrigated three times per week with 200 ml of tap water from INRA. Harvesting time was six weeks for the Mibladen study site and seven weeks for the Zaïda study.

2.4 Plants and soil analysis

The below and the above-ground parts of the plant were cut, separated, and rinsed with distilled water. The fresh weight was measured and then left in the air to dry for four days before being dried in an oven at a temperature of 70 °C for 48 hours to evaluate their dry weight. Rhizospheric soil that adhered to the roots of *L. Multiflorum* was recovered and analyzed to determine the MTEs in substrates after planting. The bioavailability of the MTEs was measured using the AAS Varian method.

2.5 Phytoremediation indices calculation

In this study, two phytoremediation indices were calculated: i) the bioaccumulation factor (BAF) that evaluates the suitability of plants to accumulate MTEs from soil (Eq. 1), and ii) the translocation factor (TF) that shows the efficiency of a plant in transferring MTEs from the below-ground to the above-ground parts (Eq. 2). These indices were calculated using the following equations (Samsuri et al. 2019).

$$BAF=\frac{[Metal]inshoot}{[Metal]insoil} \quad (1)$$

$$TF=\frac{[Metal]inshoot}{[Metal]inroot} \quad (2)$$

2.6 Statistical analysis

All data obtained in this study were analyzed by applying the ANOVA test with a significant level of 5%. The software used in this study was Excel.

3 Results and Discussion

3.1 Physicochemical characteristics of the substrates

Results presented in table 1 showed the different substrate characteristics. Except for unpolluted control soil used for the Mibladen experiment (45.90 % clay), all the studied substrates were poor in clay (ranging from 7.2 to 22.7%) and the dominance of the sandy fraction was reported. For pH, the mining soils were weakly alkaline (pH ranges from 7.83 to 8.51). However, the suitable pH for the growth and development of plants in mining areas is near to neutral pH.

For other soil properties we found that the substrates were very poor in OM contents (ranging from 0.06 to 0.38 %); very low in total nitrogen (<0.1%); and potassium (2.81-54.22 $mg.kg^{-1}$) except Cun substrate of Mibladen site, which it was high in potassium (289.2 $mg.kg^{-1}$); and very low to low in phosphorous (0.39-11.5 $mg.kg^{-1}$ to 15.91$mg.kg^{-1}$). The main parameters (OM and NPK) are generally low for both sites. This could be because of mining activities. In addition, soils without vegetation which can reduce OM and NPK levels in the site soil can suffer the effects of intense

Table1 Basic physicochemical characteristics in the experiment substrates

Parameters	Wt		Cun		Ucs	
	M	Z	M	Z	M	Z
	Avg.± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD
pH	8.51 ± 0.42	8.0 9 ± 0.81	7.89 ± 0.71	7.83 ± 0.63	7.37 ± 0.74	7.80 ± 0.70
EC (S/m)	0.033 ± 0.00	0.02 ± 0.00	1.94 ± 0.21	0.40 ± 0.03	0.07 ± 0.00	0.18 ± 0.02
Clay (%)	12.20 ± 0.73	7.20 ± 0.36	22.70 ± 2.27	18.60 ± 1.67	45.90 ± 5.97	10.60 ± 0.85
Silt (%)	15.70 ± 1.41	27.70 ± 2.49	27.30 ± 2.73	29.30 ± 2.64	31.10 ± 1.56	2.60 ± 0.18
Sand (%)	72.10 ± 4.33	65.10 ± 7.16	50.00 ± 2.50	52.10 ± 2.61	23.00 ± 2.07	86.80 ± 8.68
OM (%)	0.06 ± 0.00	0.08 ± 0.00	0.11 ± 0.01	0.38 ± 0.03	0.66 ± 0.04	1.02 ± 0.12
N (mg kg^{-1})	0.00 ± 0.00	56.62 ± 3.40	210.00 ± 14.70	134.92 ± 13.49	900.00 ± 63.00	78.30 ± 7.83
P (mg kg^{-1})	10.80 ± 0.76	0.39 ± 0.02	11.50 ± 1.04	15.91 ± 1.11	110.70 ± 5.54	41.53 ± 2.49
K (mg kg^{-1})	54.23 ± 5.42	2.81 ± 0.20	289.20 ± 17.35	9.24 ± 0.55	704.93 ± 35.25	5.62 ± 0.28

The values represent the mean of four replicates for each parameter. M: Mibladen; Z: Zaïda; Wt: waste treatment, Cun: clay uncovering, Ucs: unpolluted control soil; Avg.: Average; EC: electrical conductivity, OM: organic matter, N: nitrogen, P: available phosphorus and K: exchangeable potassium

erosion and can then be transported and dispersed and reach the water resources and the neighboring soils. Low nutrients in soils, particularly nitrogen and phosphorous, can be a limiting factor for plant growth.

In terms of soil health and physicochemical characteristics, the findings of this work indicated that the waste treatment substrates are poor (more alkaline, poor in clay content, very poor in OM and N, P, and K elements) than those of clay uncovering. These waste treatment soils are therefore the most exposed to degradation phenomena.

3.2 MTEs content before and after cultivation

The concentration of four MTEs, i.e., Cu, Zn, Cd, and Pb from the mining sites were determined to characterize the current MTEs of contaminated substrates and the effect of *L. multiflorum* on the phytoremediation of these MTEs (table 2). The results before planting showed a difference in MTEs concentrations between the tested substrates. Regardless of the site studied, the MTEs were higher in waste treatment compared to clay uncovering as confirmed by pollution index values (5.76 and 7.54 for Wt versus 1.27 and 1.18, for Cun for Mibladen and Zaïda respectively) (Table 3). This is probably attributed to the heterogeneous nature classically met for different mining substrates. A pollution index greater than one means that the soil is considered contaminated (Chon et al., 1998). In the case of this study, the substrates studied have a pollution index greater than one except for the unpolluted control soil, thus, they are all considered contaminated, particularly the waste treatment substrates. The level of pollution index is as $PI_{Z\text{-}Wt} > PI_{M\text{-}Wt} > PI_{M\text{-}Cun} > PI_{Z\text{-}Cun} > PI_{Z\text{-}Ucs} > PI_{M\text{-}Ucs}$. Similar findings were reported by EL Hachimi et al. (2013) who showed that except for the reference station all soil samples taken at the mining sites including those taken at 130 Km, have a PI higher than 1, which confirms the polymetallic contamination of the soils in the downstream area of the mine sites.

The pollution indices of Zaïdasite show that is more contaminated than the Mibladen site. This pollution is due to the very high levels of lead at Zaïda compared to Mibladen (2787.60 vs 1881 $mg.kg^{-1}$ for Wt and 350.04 vs 221.00 $mg.kg^{-1}$ for Cunat the Zaïda and Mibladen sites respectively). Further, the other MTEs (Cd, Cu, and Zn) are rather high in the Mibladen site compared to Zaïda. This finding was the same for both substrates.

From these results, it can be concluded that the plumbiferous deposit of Haut Moulouya of Morocco, which was abandoned without any pollution control or rehabilitation, constitutes an environmental and human hazard, especially when these areas are used for livestock.

Cultivation of Italian ryegrass on polluted substrates collected from both study sites resulted in a decrease in all MTEs concentrations. The greatest reductions in MTEs were achieved due to phytoremediation by the tested plant, Italian ryegrass, in the Mibladen site. This indicates that most metals had the best reductions without fertilizers for both substrates. The exceptions were Pb (for Wt), which had a better reduction when chemical fertilizers were added, and Zn (for Wt) and Cd (for Cun), which had a better reduction when OM was added. Whereas the results obtained for the Zaïda study site showed that apart from Zn of waste treatment (reduction with OM added), the best reductions in MTEs were obtained by the treatment with chemical fertilizers. However, the analysis of variance indicated that the different studied substrates

Table 2 MTEs contents ($mg.kg^{-1}$) in rhizospheric soils, before and after cultivation

MTEs ($mg.kg^{-1}$)	Bowen 1979 standards ($mg.kg^{-1}$)	Soil type	Before experiments		After experiments					
			Initial situation		No treatment		With OM treatment		With chemical Fertilizers treatment	
			M	Z	M	Z	M	Z	M	Z
			Avg.± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD
Cadmium	0.35	Wt	8.70±0.90	4.00±0.60	6.00±0.40	3.53±0.20	6.95±0.80	2.49±0.10	8.30±0.70	2.53±0.20
		Cun	6.90±0.60	2.00±0.60	6.80±0.50	1.74±0.10	6.00±0.70	1.45±0.40	6.05±0.80	1.02±0.30
		Ucs	5.40±0.70	4.60±0.30	2.50±0.20	2.99±0.70	3.70±0.70	3.03±0.60	4.00±0.50	3.03±0.20
Copper	30.00	Wt	33.00±4.30	30.30±3.60	18.00±1.30	30.23±3.80	24.50±3.40	29.92±2.30	29.50±3.40	20.27±2.30
		Cun	21.00±3.30	20.00±4.00	7.00±0.50	17.40±1.00	19.00±2.80	17.45±0.90	19.50±2.10	12.21±1.60
		Ucs	11.00±1.80	12.20±2.20	5.50±0.40	9.96±1.20	10.50±1.90	10.10±0.50	4.20±0.60	10.09±1.80
Lead	35.00	Wt	1881.00±21.00	2787.60±49.00	1632.00±114.20	2559.05±201.10	1640.00±89.00	2513.45±146.30	928.00±66.70	2442.78±132.00
		Cun	221.00±7.00	350.00±17.00	208.50±14.60	313.20±18.80	214.50±17.70	329.53±19.10	217.20±14.70	274.67±19.20
		Ucs	51.00±2.80	101.40±9.00	47.30±3.30	99.55±7.00	50.50±6.70	80.81±5.10	47.00±3.30	100.89±7.20
Zinc	90.00	Wt	300.00±23.00	191.90±21.00	296.00±20.70	181.35±8.80	265.00±14.70	149.61±8.60	273.50±21.10	131.77±8.50
		Cun	110.00±12.00	100.00±11.00	89.50±6.30	69.60±6.70	98.00±5.70	87.23±9.40	104.50±8.30	81.38±5.20
		Ucs	49.00±3.90	50.70±7.00	46.50±3.30	39.82±4.70	48.00±5.30	30.30±2.30	38.00±3.70	40.36±4.60

M: Mibladen; Z: Zaïda; Avg.: Average; Wt: waste treatment; Cun: clay uncovering; Ucs: unpolluted control soil

Table 3 pollution indices of the substrates

	Pollution index (PI)	
	Mibladen	Zaïda
Waste treatment	5.76	7.54
Clay uncovering	1.27	1.18
Unpolluted control soil	0.65	0.71

have a significant effect on the MTEs concentrations, contrary to fertilizers, which have a non-significant effect.

Generally, the decrease in MTEs content in rhizospheric versus non-rhizospheric substrates could be explained by the consumption of these elements by *L. multiflorum*.

3.3 Effectsof MTEson root and shoot biomass of plant ryegrass

Biomass production is an indicator of a plant's tolerance to different types of substrates. In contact with contaminated soils, plants react differently depending on the variety chosen. In this study, we found that Italian ryegrass can grow on substrates contaminated with MTEs (Figure 2).

When comparing the biomass of harvested plants of the studied substrates with and without added fertilizers, it was reported that the addition of fertilizers increases both root and shoot biomass in all substrates. For the Mibladen experiment, the reduction in root and the shoot biomass for each treatment was reported in the order of $B_{Ucs} > B_{Wt} > B_{Cun}$. Similar findings were reported for the Zaïda experiment when chemical fertilizer was added, while the biomass order (for root and shoot) for the rest of the results under the Zaïda experiment was variable. According to statistical analysis, significant effects of substrates on root and shoot biomass were found in the Mibladen experiment, while the Zaïda experiment showed a non-significant effect. Regarding the fertilizer's effect on the ryegrass biomass, no significant effect was recorded for both experiments but the application of fertilizers increased the biomass

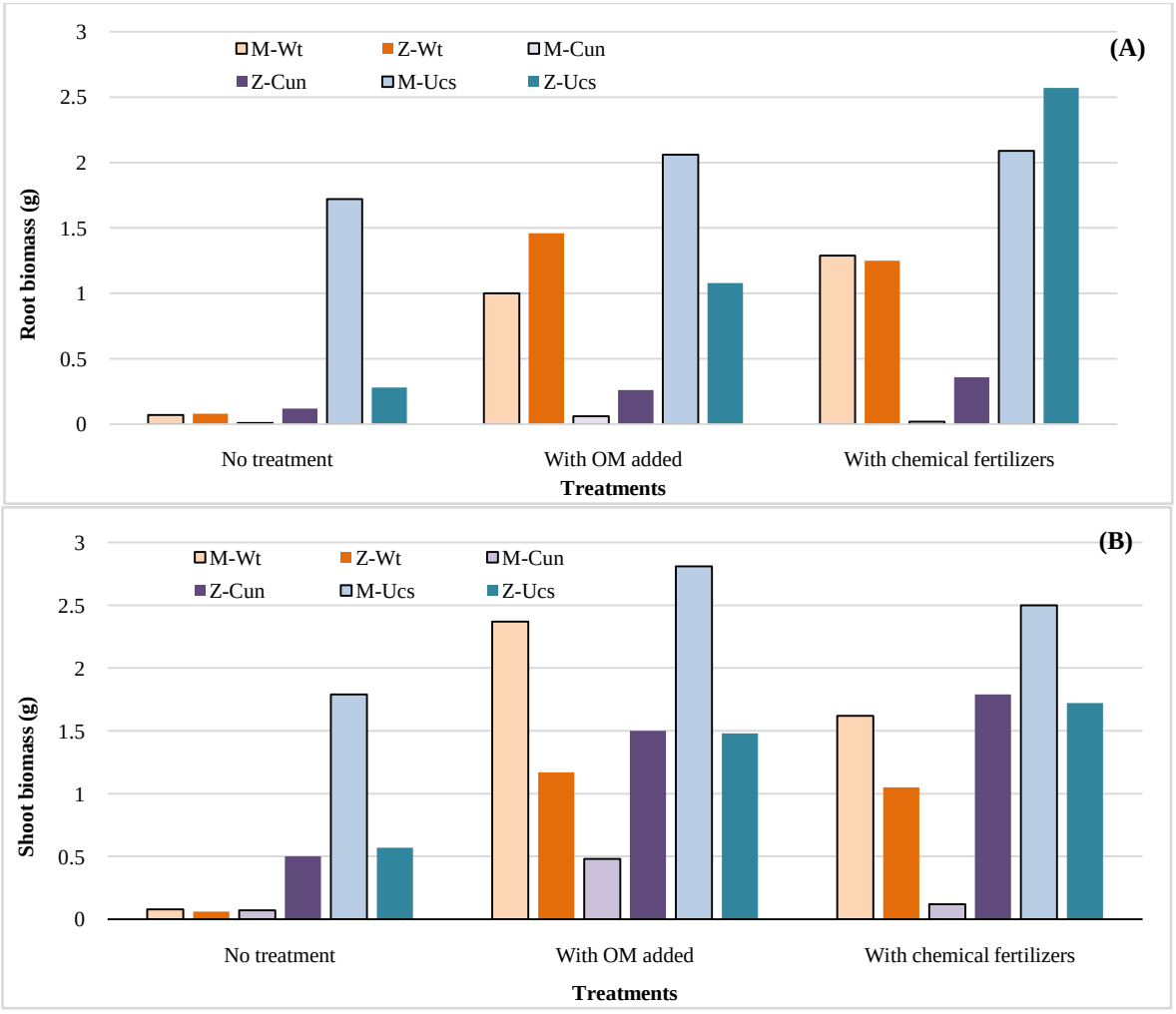

Figure 2 Plant biomass in studied substrates and under different treatments for both experiments; (A) Root biomass, (B) Shoot biomass; M: Mibladen, Z: Zaïda; Wt: waste treatment, Cun: clay uncovering, Ucs: unpolluted control soil

of below- and above-ground parts of the plant. This means that type of substrate affects the biomass of plant organs with and without fertilizers. The difference in these biomass values between substrate types is due to the nutrient contents and organic matter available to the Italian ryegrass plant in each substrate and the structure and texture of each substrate.

3.4 Phytoremediation of MTEs by Italian ryegrass

3.4.1 MTEs content in ryegrass plant organs

The MTEs concentrations in the Italian ryegrass cultivated in the substrates of the contaminated mining sites were measured to

Table 4 MTEs contents in ryegrass roots with treatment effects for both experiments

MTEs (mg.kg^{-1})		No treatment		With OM treatment		With chemical Fertilizers treatment	
		M	Z	M	Z	M	Z
		Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD
Cadmium	Wt	6.80±0.68	0.03±0.00	8.60±0.77	1.95±0.18	8.70±0.61	1.91±0.11
	Cun	7.00±0.63	0.11±0.01	7.80±0.86	0.11±0.01	6.80±0.34	0.31±0.02
	Ucs	7.90±0.47	0.10±0.01	7.25±0.36	0.62±0.06	7.45±0.60	4.73±0.33
Copper	Wt	27.00±2.97	0.10±0.01	65.50±6.55	0.00±0.00	94.00±4.70	7.63±0.53
	Cun	28.00±1.68	0.53±0.03	16.00±0.80	0.32±0.02	28.00±2.52	0.99±0.10
	Ucs	27.00±1.89	0.56±0.03	36.00±2.88	0.19±0.02	36.50±2.19	1.26±0.15
Lead	Wt	343.00±27.44	0.39±0.02	1570.00±109.90	53.63±5.36	1575.00±110.25	587.66±58.77
	Cun	49.00±4.41	3.05±0.34	100.00±9.00	3.20±0.22	49.00±4.41	5.34±0.32
	Ucs	101.50±10.15	0.56±0.04	66.00±3.96	9.37±0.56	75.00±3.75	157.77±12.62
Zinc	Wt	120.00±8.40	0.49±0.04	830.00±83.00	53.63±2.68	730.00±73.00	232.78±20.95
	Cun	79.00±7.90	3.05±0.31	530.00±63.60	0.29±0.02	79.00±5.53	5.34±0.53
	Ucs	95.00±4.13	0.67±0.05	110.00±9.90	54.64±4.37	174.00±17.40	389.17±35.03

OM: organic matter; Avg.: Average; M: Mibladen, Z: Zaïda; Wt: waste treatment, Cun: clay uncovering, Ucs: unpolluted control soil.

Table 5 MTEs contents in the above-ground part of ryegrass with treatment effects for both experiments

MTEs (mg.kg^{-1})		No treatment		With OM treatment		With chemical Fertilizers treatment	
		M	Z	M	Z	M	Z
		Avg. ±SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD	Avg. ± SD
Cadmium	Wt	6.20±0.43	0.31±0.02	7.55±0.45	0.70±0.05	8.15±0.49	0.55±0.03
	Cun	7.00±0.56	0.05±0.00	7.70±0.77	0.93±0.08	7.10±0.43	1.03±0.07
	Ucs	6.85±0.41	0.85±0.04	6.80±0.34	1.06±0.05	7.30±0.66	1.24±0.10
Copper	Wt	18.00±1.98	0.52±0.05	27.50±2.75	1.00±0.08	41.50±2.08	1.09±0.08
	Cun	18.00±1.08	2.00±0.12	40.00±2.00	4.64±0.42	23.00±2.07	5.89±0.41
	Ucs	15.50±1.09	1.13±0.06	23.50±1.88	1.77±0.12	18.00±1.62	3.56±0.32
Lead	Wt	120.00±12.00	0.52±0.04	180.00±12.60	12.00±1.20	168.50±11.80	8.72±0.87
	Cun	81.00±7.29	13.00±11.70	87.00±7.83	4.64±0.32	109.00±10.90	76.57±8.42
	Ucs	62.00±6.20	2.26±0.16	78.00±4.68	5.32±0.32	61.50±3.08	5.33±0.32
Zinc	Wt	131.00±11.79	2.08±0.19	410.00±36.90	18.00±1.62	450.00±45.00	29.43±2.65
	Cun	350.00±42.00	9.00±0.90	300.00±33.00	11.60±0.70	250.00±22.50	23.56±2.12
	Ucs	94.50±9.45	5.65±0.40	72.00±5.76	14.18±1.13	84.50±6.76	14.22±1.28

OM: organic matter; Avg.: Average; M: Mibladen, Z: Zaïda; Wt: waste treatment, Cun: clay uncovering, Ucs: unpolluted control soil.

evaluate the capacity of this plant to absorb these elements by their organs. The MTEs content in the root and the above-ground parts are summarized in tables 4 and 5 respectively.

From these tables, it can be seen that very high levels of MTEs were found in both parts of the plant grown at the Mibladen site compared to the Zaïda site. For the Mibladen site, the MTEs ranged from 6.80 to 1575.00 $mg.kg^{-1}$ for below-ground ryegrass parts and from 6.20 to 450.00 $mg.kg^{-1}$ for the above-ground parts. For the Zaïda site these elements ranged from 0.00 to 587.66 $mg.kg^{-1}$ for the below-ground parts and from 0.05 to 76.57 $mg.kg^{-1}$ for above-ground ryegrass parts. In addition, in the Mibladen substrates for the remaining treatments, most of the MTEs contents are higher in the below-ground parts compared to the above-ground parts of *L. multiflorum*. A contrary finding was observed in the substrates of the Zaïda site without fertilizers. Some MTEs concentrations are reversed in favour of the roots with the application of fertilizers, especially in the case of chemical fertilizers.

In general, it was reported that Cd, Pb, Zn, and Cu accumulations in the Italian ryegrass organs depend on the type of MTEs, soil characteristics, plant species, and climatic conditions. To evaluate their accumulation and their transfer to each ryegrass plant organ, the calculation of phytoremediation indices is necessary.

3.4.2 Bioaccumulation and translocation factors

The BAF and TF values of MTEs in the study areas are shown in Tables 6 and 7 respectively. Comparing the effect of the three treatments and the type of contaminated substrates, similar findings are noticed for the Zn and Cu elements. Indeed, for these elements, the highest BAF were found in the substrates of clay uncovering without fertilizers for the Mibladen site (3.911 and 2.571 for Zn and Cu respectively) whereas, in the Zaïda site, these elements were found to be high in waste treatment substrates when chemical fertilizers were applied (0.290 and 0.482 for Zn and Cu respectively). For the Mibladen experiment, the highest BAF was found in the unpolluted control soils with chemical fertilizers added (4.286 and 2.224 for Cu and Zn respectively). In the Zaïda experiment, the BAF was highest in the control soils when OM was added (0.468 for Zn). Regardless of the studied site, the highest BAFs were for Cd (1.283 with OM added and 1.010 with chemical fertilizers for the Zaïda site) and Pb elements (0.505 and 0.279 in the Mibladen and Zaïda sites respectively when chemical fertilizers were applied) recorded in clay uncovering substrates when fertilizers were added. Regarding the highest concentrations of these last toxic elements in the unpolluted control soils, they were recorded for the Pb with OM added (1.545 and 0.066 in Mibladen and Zaïda respectively), and for Cd without fertilizers (2.740) in Mibladen site and when chemical fertilizers were applied (0.409) in Zaïda site.

The huge difference in the concentrations of the MTEs accumulated by the plant can be attributed to the MTEs content in substrates. Previous studies reported that a plant species can be considered as an accumulator and hyperaccumulator for MTEs when BAF and BAF/TF are greater than one respectively (Samsuri et al. 2019; Laghlimi et al. 2022). In addition, Samsuri et al. (2019), reported that plants can be tolerant when they can grow well in the presence of high concentrations of toxic metals. This can lead to the immobilization of MTEs in the soils.

Table 6 Bioaccumulation factor in ryegrass plant grown on contaminated substrates with treatment effects for both experiments

		No treatment		With OM added		With chemical Fertilizers	
		M	Z	M	Z	M	Z
Cadmium	Wt	1.04	0.09	1.09	0.28	0.98	0.22
	Cun	1.04	0.03	1.28	0.64	1.17	1.01
	Ucs	2.74	0.28	1.84	0.35	1.83	0.41
Copper	Wt	1.00	0.02	1.12	0.03	1.41	0.05
	Cun	2.57	0.11	2.11	0.27	1.18	0.48
	Ucs	2.82	0.11	2.24	0.18	4.29	0.35
Lead	Wt	0.07	0.00	0.11	0.00	0.02	0.00
	Cun	0.39	0.04	0.41	0.01	0.50	0.28
	Ucs	1.31	0.02	1.54	0.07	1.31	0.05
Zinc	Wt	0.44	0.01	1.55	0.12	1.65	0.22
	Cun	3.91	0.13	3.06	0.13	2.39	0.29
	Ucs	2.03	0.14	1.50	0.47	2.22	035

M: Mibladen; Z: Zaïda; Wt: waste treatment; Cun: clay uncovering; Ucs: unpolluted control soil.

Table 7 Translocation factor in ryegrass plant grown on contaminated substrates with treatment effects for both experiments

		No treatment		With OM added		WithchemicalFertilizers	
		M	Z	M	Z	M	Z
Cadmium	Wt	0.91	10.33	0.88	0.36	0.94	0.29
	Cun	1.00	0.45	0.99	8.45	1.04	3.32
	Ucs	0.87	8.50	0.94	1.71	0.98	0.26
Copper	Wt	0.67	5.20	0.42	-	0.44	0.14
	Cun	0.64	3.77	2.50	14.50	0.82	5.95
	Ucs	0.57	2.02	0.65	9.32	0.49	2.83
Lead	Wt	0.35	1.33	0.11	0.22	0.11	0.01
	Cun	1.65	4.26	0.87	1.45	2.22	14.34
	Ucs	0.61	4.04	1.18	0.57	0.82	0.03
Zinc	Wt	1.09	4.24	0.49	0.34	0.62	0.13
	Cun	4.43	2.95	0.57	40.00	3.16	4.41
	Ucs	0.99	8.43	0.65	0.26	0.49	0.04

M: Mibladen; Z: Zaïda; Wt: waste treatment; Cun: clay uncovering; Ucs: unpolluted control soil

Phytostabilization aims to stabilize pollutants chemically and physically by reducing bioavailable MTEs from sand preventing erosion (El Aafi 2016).

In the Mibladen experiment, *L. Multiflorum* can be classified as a hyperaccumulator plant especially in the clay uncovering substrates without fertilizers for Zn (3.911) and Cu (2.571) and Cd (1.283) with OM added. The *L. Multiflorum* had a low BAF value for Pb (BAF < 1) in the same experiment for all the treatments and contaminated substrates of the Zaïda experiment.

Regarding the highest TF, it was reported very high (TF>>1) in clay uncovering for the Zaïda experiment (40.00; 14.50, and 8.46 when OM was added for Zn; Cu, and Cd respectively and 14.34 for Pb with chemical fertilizers). Generally, for the contaminated substrates and treatments effect, most of the TFs are higher than one for the Zaïda experiment, while in the case of the Mibladen site TF value was lower than 1 for most of the results.

4 Conclusion

Phytoremediation allows the treatment and stabilization of mine tailings. The findings of this study showed that the Zaïda and Mibladen areas are highly contaminated with MTEs. This is because they were abandoned without any pollution control or rehabilitation. These MTEs accumulate with time and will seriously affect the food chain. From these results, it can be concluded that *L. Multiflorum* is more suitable for Zn, Cu, and Cd phytoextraction from the soils of Mibladen than those of Zaïda, especially in clay uncovering versus waste treatment. Meanwhile, this plant grown under clay uncovering with OM added is shown as a better phytotranslocator for the three MTEs (Zn > Cu > Cd) in the Zaïda experiment than in the Mibladen experiment. Further, the overall observation of BAF and TF of Pb indicated that *L. Multiflorum* is more tolerant and less of a phytotranslocator for this metal in Mibladen than Zaïda, especially in clay uncovering with chemical fertilizers. The phytoremediation results indicated the ability of *L. Multiflorum* to tolerate, hyperaccumulate, and translocate MTEs in the mining soils of Haut Moulouya (Morocco). This ryegrass plant can be used as a phytoremediator plant to establish an ecological cover in this area by accumulating or preventing the migration of Zn, Cu, Cd, and Pb with the slope and their diffusion in the neighboring agriculture areas.

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Conflict of Interest

The authors attest that there is no conflict of interest regarding the publication of this manuscript.

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Synergistic anticancer effect of combination treatment of vitamin D and pitavastatin on the HCC1937 breast cancer cells

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Cell cycle arrest

ABSTRACT

Vitamin D (Vit D) has anticancer properties including activating cell senescence inhibiting cancer cell proliferation, inducing apoptotic cell death, and decreasing cancer cell migration. On the other hand, statins showed favorable anticancer activities including anti-survival, anti-proliferation, and anti-migration effects. The current study aimed to investigate the synergistic anticancer effect of Vit D and statins against HCC1937 triple-negative breast cancer cells. The antiproliferative effect was tested by MTT assay after 48 hours of the treatments. Trypan blue test and clonogenic assay were used to test the anti-survival activities of the treatments. The ability of the treatments to inhibit the migration ability was tested by scratch assay. Levels of the cell cycle and apoptotic markers were determined by western blotting. Results of the study revealed that all the tested compounds including Vit D, atorvastatin (Ator), simvastatin (Simv), and pitavastatin (Pita) inhibited HCC1937 breast cancer cell growth with different IC_{50} values ranging from 4.49-12.95 µM. Combined application of Pita and Vit D showed potent synergistic antiproliferative activities against HCC1937 breast cancer cells. The combined therapy of (1µM Vit D and 2 µM Pita) inhibited HCC1937 cell proliferation by cell cycle arrest and apoptosis as evidenced by increasing p21, p53, and cleaved PARP. Finally, the combined treatment decreased the p-STAT3 level in HCC1937 breast cancer cells. The results of the study can be concluded that the combined treatment of Pita and Vit D has a synergistic anticancer effect against HCC1937 breast cancer cells.

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1 Introduction

Nowadays, breast cancer is the most common invasive cancer in worldwide women. Based on WHO 2021 statistics, cancer is the second leading cause of death globally, and among the most commonly reported cancers, female breast cancer (**12**%) is the most frequently diagnosed cancer in the world (Sung et al. 2021). In Palestine, Breast cancer was the third largest cause of cancer mortality in 2018 at 12% and a more than 135% rise in breast cancer cases is expected by 2040 (AlWaheidi 2019). Immunohistochemical analysis revealed that breast cancers that express the estrogen receptor, progesterone receptor or both respond well to hormone therapy(Aliwaini et al. 2019; Porras et al. 2021). Another important progress in breast cancer therapy was identifying and targeting the Her2 subtype of epidermal growth factor receptors (EGFR) which improved the outcome of Her2-positive patients (Aliwaini et al. 2021). Triple-negative breast cancers (TNBCs) are called such because they lack receptors for estrogen, progesterone, and Her2. TNBCs are highly aggressive and resistant to conventional chemotherapy and are more common in individuals of African descent (Seachrist et al. 2021). It is important to note that more than 70% of TNBCs overexpress genes implicated in metastasis and invasion as well as genes involved in proliferation and resistance to apoptosis including AKT, PI3K, RAS, and NF-Kb (Porras et al. 2021). More importantly, mutations in p53 is reported to be one of the most common features of TNBCs and several studies indicate that these mutant p53 proteins enhance tumorigenesis and treatment resistance (O'Grady et al. 2020). Nowadays many researchers studied several anticancer agents and evaluated both monotherapy and combination therapy with currently used therapies to treat cancers and reduce the development of cancer cells in many organs (Aliwaini 2020).

Vitamin D (calcitriol) can be either endogenously synthesized in the skin from 7-dehydrocholesterol, upon exposure to the sun's ultraviolet light, or, in a small portion, can be absorbed from the diet (Young et al. 2021). There are two types of Vit D; the main form 25-hydroxycholecalciferol (25 (OH) D) and hormonal form 1, 25-dihydroxycholecalciferol (1, 25 $(OH)_2D$) in the liver and kidneys (Young et al. 2021). Several diseases have been found in animal studies to respond positively to Vit D and 1, 25 $(OH)_2D$ or their analogs, there have also been promising observations regarding adequate Vit D nutrition and cancer prevention that affects the development of cancer (Liu et al. 2021).

O'Brien et al.(2022) stated that statins are routinely used to treat hyperlipidemia, but may also have antineoplastic effects. It can also be applied to the treatment of other diseases, particularly cancer, in which progression depends on increased migration, survival, and ultimately proliferation (Göbel et al. 2019). Statins competitively inhibit the rate-limiting enzyme of the mevalonate pathway, 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) leading to low levels of isoprenoids geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) (Guerra et al. 2021; Razali et al. 2018). The current study tested the possible synergistic cytotoxic effect between statins (simvastatin, atorvastatin, and pitavastatin) and Vit D. The most potent combination of Vit D and pitavastatin was further investigated.

2 Materials and methods

2.1 Cell culture

Breast cancer cells HCC1937 were maintained in RPMI1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 IU/Ml penicillin, and 100 µg/mL streptomycin. Cells were cultured at 37°C in a humidified CO_2 incubator and subcultured every 3-5 days.

2.2 Cytotoxicity assay

Cell viability was determined by measuring the capacity of reducing enzymes present in viable cells to convert MTT to formazan crystals as described by Aliwaini et al. **(**2021). Briefly, cells were incubated with increasing concentrations of the following combinations Vit D + simvastatin, Vit D + atorvastatin, or Vit D + pitavastatin and incubated for 48 hrs, then 10 µL MTT was added to each well. After 4 hrs of incubation at 37°C, 100 µL of the solubilizing solution was added to each well, with shaking for 1 hr to dissolve the formazan crystals. The color intensity of the blue formazan solution formed in each well was measured at 570/690 nm using a BIO-TEK Instruments EL 312e microplate reader (Bio-Tek Instruments, Winooski, VT). The percentage of cell viability was calculated relative to vehicle-treated control designated as 100% viable cells. Data were fitted to a sigmoidal dose-response model (GraphPad Prism®, GraphPad Software Inc).

2.3 Clonogenic survival assay

The clonogenic survival assay was performed to determine the long-term survival of HCC1937 cells after different treatments. Cells were seeded and treated with statins, Vit D, or the combination of Vit D and Pita Twenty-four hours after treatment, cells were trypsinized, re-suspended in fresh medium, and re-plated at a low density of 1000 cells per well in 6-well plates. Cells were allowed to grow and monitored for colony formation for 14 days. Media were routinely changed every 2 to 3-day intervals. Live cells were washed 3 times with 1 X PBS, fixed for 15 minutes in methanol: acetic acid (3:1), and excess fixative 3 times washed off with 1 X PBS. Thereafter cells were stained for 15 minutes with 0.5% crystal violet (Sigma-Aldrich, USA) in 100% methanol (Aliwaini et al. 2015). Colonies were imaged and both size and number of colonies were quantified using Image J together with Origin Pro 2021 software. The plating efficiency was calculated and presented ±SEM.

2.4 Growth curve assay

Breast cancer cells were seeded for 24 hours to settle and then treated with Vit D, Pita, the combination (Vit D and Pita), or vehicle. The number of cells was assessed after 24, 48, and 72 hours of the treatment.

2.5 Scratch motility assay

Cells were grown to confluence and a linear scratch was made through the monolayer using a sterile 200 µl pipette tip. To remove cell debris, the growth medium was replaced and several markings were made along the edges of the scratch line which were used as reference points and the wound widths were measured at the time of the scratching (0 hours) and after the indicated treatments. Pictures were taken using an inverted light microscope (Olympus 1X71, USA) and camera (Zeiss AxioCam, Germany) respectively. Migration distances were measured using Axiovert software (Zeiss, Germany).

2.6 Western blotting

Cells were harvested and protein was prepared as described previously (Aliwaini et al. 2021). Primary antibodies used for western blotting are anti-p-Stat3 (sc-8059), anti-PARP1/2 (sc-7150), anti-caspase-9 (sc-56076), anti-p53 (sc-126), anti-p21 (sc-756) and anti-α-Tubulin (sc-8035) (Santa Cruz, California, USA).

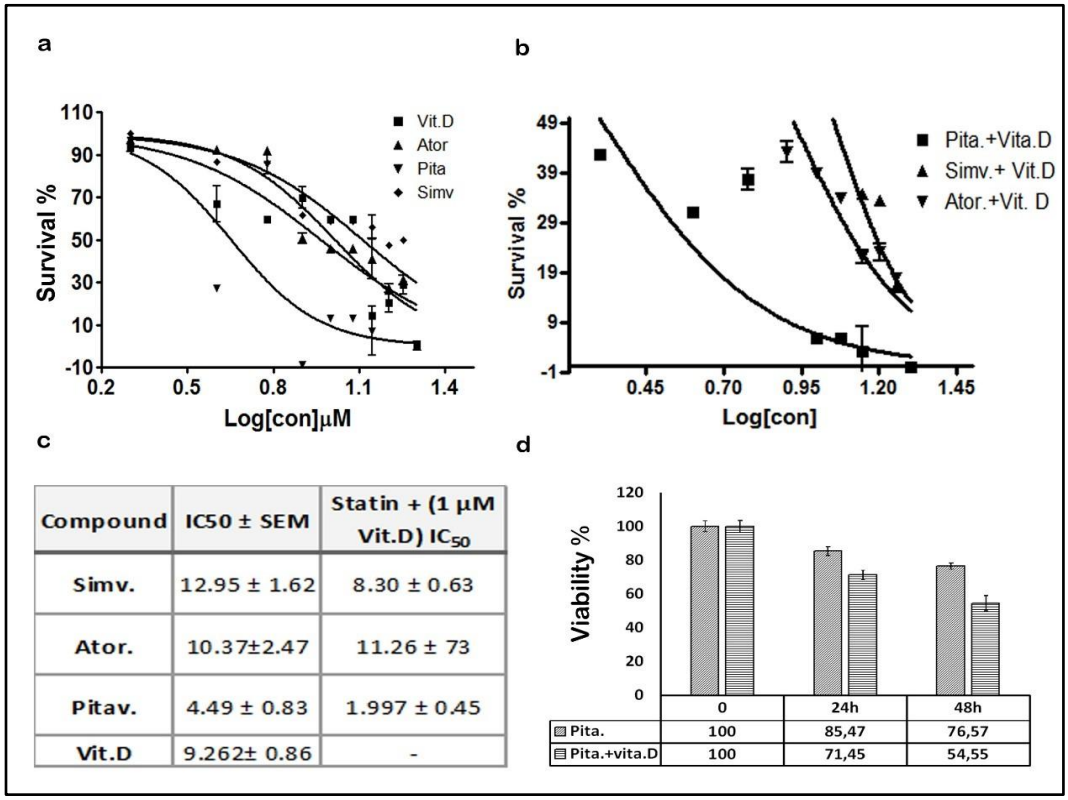
2.7 Statistical analysis

Data presented are mean ± SEM (Standard error of the means) of appropriate replicates. Statistical significance was assessed between the groups using the Student‘s t-test. A value of $P< 0.05$ was accepted as statistically significant.

3 Results

3.1 Effect of Statins and Vit D on HCC1937 breast cancer cell line proliferation

We initially tested the antiproliferative effect of three widely used statins including atorvastatin (Ator), simvastatin (Simv), and Pita (Pita) and Vit D (Vit D) on the viability of HCC1937 breast cancer cells. Results presented in figure 1 (a) revealed that low concentrations (less than 20 µM) of all tested statins and Vit D (less than 10 µM) induced significant anti-proliferative effects in a dose-dependent manner. In



Compound	IC50 ± SEM	Statin + (1 µM Vit.D) IC_{50}
Simv.	12.95 ± 1.62	8.30 ± 0.63
Ator.	10.37±2.47	11.26 ± 73
Pitav.	4.49 ± 0.83	1.997 ± 0.45
Vit.D	9.262± 0.86	-

Figure 1 Cytotoxicity of Vit D and statins against breast cancer cells (1 a and b); Cell viability was tested using the MTT assay and data were expressed as a percent of vehicle-treated control ± SEM ; (1 c) table summarizes the IC_{50} values of different treatments; (1 d) Cell viability was assessed by trypan blue assay after 48 hours of the indicated treatments. The data represents pooled results of at least three independent experiments performed in quadruplicate.

comparison to other statins, Pita showed the most potent antiproliferative effect with an IC_{50} of 4.49 µM. To test the possible synergistic effect of different statins with Vit D, cells were pretreated with a low dose of Vit D (1µM) and then treated with Simv, Atoror, and Pita, and were tested by MTT assay. Figure 1 (b, c) shows that both Pita and Simv have more potent cytotoxic effects in the presence of Vit D than the single treatment of each of them. However, the most synergistic effect was observed by combining Pita and Vit D treatments. The IC_{50} value of Pita in the presence of Vit D was 1.997 µM. While Pita treatment induced a significant cell death of about 24 % after 48 hours of the treatment, the combined treatment (Pita and Vit D) killed more than 45 % of breast cancer cells (Figure 1d). Altogether, these data indicate that the combined treatment of Pita and Vit D has a significant cytotoxic effect against HCC1937 breast cancer cells.

3.2 Effect of combined treatment of Pita and Vit D on growth and colony formation ability of breast cancer cells

Both Vit D and Pita were found to effectively reduce the clonogenic formation of cancer cells in vitro (Figure 2 a, b). However, this reduction was augmented by combining Pita and Vit D. The percentage of plating efficiency decreased from 37% for control cells to about 10% for the combination (Vit D + Pita) treated cells. To examine the synergistic effect of Vita D and Pita on the growth of human breast cancer cells, a growth curve assay was performed. Results of this study revealed that both Vit D and Pita treatments inhibited the growth rate after four days of the treatment. However, the combined treatment inhibited cancer cells more significantly (Figure 2 c). Taken together, these data show that the combined treatment of Vit D and Pita inhibits the growth and colony formation ability of HCC1937 breast cancer cells.

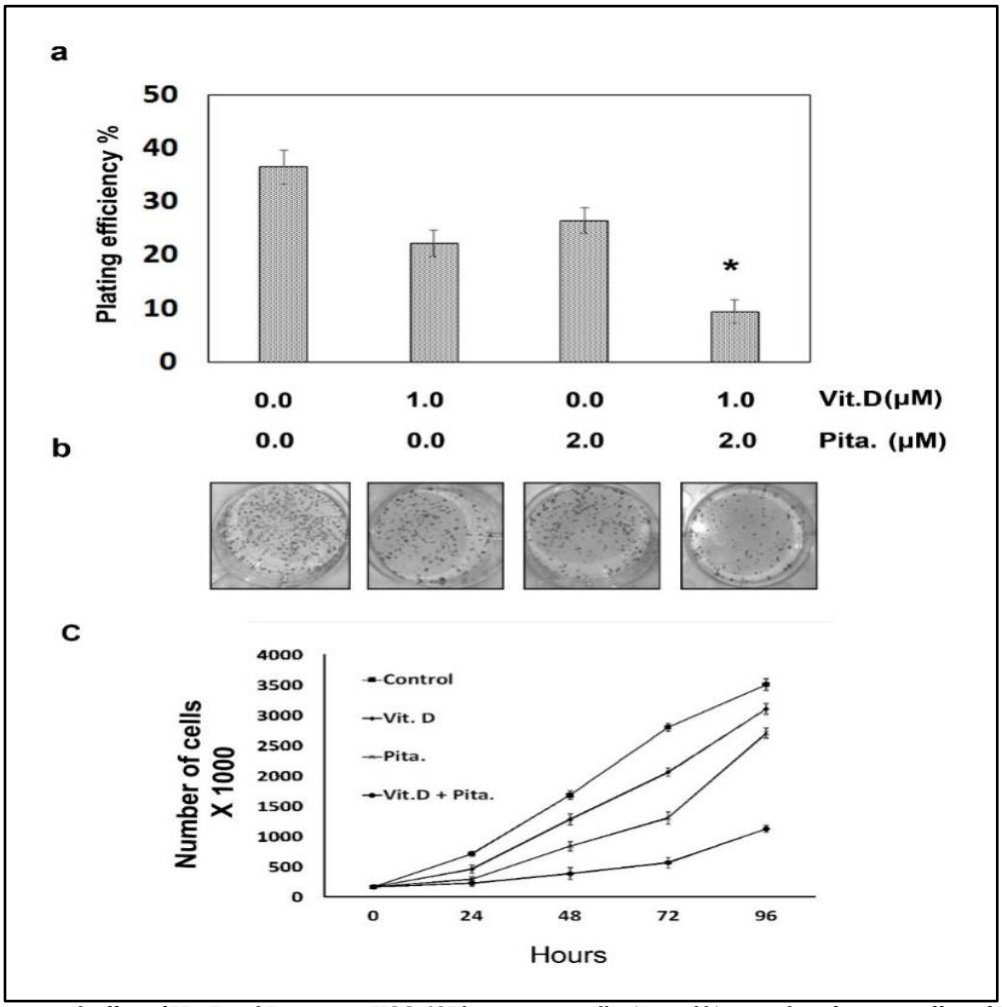


Figure 2 Anti-growth effect of Vit. D and Pita against HCC1937 breast cancer cells; (2 a and b) anti-colony formation effect of Vit D and Pita against HCC1937 cells (2a) indicated the effect of the treatment on the plating efficiency of the cell lines, (2b) Representative images of clonogenic results for HCC1937 cells treated as indicated on the figure, (2c) The curves of HCC1937 breast cancer cell line treated as indicated in the figure. The difference was significant after the sixth day ($P<0.05$).

3.3 Combined application of Pita and Vit D on the migration ability of breast cancer cells

To further explore the anti-migration activity of the combined treatment (Vit D and Pita), a wound-healing assay was performed. HCC1937 cells were exposed to Pita, Vita D, or the combination of Vit D and Pita for 48 hours. Results of the study revealed a significant reduction in cell motility in all tested treatments; however, the combination of Vit D and Pita showed the most potent anti-migratory effect (Figure 3 a, b). Results of the study also suggested that the combination of these two agents inhibits the migration ability of HCC1937 cells by more than 50% after 48 hours of the treatment.

3.4 Effect of combined treatment of Vit D and Pita on cell cycle arrest in HCC1937 cells

Application of Vit D and Pita as a single treatment or in combination can also induce cell cycle arrest. Levels of the cell cycle markers were determined by western blotting for a protein harvested from HCC1937 breast cancer cells treated with single or combined treatments of (1µM Vit D or 2µM Pita). Results presented in Figure 4 show that Vit D as a treatment or combination with Pita inhibits the phosphorylation of p-STAT3 protein in HCC1937 cells. Importantly, combined treatment of Vit D (1µM) and Pita (2µM) induced lower levels of p-STAT3 than the single treatment. Inhibition of p-STAT3 led us to test the possible effect of single or combined treatments on the expression of P53 protein which plays an important role in cell cycle regulation by stimulating the p21 gene to express P21 protein. P21 protein inhibits Cdk and induces G1 cell cycle arrest. The results presented in Figure 4 revealed that the combined treatment induces high levels of P53 and P21 proteins in the HCC1937 cell. Altogether, these findings clearly show that combined treatment of Vit D and Pita induces G1 cell cycle arrest by inhibiting the STAT3 pathway and inducing P53 and P21 expression in HCC1937 breast cancer cells.

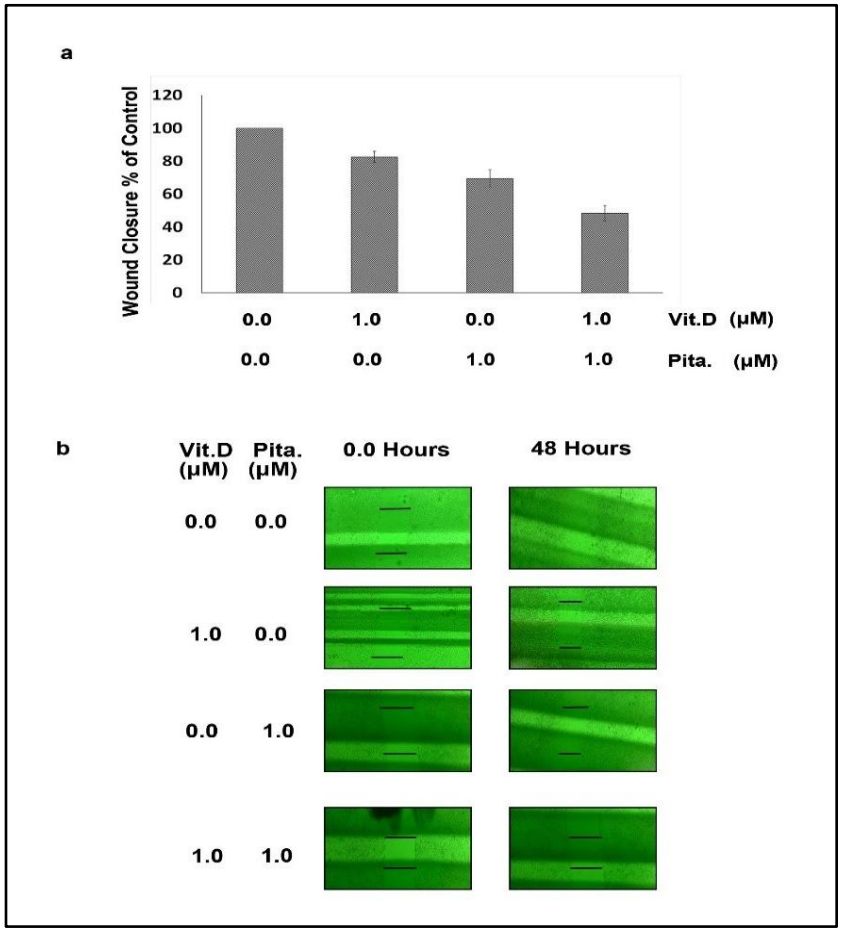


Figure 3 Vit D and Pita inhibited the migration of breast cancer cells; (3a) The graph shows the ability of the treatments to inhibit the migratory ability of HCC1937 breast cancer cells (3b) At specified time points (0 and 96) hours cells were photographed using (10x; Olympus 1X71). Assays were done in duplicate and two independent experiments were performed.

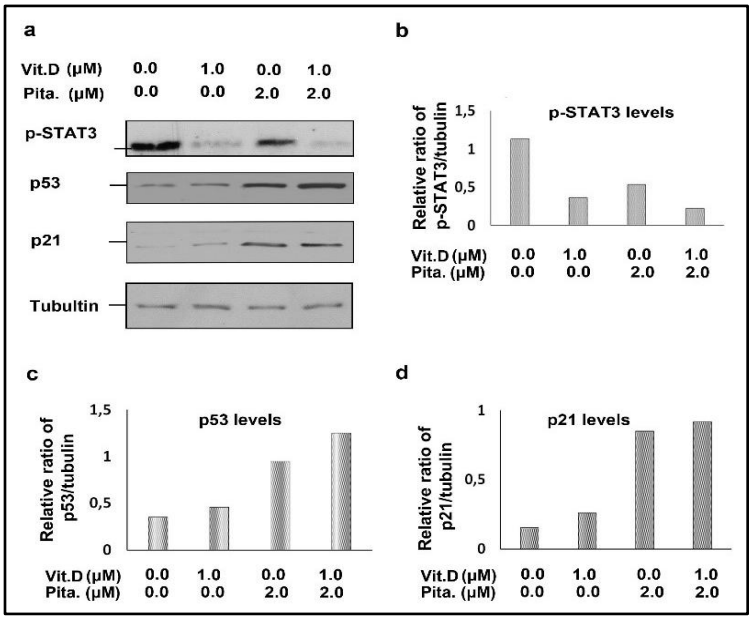


Figure 4 Vit D and Pita treatments induced cell cycle arrest in HCC1937 breast cancer cells. (4a) Western blotting of proteins from the cancer cells treated with indicated treatments for 48 hours and analyzed with antibodies to p STAT-3, p53, and p21. Tubulin was used as a loading control; (4 b,c,d) Densiometric readings of the specific proteins relative to tubulin

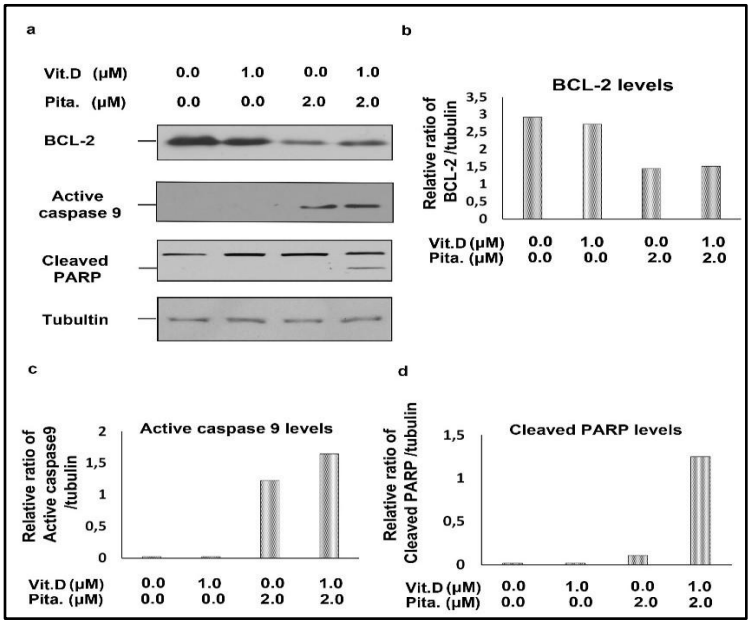


Figure 5 Vit D and Pita treatments induced apoptosis in HCC1937 breast cancer cells, (5a) Western blotting of proteins from the cancer cells treated with indicated treatments for 48 hours and analyzed with antibodies to BCL-2, caspase 9 and cleaved PARP. Tubulin was used as a loading control; (5 b,c,d) Densiometric readings of the specific proteins relative to tubulin

3.5 Combined treatment of Vit D and Pita induces apoptosis in HCC1937 cells

To test whether Vit D, Pita, or the combination (Vit D and Pita) also induces apoptosis in cancer cells, in this study, western blotting was performed and apoptotic markers were detected. Results presented in Figure 5 showed that the combined application of Vit D and Pita downregulated BCL-2, the anti-apoptotic protein, and induced both active caspase 9 and cleaved PARP after 48 hrs of the treatment. These findings indicate that combined treatment of Pita and Vit D induces intrinsic apoptosis in HCC1937 cells.

4 Discussion

Several previous studies showed that statins have effective anticancer properties on several types of cancer cells. In 2021, Rezano et al. (2021) showed that simvastatin has a cytotoxic effect on MDA-MB-231 and MCF7 with an IC_{50} of 4.5μM and 8.9μM respectively (Rezano et al. 2021). Similarly, O'Grady et al. (2020) found that simvastatin has an IC_{50}value of 40.8 μM while it was reported 49.1μM for atorvastatin on triple-negative breast cancer cell lines compared to non-triple negative breast cancer cell lines (O'Grady et al. 2020; Rezano et al.2021). Tilija Pun et al. (2022) showed that Pita induced apoptosis in oral (SCC4 and SCC15) and colon (HT29, HCT116, and SW480) cancer cell lines when treated with 0.25–0.5 μM of Pita In 2020, in vitro study by Aliwaini et al. (2021) showed that combined treatment between Pita and doxorubicin has a synergistic cytotoxic antiproliferative effect against MCF7 breast cancer cells with an inhibitory concentration of 1 μM.

Based on the results of this study, it can be suggested that Vit D has strong effects on the cell cycle where it plays a critical role in regulating proteins related to the cell cycle checkpoints such as P53, P21, P27, and Cdk. Many researchers and reviewers had highlighted that there was an association between Vit D and cell cycle arrest. Li et al. (2019) reported that Vit D3 could increase gene expression of Vit D receptor (VDR) and p21 in pancreatic cancer cells (PC). Similarly, Bao et al. (2014) also investigated the antiproliferative properties of Vit D and cisplatin with single or combined treatment on gastric cancer cells. The same study showed that Vit D had a synergistic effect on gastric cancer cells (GC) by inducing cell cycle proteins such as p21 and p27 and there was a larger number of cells in the G0/G1 in the combined treatment than the single treatment (Bao et al. 2014)**.** Ya-li zhang et al.(2021) have reported that Vit D had a synergistic effect on the K562 cell line and its effect was significantly increased after being treated in a combination with arsenic trioxide. Vit.D treatment induced G_0/G1 cell cycle arrest and elevated the mRNA levels of VDR, p21, and p27 significantly (Charoenngam and Holick 2020; Morris 2005). Along with the same line, other researchers such as Zheng et al. (2019) evaluated the anticancer activity of different concentrations of Vit D on MCF7 and MDA-MB-453 breast cancer cell lines after 48 hours. They observed that Vit D treatment downregulated the gene expression of Ras and ERK and induced cell cycle arrest in both breast cancer cell lines (Zheng et al. 2019).

Several publications like Lee et al. (2020) evaluated the anticancer activity of Pita on oral squamous cell carcinoma cell lines (OSCC15 and OSCC4) they concluded that Pita treatment upregulated FOXO3a by modulating the AMPK and Akt pathways. Similarly, Wang et al. (2016) found that simvastatin arrested the cell cycle of bladder cancer cell lines (BCa) in G_0/G1(Wang et al. 2016). In agreement with these findings, Al-Qatati and Aliwaini (2017) showed that Pita induced cell cycle arrest on human melanoma cell line A375 and WM115. Western blotting assay of the treated cell lines with Pita showed a significant increase in the expression of p53 and p21. Furthermore, the combined treatment of dacarbazine and Pita synergistically increased p53 and p21. Another study by Wang et al.(2020) examined different concentrations of atorvastatin treatment on hepatocellular carcinoma (HCC) and demonstrated that there was a clear increase in the expression of the inhibitory proteins p21 and p53 in a dose-dependent manner which played a vital role in the suppression of cell growth (Wang et al. 2020). Our present study shows that the combination of Vit D and Pita inhibited the p-STAT3 and increased p53 and p21 proteins in HCC1937. The present study also showed that a significant reduction in cell migration (30%) was observed for HCC1937 cells exposed to a combination of 1μM Vit D and 1μM Pita after 48 hours.

Conclusion

Taken together these observations indicate the potent synergistic anticancer effect of Vit D and Pita against the HCC1937 triple negative breast cancer cells. This combined treatment showed a significant anti-proliferative effect by inducing cell cycle arrest and apoptosis in breast cancer cells.

Conflict of Interest Statement

There are no conflicts of interest.

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Characterization of arsenic-resistant endophytic *Priestia megaterium* R2.5.2 isolated from ferns in an arsenic-contaminated multi-metal mine in Vietnam

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KEYWORDS

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Bioremediation

Arsenic

Priestia megaterium

ABSTRACT

Bioremediation is a biological process to remove or neutralize environmental pollutants. This study was carried out to investing at the efficacy of arsenic resistant endophytic bacteria isolated from *Pteris vittata*, *Pityrogramma calomelanos*, *Blenchum orientale*, and *Nephrolepis exaltata*, which grow in a highly arsenic (As) contamination mining site in Vietnam. Their segmented roots, stems, and leaves were homogenized separately and inoculated on LB agar plates containing 5mM As(III) and As(V). A total of 31 arsenic resistant endophytic strains were selected, in which strain R2.5.2 isolated from the root of *P. calomelanos* had the highest arsenic resistant capability. Strain R2.5.2 tolerated up to 320 mM and 160 mM of arsenate and arsenite, respectively. The strain developed well on a media of 0.1−5% NaCl, at 20-40ºC and pH 5−9, and actively utilized most of the sugar sources. It had a high IAA biosynthesis capacity with an average concentration of 19.14 mg/L, tolerated to 0.5-16 mM concentration of Ag^{+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cr^{4+}, and reduced As(V). Based on 16s rDNA, R2.5.2 was identified as *Priestia megaterium*. The *ars C* gene coding for arsenate reductase catalyzing reduction of As(V) was successfully amplified in *P. megaterium* R2.5.2. The selected strain may have potential use for bioremediation practice.

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1 Introduction

Arsenic (As) is a naturally occurring mineral that is widely distributed in the Earth's crust and ubiquitous in the environment with origins from both geogenic and anthropogenic sources such as excessive usage of herbicides and pesticides, mining, burning fossil fuels, etc. As a consequence, arsenic concentration in air, soil, and groundwater has increased worldwide and raises public concern. According to World Health Organization (WHO) and International Agency for Research on Cancer (IARC), arsenic and arsenic compounds have been classified as Group 1 human carcinogens (IARC 2022). Additionally, it ranks top on the agency for toxic substances and disease registry's priority list of hazardous substances (Yang and Rosen 2016).

Although the biggest threat to human health comes from As-contaminated drinking water (Chung et al. 2014), As-polluted soil should not be overlooked. The average amount of arsenic in soil throughout the world is 5 mg/kg (Stafilov et al. 2010) and the permissible limit of arsenic in soil is 24 mg/kg according to the U.S. Environmental Protection Agency (Singh et al. 2015). However, in many countries, soil can be contaminated with concentrations of much arsenic higher than the permissible limit, for example, 18,100 mg/kg in an Au-enriched metallogenic of lower Silesia-Southwestern Poland, 200–860 mg/kg in Southeastern Brazil, 660 mg/kg in Simav plain-Turkey and 489 mg/kg in Esquiña-Chile (Singh et al. 2015). In the Nui Phao mine of Vietnam, the total concentrations of arsenic in the soil vary from 34–3,390 mg/kg, with a mean value of 50.93-55.44 mg/kg which exceeds the maximum allowable limit of the EPA by a factor of 1.4-141.25 (Nguyen et al. 2020) and directly or indirectly affected the health of local communities.

To remediate As-contaminated soil, mechanical or physio-chemical techniques such as soil incineration, excavation and landfill, soil washing, solidification, and electric field application have been effectively used (Lim et al. 2014), but along with their benefits, these methods might adversely affect soil fertility due to removal of basic cations. On the contrary, phytoremediation attracts great interest as a better option because of its environmentally friendly and cost-effective approach to treating two predominant inorganic oxidation states of As, arsenate (As(V)) and arsenite (As(III)) (Bali and Sidhu 2021). Although arsenic is non-essential and generally toxic to plants (Finnegan and Chen 2012), some As hyperaccumulators are capable of absorbing a large amount of As and translocating it to their aboveground biomass. The Chinese brake fern (*Pteris vittata L.*) was the first As hyperaccumulator to be reported in 2001. Since then, other As hyperaccumulators have been reported, but most of them are known to be *Pteris* ferns and *Pteris vittata* are the most promising models for phytoremediation. Extensive work has explored arsenic phytoextraction with the fern *P. vittata* as an *in situ* alternative to soil excavation-based arsenic remediation methods. Arsenic accumulated by ferns can be found in a range of soil physicochemical conditions (Danh et al. 2014). However, phytoextraction rates are slow even at a moderate arsenic concentration in soil (~100 mg As/kg soil), and the remediation time could take several decades to reduce arsenic contamination (Chen et al. 2002). Furthermore, the efficiency of phytoremediation is influenced by several factors such as plant growth rate, phytotoxicity, plant nutrition, and root exudation (Yang and Rosen 2016). Therefore, manipulations to increase soil arsenic availability, fern biomass, and fern arsenic uptake have been investigated to increase phytoextraction rates (Matzen et al. 2021).

The interaction between plants and endophytes that colonize in the plant's internal tissue are beneficial in the accumulation of heavy metals. The plant endosphere provides nutrients and serves as a habitat for the endophytes, while the endophytic microorganisms contribute in the plant growth by secreting plant growth-promoting substances such as organic acids, ACC deaminase, indole-3-acetic acid (IAA) and siderophores in turn (Titah et al. 2018). They share all the important characteristics to promote the growth of host plants found in rhizobacteria. Nevertheless, the valuable effects of the endophytic bacteria to host plants are usually greater than those provided by many rhizospheric bacteria, especially when plants are challenged by stress conditions (Afzal et al. 2019). Therefore, with the beneficial properties of endophytes isolated from arsenic-accumulating plants, their application in phytoremediation may also show great potential.

Some microorganisms have made necessary genetic adaptations to create resistance toward As, allowing them to survive and thrive in environments containing arsenic concentrations that are toxic to most other organisms. For bacteria, resistance to arsenite and arsenate compounds comes from the arsenical resistance (ars) operon. Bacterial *ars* operons consist of three to five genes (*ars* R, D, A, B, and C), which are located in the plasmids (Owolabi and Rosen 1990), or in the chromosomes (Diorio et al. 1995). *Ars* R and *ars* D are regulatory genes, while the complex of *ArsA* and *ArsB* forms an anion-translocating ATPase that catalyzes the extrusion of arsenite (As(III)) from the cytoplasm, lowering the intracellular concentration of the toxic arsenic (Mukhopadhyay et al. 2002, Owolabi and Rosen 1990). Arsenate (As(V)) is enzymatically reduced to arsenite (As(III)) by small cytoplasmic arsenate reductase, which is the product of the ars C gene. However, there are limited researches on endophytes associated with ferns and their roles in As tolerance and during the critical phase of reduction in roots before the translocation process (Gu et al. 2018).

In this study, endophytic bacteria were isolated from four common fern species *Pteris vittata*, *Pityrogramma calomelanos*, *Blenchum*

orientale, and *Nephrolepis exaltata* grown naturally on Nui Phao multi-metal mine located in Thai Nguyen province-Northern Vietnam, where the soil was highly contaminated with As due to mining activities. This study aimed to select the best endophyte and to evaluate its biological characteristics. The study suggests a potential strain for bioremediation.

2 Materials and Methods

2.1 Isolation and selection of highly arsenic-resistant endophytic bacteria

Four arsenic hyperaccumulating fern species, *Pteris vittata*, *Pityrogramma calomelanos*, *Blenchum orientale,* and *Nephrolepis exaltata* were collected at three locations including S1 - hamlet 2; S2 - hamlet 4, S3 - hamlet 11 around Nui Phao mine, Thai Nguyen province, Vietnam (21º38'15"N-21º38'54" N and 105º40'35"E-105º41'4" E).

Roots, stems, and leaves of each species were prepared separately and surface sterilized after Shutsrirung et al. (2013) and Phan et al. (2016). Next, plant samples were washed under running water for 10 min to remove all soil and dirt, and then the leaves, stem, and roots were cut into small pieces (1 × 1 cm for leave and 1cm segments for others) and soaked in 1% sodium hypochlorite for 1 min, followed by 70% ethanol for 5 min. The samples were further washed four to five times with sterile water. They were homogenized in a sterile laboratory porcelain mortar with a small amount of sterile distilled water. The obtained solutions were diluted to appropriate concentrations and inoculated on LB agar plates containing 5mM As(III) and As(V). After an incubation period of 3-5 days at 27-30°C, colonies with different morphotypes were selected and purified via multiple subcultures on LB agar.

The minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) of arsenate [As(V)] and arsenite [As(III)]-resistant endophytic bacteria were determined to evaluate their tolerance level to As as per Andrews (2001). For this, isolated bacterial strains were cultured in the shake cultural flasks containing LB medium supplemented with 5-320 mM arsenite ($NaAsO_2$) and arsenate ($Na_2HAsO_4.7H_2O$), at 30ºC and 200 rpm for 24 h. In all experiments, bacterial growth in shake flask cultures was assayed by measuring the optical density of the culture broth at 600nm (OD_{600}) using a Shimadzu UV-2550 UV/VIS spectrophotometer. The endophytic bacterium showing the highest As-tolerance was selected for further study.

2.2 Determination of IAA biosynthesis and As transformation capacity

The concentration of IAA in the culture broth was determined by Bric et al.(1991). The production of IAA was carried out in LB medium supplemented with 2 g/L tryptophan. The isolate was incubated at 30ºC, 200 rpm for 24 hours, and the supernatant was collected by centrifugation at 10,000 g for 10 min. For 1 mL of supernatant, 2 mL of Salkowski reagent (50 mL of 35% $HClO_4$ + 1 mL of 0.5 M $FeCl_3$) was added and the mixture was incubated at room temperature in the dark for 30 minutes, then light absorbance was measured at 530 nm and IAA concentration was calculated.

The ability of the bacteria to reduce As (V) or to oxidize As (III) was evaluated using the silver nitrate method after Simeonova et al. (2004). Endophytic bacteria were cultured on LB agar supplemented with 5 mM As(III) and As(V), respectively, at 30ºC for 72 h. The plates were then flooded with 0.1M silver nitrate solution. The appearance of a light yellow halo around the colony would indicate precipitation of silver ortho-arsenite (Ag_3AsO_3), while a light brown-red halo would relate to silver-ortho-arsenate (Ag_3AsO_4).

2.3 Characteristics of isolated strains

After 48 hours of bacterial isolates cultivation on LB medium plates at 37ºC, colony and cell morphology were investigated under optical microscopy. Photography of the cultured bacterial isolates was carried out using a scanning electron microscope JSM-5410LV (Jeol - Japan), at the voltage of 15 kV and under a high vacuum.

2.3.1 Determination of salt tolerance

The selected isolate was cultivated in LB medium supplemented with different concentrations (0.1-10%) of NaCl at 37ºC and 200 rpm for 48 hours.

2.3.2 Determination of favorable pH and temperatures for growth

The cultivation conditions were similar to that mentioned above, except that the initial pH of the LB medium was adjusted to different values from 3 to 12. In another set of experiments, the bacterial isolate was cultivated at different temperatures ranging from 10-55ºC.

All experiments had three replications. The growth, expressed in OD_{600}, was recorded and compared to find the range of salt tolerance and suitable pH and temperature conditions.

2.3.3 Biosynthesis of extracellular enzymes

The bacterial isolate was cultured on LB medium supplemented with 1% of one of the following substrates: soluble starch, casein, cellulose, chitin, xylan, and CMC (carboxymethylcellulose). Respective enzyme activity was assayed as per Phan et al. (2021).

2.3.4 Carbon source utilization

The isolate was grown on ISP9 mineral medium supplemented with 1% of one of the following carbon sources: D-Glucose, L-Arabinose, D-Xylose, D-Manitol, D-Fructose, D-Cellulose, D-Rafinose, and Sucrose. The sugars were sterilized by the Tyndall method. Glucose was used as a positive control and ISP9 medium as a negative control (Nonomura 1974).

2.4 Investigation of metal tolerance

The ability of isolates to tolerate other heavy metals including Ag^{+}, Hg^{+2}, Cr^{4+}, Co^{+2}, $Ni^{+2,}$ and Cu^{2+} was investigated by adding $AgNO_3$, $HgCl_{2,,}$ $K_2Cr_2O_7$, $CoCl_2.6H_2O$, $Ni(NO_3)_2.6H_2O$ and $CuSO_4.5H_2O$, respectively was also checked at a concentration ranging from 0.5 to 16mM to LB medium. Shake flask cultivation was carried out at 200 rpm at 37°C. Bacterial growth (OD_{600}) was recorded after 48 hours. All experiments were performed in triplicate. The sterile medium was used as a reference.

2.5 Strain identification and phylogeny analysis

The DNA was extracted after Sambrook (2001)and subjected to PCR to amplify the 16S rDNA gene using two primers: 27f (5'-TAACACATGCAAGTCGAACG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The thermal cycling program was initially denaturation at 94°C for 5 min, followed by 35 cycles: 94°C for 60 sec, 60°C for 60 sec, and 72°C for 90 sec, with a final step of 72°C for 10 min before keeping the sample at 4°C. The PCR products were analyzed using ABI PRISM 3100 Avant Genetic Analyzer sequence reader, processed with SeqAssem version 01/2005 and Sequencher version 4.0.5 software. The 16S rDNA nucleotide sequences were analyzed according to NCBI Gene Bank data. Sequence similarity was determined and compared with other sequences compared on the GenBank using BLAST (www.ncbi.nlm.nih.gov). The genetic similarity of the strains was constructed using CLC DNA workbench 6.6 software. The phylogenetic tree was created with Mega 6.0.

2.6 Arsenic-resistance gene amplification

The primer set BmegaarsC was used for PCR amplification of the Arsenic-resistant gene (Table 1). The PCR reaction mixture consisted of 1 µl of 10 pM of each primer, 2 µl of the DNA template, 10 µl of PCR buffer, and deionized water to a final volume of 20 µl. PCR amplification conditions were set as follows: 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 s, incubation at 53ºC for 30 s and elongation at 72ºC for 30 s, and extension at 72ºC for 5 minutes. PCR products were determined by gel electrophoresis using 1% agarose (Sambrook 2001).

2.7 Statistical analyses

All treatment data were presented as the mean of three replicates ± standard deviation (SD) using Microsoft Excel 2010. Duncan's multiple range test was employed to analyze differences between means using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.).

3 Results

3.1 Isolation and selection of arsenic-resistant endophytes

The study showed that arsenic accumulation in the rhizosphere (0 – 20 cm deep) at this region varied from 316-1606 mg/kg of soil. From the 20 collected fern plants at the Nui Phao mine, 26 As(V) tolerant and 5 As(III) tolerant endophytic bacterial strains were isolated. Among 31 strains obtained from the incubation, isolate R2.5.2 from the root of *P.calomelanos* had the highest tolerance to arsenate (320 mM) and arsenite (160 mM). Therefore, this strain was selected for further examination of its characteristics.

3.2 Biological characteristics and growth analysis of selected strain

Bacterial isolate R2.5.2 was gram-positive with rod-shaped smooth cells and had a size of 0.9 x 3.6 µm (from SEM images of this isolate at 10000 x magnification, Figure 1). The strain had opaque white and non-convex spherical colonies of 1 mm diameter and produced brown pigment.

Furthermore, the medium with 0.1% to 2% NaCl did not have any significant effect on the growth of isolate R2.5.2 (Figure 2). Higher NaCl concentrations decreased the growth of the strain and its growth rate stopped at 10% NaCl. pH values between 5-9 were suitable for isolating R2.5.2. Similar to pH, temperature also affected the growth of isolate R2.5.2, and a temperature range of 20-40ºC was suitable for the growth of isolate R2.5.2 (Figure 2).

The isolate R2.5.2 was able to produce endospores and synthesized enzymes such as cellulase, chitinase, CMCase, xylanase, and protease with hydrolysis zone diameters of 8, 12, 15, 23, and 35 mm, respectively (Table 2). This isolate actively utilized most of the sugar sources and grew well on agar plates containing D-fructose, D-manitol, D-cellulose, sucrose, and D-rafinose (Table 2).

Table 1 Primer set used for PCR amplification

Primer	Primer sequence (5' to 3')	T_m(ºC)
Bmega-arsC-F	GGAATTCCATATGTCTAAAAAAACACTTTATTTC	53
Bmega-arsC-R	CGCGGATCCTTAGTGATGGTGATGGTGATGTTTACCTGTTTCAGCAAAACG	

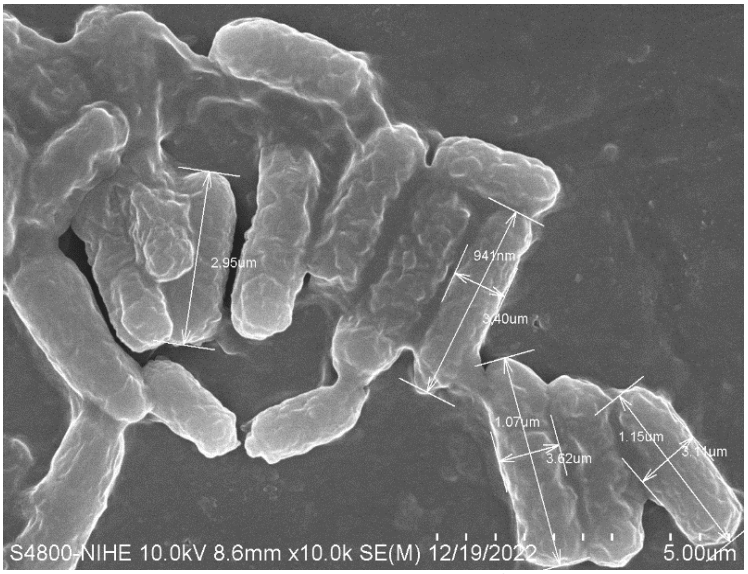


Figure 1 Scanning Electron Microscopy (SEM) image of isolate R2.5.2 (x10,000)

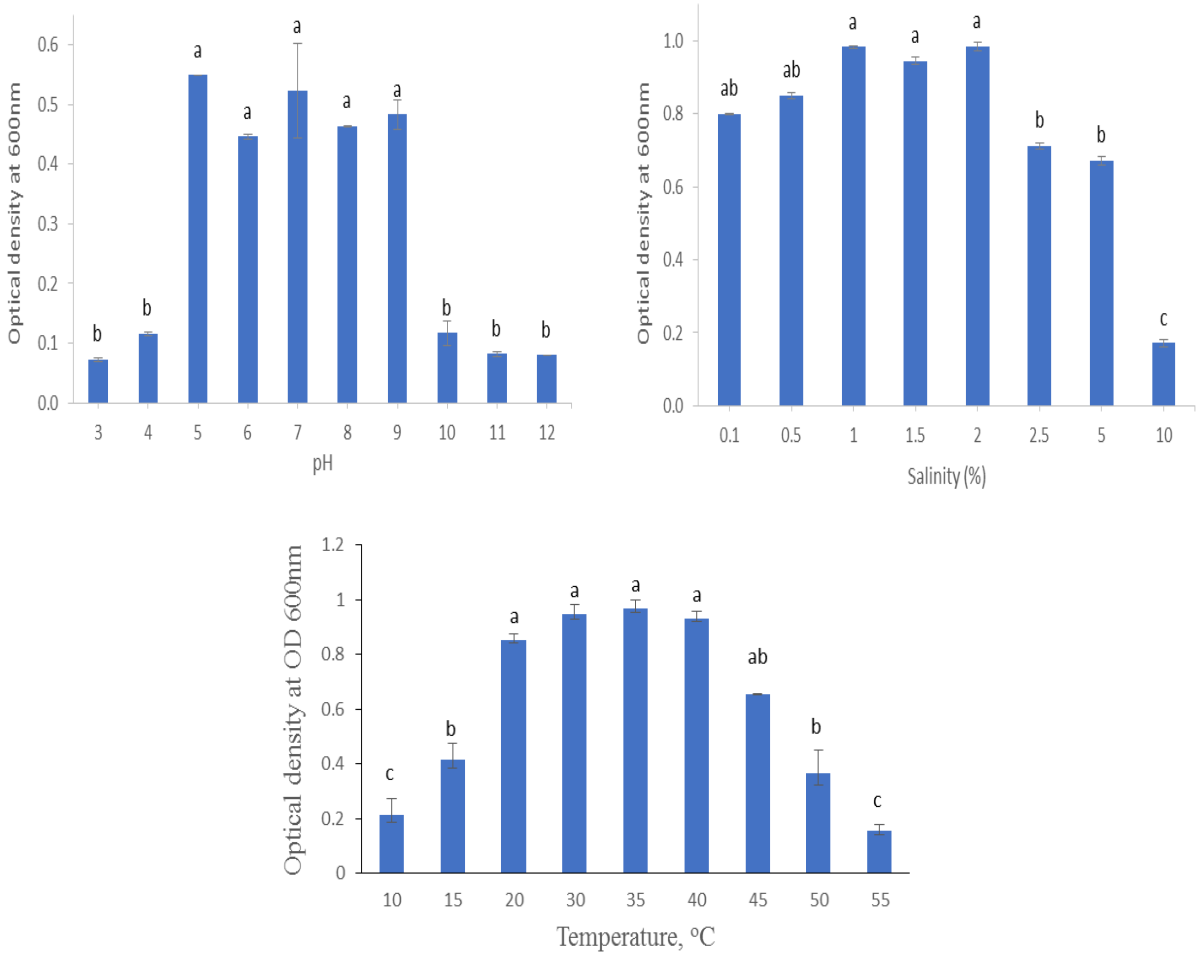


Figure 2 Effects of pH, NaCl, and temperature on the growth of strain R2.5.2. The bars stand for the standard error of three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's test).

Table 2 Biological characteristics of strain R2.5.2

Experiment	Carbon source	Growth
Carbon source utilization	D-Glucose	+
	L-Arabinose	+
	D-Xylose	+
	D-Manitol	++
	D-Fructose	++
	D-Cellulose	++
	D-Rafinose	++
	Sucrose	++
	Mineral	+-
	Extracellular enzyme	Diameter (D-d) mm
Extracellular enzyme	Amylase	-
	Protease	35
	CMCase	15
	Chitinase	12
	Xylanase	23
	Cellulase	8

3.3 IAA biosynthesis and As transformation capacity

In this study, the strain R2.5.2 possessed high IAA biosynthesis capacity with an average concentration of 19.14 mg/L (Figure 3). The test results indicated that strain R2.5.2 reduced As(V) and As(III) due to the formation of light yellow color and light brown-red color halo around the colony, respectively, in LB agar medium supplemented with As(V) and As(III) (Figure 4).

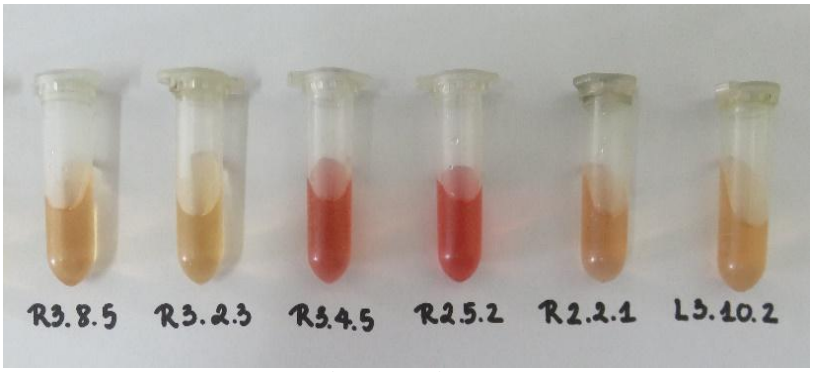
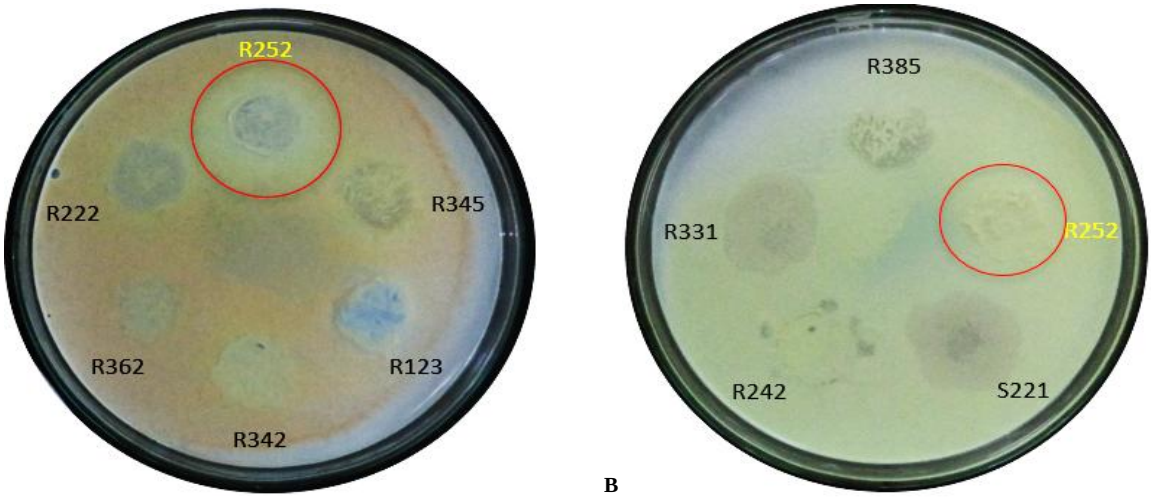


Figure 3 The ability of synthesis of IAA by the R2.5.2 strain



A **B**

Figure 4 The ability of the R2.5.2 strain to reduce As (V) or to oxidize As (III); LB agar supplemented with 5 mM As(V) (A) and As(III) (B)

Isolated strain R2.5.2 was tested for its ability to grow under the presence of some heavy metals and the results revealed that strain R2.5.2 effectively grew in the presence of other toxic heavy metals. Strain R2.5.2 was resistant to Ag^{+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}và Cr^{4+} heavy metal in the range of 0.5-16 mM, but at 16 mM concentration, strain R2.5.2 showed little or no positive sign of growth (Figure 5).

3.4 Strain identification of the isolate R2.5.2

The total DNA of the selected strain R2.5.2 was extracted using a total DNA extraction kit (NucleoSpin® Tissue extraction kit, Macherey-Nagel, Germany). The 16S rRNA gene of the bacterial strain was amplified by PCR using primer pair 27F-1492R (Figure 7a) and showed high homology of over 99% with the corresponding genes of several bacteria belonging to *Priestia megaterium* species (Figure 6). From the results of 16S rDNA gene sequencing combined with biological and physiological characteristics, strain R2.5.2 was named *P.megaterium* R2.5.2 and submitted to GenBank under accession number OL662937.1.

3.5 Arsenic-resistance gene of strain *Priestiamegaterium* R2.5.2

The *arsC* gene of strain *P. megaterium* R2.5.2 was amplified by PCR from a genomic DNA (gDNA) template using primer pairs

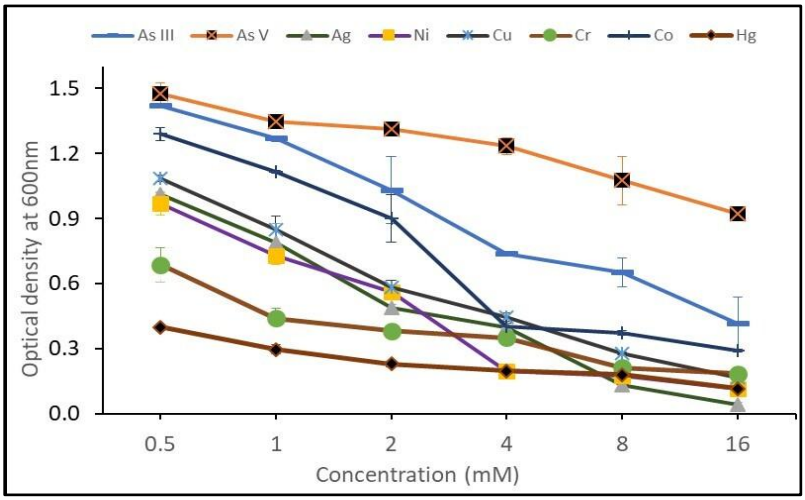


Figure 5 Influence of heavy metals on growth of strain R2.5.2.The bars stand for the standard error of three replicates

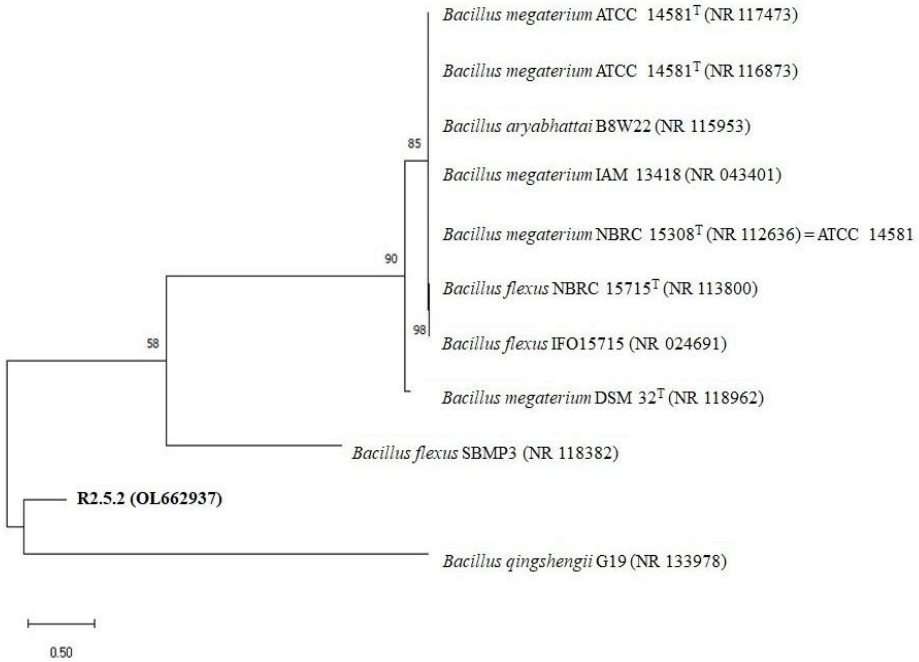


Figure 6 Neighbor-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequence of the strain R2.5.2 and closest species. Numbers on branches correspond to bootstrap values obtained with 1000 replicates.

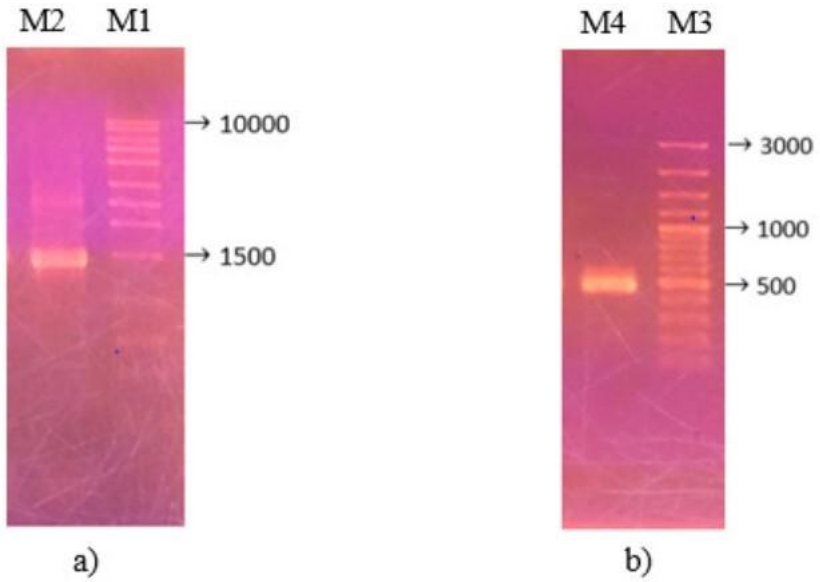


Figure 7 (a) PCR amplification of 16S rRNA gene (M1: 1 kb DNA Ladder, M2: 16S rRNA gene) and (b) arsC gene of strain R2.5.2 (M3: GeneRuler 100 bp DNA Ladder (G Biosciences, America), M4: ars C gene)

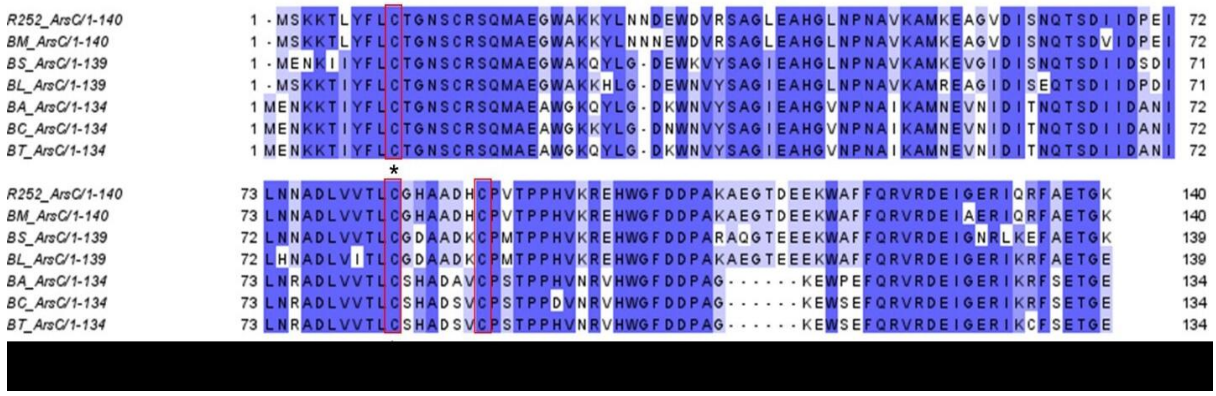


Figure 8 Comparison of protein sequences of ArsC gene from strain R2.5.2 with corresponding protein sequences of other *Bacillus* strains

Bmega-arsC-F and Bmega-arsC-R (Table 1), giving a single gene cassette with the size of 500 bp on 1% (w/v) agarose gel, which corresponded to the expected size when designing primers for amplifying the *arsC* gene from *Bacillus megaterium* (Figure 7b). The *arsC* gene of *P. megaterium* R2.5.2 was successfully amplified and the PCR product (Figure 7b) was purified and used for paired-end sequencing using primer pairs Bmega-arsC-F and Bmega-arsC-R. The *arsC* gene of *P. megaterium* R2.5.2 had a length of 423 bp, encoding a protein of 140 amino acids. The arsC gene sequence of strain *P. megaterium* R2.5.2 was submitted to the GenBank database under accession number OM055827.

A comparison between the amino acid sequence of *arsC* product from *P. megaterium* R2.5.2 with corresponding *arsC* products of other *Bacillus* strains (*B. megaterium*, *B. subtilis*, and *B. licheniformis*) revealed that the similarity was high (83% - 97%) (Figure 8).

ArsC of strain *P. megaterium* R2.5.2 was compared with that of *B. megaterium* DSM 319 (BMD_1727, 97.8%), *B. subtilis* 168 (P45947, 83.4%), *B. licheniformis* (Q65IV4, 87.05%), *B. anthracis* (Q81NJ6, 72.9%), *B. cereus* (A0A1Y6B0W9, 72.9%) and *B. thuringiensis* (Q6HGP0, 72.1%). The active site of ArsC was framed and marked with an asterisk (*). Protein sequence comparisons were performed with Clustal Omega and presented in Jalview. The intensity of the blue color gradient was encoded based on 50% sequence similarity.

4 Discussion

There are numerous arsenic-resistant bacteria have been collected from arsenic-rich environmental plants. In this study, 26 As(V) tolerant and 5 As(III) tolerant endophytic bacterial strains were isolated. Most of the As-resistant endophytic bacteria were found in the fern roots. Among them, *P. megaterium* R2.5.2 from the root

of *P. calomelanos* had the maximum tolerance to arsenate (320 mM) and arsenite (160 mM). Román-Ponce et al. (2018) reported that 27 strains isolated from roots of *Prosopis laevigata* and *Sphaerealcea angustifolia* were able to tolerate high concentrations of arsenic with MIC from 20 to over 100 mM for As(V) and 10–20 mM for As(III). According to Ghosh et al. (2018), *Bacillus aryabhattai* MCC3374 exhibited high resistance to arsenate (MIC: 100 mM) and arsenite (MIC: 20 mM). Furthermore, *K. palustris* NE1RL3 was an arsenic-resistant bacterium with MIC of 14.4 mM and 300 mM for As(III) and As(V), respectively, in the LB medium (Zacaria Vital et al. 2019). Similarly, Bermanec et al. (2021) isolated three bacterial strains from an arsenic-contaminated CrvenDol mine in North Macedonia with a MIC of 209 mM for arsenite and 564 mM for arsenate.

The results of this study showed that temperatures in the range of 10-50ºC was suitable for R2.5.2, and the optimal temperature is between 20 and 40ºC. A similar finding revealed that arsenite-oxidizing bacteria could also grow at a temperature between 40ºC and 50ºC (Kinegam et al. 2008).

Among the sugar sources that this isolate could actively consume, L-arabinose was considered as a major plant saccharide that could not be found in animals (Crozier et al. 2021). Hence, the ability to utilize L-arabinose was regarded as a trait contributing to an endophytic lifestyle, similar to the case of *Pseudomonas* endophytes of cucumber (Podolich et al. 2015). The capacity to consume a wide range of nutrient sources and produce numerous extracellular enzymes is likely to help this strain easily adapt to environmental conditions with diverse substrates ranging from sugars to complex organic compounds. In this study, isolate R2.5.2 could synthesize some extracellular enzymes, such as cellulase, chitinase, CMCase, xylanase, and protease. This isolate could actively utilize most of the sugar sources and grew well on agar plates containing D-fructose, D-mannitol, D-cellulose, sucrose, L-arabinose, and D-raffinose.

The biosynthesis of plant hormones such as IAA in endophytic bacteria is one of the vital mechanisms that can affect the growth of host plants, and increase the biomass of leaves, roots, and root length. Many studies have shown that metal-resistant endophytes were capable of producing IAA, which promoted plant growth. In this study, strain R2.5.2 possessed high IAA biosynthesis capacity with an average concentration of 19.14 mg/L. According to Luo et al. (2011), 30 Cd-resistant endophytes belonged to 4 groups i.e. *Actinobacteria* (43%), *Proteobacteria* (23%), *Bacteroidetes* (27%) and *Firmicut*es (7%), which produced 0.6-122 mg/LIAA. Similarly, a higher concentration of IAA biosynthesized by two Pb-resistant endophytes from the roots of *Alnus firma* was 15.8-27.9 mg/L was isolated by Sheng et al. (2008). According to Xu et al. (2016), bacterial strains isolated from *Pteris vittata* were also able to biosynthesize IAA ranging from 2.43-32.4 mg/L, which was higher than the findings reported by Zhu et al (2014), where IAA concentration was 0.2-10.8 mg/L. This is an important characteristic of endophytic bacteria, which increases the plant biomass to enhance arsenic resistance and accumulation.

As for As-tolerant/non-hyperaccumulator plants, evaporation or efflux of arsenic in roots can decrease arsenic translocation to shoot. In their roots, the conversion of arsenic to less toxic organic forms or transportation to vacuoles as As(III) or As(III)-glutathione/phytochelatin complexes occurs to prevent arsenic translocation to shoots. In contrast, the translocation of arsenic to shoots and reduction of As(V) to As(III) in hyperaccumulators are reported to be highly efficient, and efflux levels are insignificant. As(III) has been identified as the principal form of arsenic transported from root to shoot, regardless of whether As(V) or As(III) was supplied to the plants, even though As(III) was more toxic than As(V) in their organic forms (Gupta 2018). The reduction of As(V) to As(III) in roots may be a reason for translocating high amounts of arsenic in hyperaccumulators such as *I. cappadocica* and *P. vittata*. They were able to accumulate 60–80% of arsenic in shoots, while only 5–10% of total arsenic is found in non-accumulating species such as *P. tremula* and rice. Additionally, the reduction of As(V) to As(III) via arsenate reductase was accepted as the first step in the detoxification of arsenic in hyperaccumulators (Souri et al. 2017). Consequently, endophytic bacterial strains capable of converting As(V) into As(III) will increase the arsenic accumulation capacity of the plant by translocating arsenic from root to shoot, the part with the highest proportion of biomass of the plant. This study confirmed that isolate R2.5.2 belonged to the *Priestia megaterium* species. In previous studies, many members of the genus *Priestia* have demonstrated their ability to stimulate plant growth as well as tolerance to high arsenic concentrations. Some other studies also concluded that *P. megaterium* YC4-R4 and TG1-E1 showed high salt tolerance as well as plant growth-promoting characteristics (Biedendieck et al. 2021), *Exiguobacterium auranticum* SV7, *Paenibacillus sp.* SV10 and *Priestia koreensis* LV19 possessed an extensive range of antifungal as well as plant growth promoting activities (Bashir et al. 2021). Further, Gupta et al. (2020) reported that *Priestia aryabhattai* is resistant to arsenic and UV radiation. Similarly, Titah et al (2018) isolated *Bacillus megaterium* species from the roots of *Ludwigia octovalvis* and reported that it was resistant to arsenic and was also capable of absorbing arsenic.

In As-resistant bacteria, arsenate reductase coded by gene *arsC* mediated the reduction of As(V) to As(III) in the cytoplasm, and then As(III) was expelled from the cell via an ATP-independent ArsB, which facilitated arsenic detoxification (Stolz et al. 2006; Rosen 2002). Thus, the higher the As(V) reduction capacity of endophytes was, the higher their resistance to As(V) was. Xu et al.

(2016) also concluded that As(V) reduction by *Pretis vittata* endophytes had a positive relationship with arsenic tolerance. From this study, strain R2.5.2 possessed the arsC gene, which showed its potential in bioremediation of As-polluted soil, when used with As-hyperaccumulator plants.

In addition to arsenic, endophytic bacteria have been recognized for their contribution to plant growth and tolerance towards a wide range of heavy metals (Rajkumar et al. 2009). Das and Barooah (2018) studied the MIC of *Staphylococcus sp*. TA6 strain for Hg^{+2}, Cd^{+2}, Co^{+2}, Ni^{+2}, Cr^{+2} and found that the strain could survive at 0.5mM, 0.8mM, 1.0mM, 4mM, and 6mM, respectively. According to Manzoor et al. (2019), *P. aeruginosa* had high tolerance against Cd (20 mM), Zn (28 mM); *P. fluorescens* JH 70-4 displayed high tolerance to As (8 mM), Cu (6 mM), Ni (6 mM), and Cd (0.9 mM); *Pseudomonas spp*. PG-12 was efficient in resisting up to 0.6 mM Cd. In this study, strain R2.5.2 was also resistant to Ag^{+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, andCr^{4+} in the range of 0.5-16 mM, but at 16 mM concentration, strain R2.5.2 showed little to no positive reaction for growth. In particular, strain R2.5.2's growth was healthy with an increase in the presence of As (V) and As (III) at both 0.5mM (17.74% and 13.07%, respectively) and 1mM (7.29% and 1.12% in turn). Karn and Pan (2016) also showed that *Bacillus sp*. XS2 grew better at low As (III) concentration.

Conclusion

The selected strain R2.5.2 isolated from the root of *P. calomelanos* showed tolerance to high levels of arsenate (320 mM) and arsenite (160 mM) and was identified as *P. megaterium* R2.5.2. The strain grew well within a wide range of salinity (0.1-5% NaCl), pH (5-9), and temperature (20-40ºC). Apart from its ability to reduce As(V), strain *P. megaterium* R2.5.2 was tolerant to 0.5-16 mM of several heavy metals, including Ag^{+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Cr^{4+}. In addition, strain *P. megaterium* R2.5.2 produced on average19.14 mg/L of indole-3-acetic acid, suggesting its ability to promote host plant growth. The findings of this study may contribute to further understanding of As-hyperaccumulator plants and their endophytic bacteria in bioremediation of As-polluted soil.

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Conflict of interest

The authors of this article declare no conflict of interest.

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Screening, identification, and antibiotic activity of secondary metabolites of *Penicillium sp.* LPB2019K3-2 isolated from endemic amphipods of Lake Baikal

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ABSTRACT

This study aimed to assess the influence of nutrient media content on the production of antibiotics and the ability of water fungi isolated from lake Baikal to synthesize novel natural products. Interest in this topic stems from the high demand for new drugs, and studies are carried out via the screening of new natural products with biological activity produced by unstudied or extremophilic microorganisms. For this study, a strain of *Penicillium* sp. was isolated from endemic Baikal phytophagous amphipod species. Here, we identified natural products using the following classical assays: biotechnological cultivation, MALDI identification of the strain, natural product extraction, antimicrobial activity determination, and modern methods such as HPLC-MS for the dereplication and description of natural products. It was found that many detected metabolites were not included in the most extensive database. Most of the identified metabolites were characterized by their biological activity and demonstrated antibiotic activity against model Gram-positive and Gram-negative bacteria. The isolated strain of water fungus produced penicolinate B, meleagrin A, austinoneol A, andrastin A, and other natural products. Additionally, we show that the synthesis of low-molecular-weight natural products depends on the composition of the microbiological nutrient media used for cultivation. Thus, although the golden age of antibiotics ended many years ago and microscopic fungi are well studied producers of known antibiotics, the water fungi of the Lake Baikal ecosystem possess great potential in the search for new

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natural products for the development of new drugs. These natural products can become new pharmaceuticals and can be used in therapy to treat new diseases such as SARS, MERS, H5N1, etc.

1 Introduction

The chemistry of low-molecular-weight natural products is a well-known branch of pharmaceutical chemistry and is a historically effective method for translating the biotechnological potential of molecules found and extracted from different organisms such as plants, animals, fungi, or microorganisms (Newman and Cragg 2020). Microorganisms produce thousands of secondary metabolites with biological activity. Among the most identified natural products, most of them are produced by actinobacteria (65-70%), non-filamentous bacteria (10-15%), and microscopic fungi (20%). The biological activity of microbial natural products is often characterized by antibiotic activities (Demain 2014).

Nowadays, humankind needs a source of new natural products with biological activity. This is due to the increased number of diseases and the resistance of many pathogenic microorganisms to existing antibiotics (Hasan et al. 2015; Aslam et al. 2018). As the antibiotic crisis continues to grow, it will lead to the creation of new pharmaceutical ingredients using new assays, techniques, and substances. Additionally, the antibiotic crisis can be partially solved by intensifying the screening of new natural products, antibiotics, and their producers (Talebi et al. 2019; Jorgensen et al. 2022; Bhomwick et al. 2022).

The aquatic environment requires special attention because of the huge variety of free-living and symbiotic microorganisms and their secondary metabolism products (Aleruchi et al. 2018; Dat et al. 2021; Jamal and Sathianeson 2022). The biotechnological potential of aquatic microorganisms is great, and this is demonstrated by their ability to synthesize enzymes, antibiotics, terpenes, carbohydrates, and other organic molecules with biological activity (Imhoff 2016; Hitora et al. 2021).

The first antibiotic obtained from the aquatic fungus *Acremonium chrysogenum* was cephalosporin (Hu and Zhu 2016). Advances in the chemistry and biotechnology of cephalosporin C led to the synthesis of cephalosporin based drugs, which are used in regular medical practice to prevent the pathogenic spread and *Staphylococcus* infection. The discovery of penicillin led to studies on microorganisms as a great source of antibiotics, which, consequently, resulted in the development of biotechnological methods and the synthesis of biologically active natural products (Dembitsky 2014; Zhu et al. 2014; Richter et al. 2014; Gonçalves and Romano 2018). In the past 5-6 decades, interest in fungi has decreased (Kavanagh and Sheehan 2018; Yadav et al. 2019; Agrawal et al. 2022). There are significant barriers to the development of pharmaceutical studies with fungi, as these microorganisms are described as having an extremely low cultivation efficiency and high pathogenicity (Gupta et al. 2020). Such limitations restrict the discovery of antibacterial compounds that are synthesized by various unknown fungi. Studies on microorganisms that typically inhabit shallow water or soils led to the isolation of similar microorganisms with equal and well-studied biosynthetic capabilities (Hamza et al. 2015; Keller 2019). However, the studies performed in recent years testify to the intensification of studies using fungi for pharmaceutical biotechnology (Guo et al. 2022; Chen et al. 2022; Smith et al. 2023).

The commercial interest of pharmaceutical companies and the increase in of amount of clinical infections has led to the discovery of natural products produced by aquatic fungi. These compounds can be used to treat various diseases such as cardiovascular disease, diabetes, cancer, etc.(Grossart and Rojas-Jimenez 2016; Das et al. 2022; Miri et al. 2022). These compound-producing fungi were isolated from seawater, freshwater, and deep-sea organisms such as corals, macroalgae thickets, and other aquatic organisms (Frisvad 2015; Durand et al. 2019; Fernandes et al. 2022; Rad et al., 2022).

Lake Baikal is characterized as having high biotechnological and biomedical potential due to its diverse and well-studied flora, fauna, and associated microorganisms (Axenov-Gribanov, 2016a, 2016b, 2020; Protasov 2017; Shishlyannikova et al. 2017; Sukhanova et al. 2017; Zemskaya 2020; Voitsekhovskaia et al. 2020; Lipko and Belykh 2021). Amphipods (crustaceans) represent the main group of aquatic organisms in Lake Baikal, as they are well distributed and reflect the highest diversity in the lake (Rabosky 2022).

Until now, no studies have been conducted on the pharmaceutical properties of the aquatic fungi of Baikal. This study aimed to assess the influence of the nutrient media content on the production of biologically active natural antibiotic products and to estimate the Baikal *Penicillium* sp. strain's ability to synthesize novel natural products.

2 Materials and Methods

2.1 Animal samples and isolation and identification of strain *Penicillium* sp. LPB2019K3-2

The strain *Penicillium* sp. LPB2019K3-2 was isolated from the endemic species of the amphipod *Eulimnogammarus cyaneus*. *E. cyaneus* is a relatively small (adult body size: 11-15 mm) phytophagous species that is widespread around the shoreline of

Lake Baikal (Jakob et al. 2017; Takhteev 2019;). The amphipods were collected from Listvyanka village (N 51.867936, E 104.829715, South Baikal) using a benthic dragnet. The amphipods were then washed with 70 % ethanol and sterile distilled water. Then, animals were homogenized manually in 20 % sterile glycerol at a ratio of 1:10. One liter of water from the sampling point was collected as a negative control. This water was filtered through a syringe bacterial filter with a 0.45 µm pore diameter.

Strain of *Penicillium* sp. were isolated and cultured on solid nutrient medium mannitol–soya flour (MS) agar (D-mannitol, 20 g/L; soy flour, 20 g/L; agar, 20 g/L; pH 7.2) at 28 °C (Zhao et al. 2019). The culturing medium was supplemented with the antibiotic phosphomycin (100 µg/mL). Homogenates were diluted from 10 to 1000 times in sterile 1 % saline solution, and the prepared dilutions were plated on MS medium; each dilution was replicated thrice. The prepared Petri dishes were incubated for 14 days at 28 °C and were checked every 24 h to determine the appearance of fungal colonies. Fungi were recognized based on the morphology of the colonies and aerial mycelium (Suhaib et al. 2011).

The isolated strain was identified using the MALDI BIOTYPER system (Massachusetts, USA). For this, 12-18 h colonies were used (Sogawa et al. 2011). The direct load method was implemented using α-cyano-4-hydroxycinnamic acid. Triplicate identification was performed until samples of the strain were in the "Green zone" (high-reliable identification) (Ferreira et al. 2010).

2.2 Cultivation of strain and extraction of secondary metabolites

The isolated and identified fungal strain was cultured to evaluate the primary synthesis of secondary metabolites. Cultivation was performed in a selected liquid media such as HMP-broth (HMP-base, 21g/L; NaCL, 9 g/L) or TSB (casein peptone/pancreatic 17 g/L; K_2HPO_4 2.5 g/L; glucose, 2.5 g/L; NaCL 5 g/L; soy peptone3 g/L). Cultivation was performed in 100 mL of liquid nutrient media for 7 days at 28 °C (UI Hassan et al. 2019).

After 7 days of cultivation, liquid cultures were centrifuged at 3000 rpm for 10 minutes. Metabolites were extracted from the supernatant with ethyl acetate (Sigma Aldrich, Darmstadt, Germany) in equal proportion. Crude extracts from cell biomass were obtained using an acetone: methanol mixture (1:1 ratio). Natural products were extracted according to the general manual for the isolation of natural products (Nahar and Sarker 2012). The resultant extracts were evaporated and dissolved in 500 µL of methanol (Sigma Aldrich, Darmstadt, Germany).

2.3 Estimation of antibiotic activity

Three test cultures, namely *Bacillus subtilis* ATCC 66337, *Pseudomonas putida* KT 2440, and *Saccharomyces cerevisiae* BY4742, were selected to test the antibiotic activity of the crude extracts. The antibiotic activity of the crude extracts was qualitatively analyzed using the disk diffusion method (Surabhi et al. 2018). To assess antimicrobial activity, 30 µl of the extract was loaded onto 5 mm paper disks and dried at room temperature. Then, the disks were transferred onto solid LB and YPD media with inoculated test cultures. Experimental Petri plates were incubated for 12–24 h at 37 °C until growth inhibition zones appeared.

2.4 Screening and identification of secondary metabolites

To screen and identify the secondary metabolites in the fungi cultures, we used the modern and often used HPLC-MS method and further dereplication analysis. The HPLC-MS method allowed us to perform a separation of the crude extract for detailed chemical analysis or analysis of natural products. The dereplication analysis of natural products allowed us to estimate the chemical composition of the primary and secondary metabolites using a database of natural products.

For HPLC-MS analysis, samples were chromatographically separated using the UHPLC system (Ultimate 3000, Dionex, Sunnyvale, USA). The C18 UPLC column (ACQUITY UPLC BEH 100 mm x 2.1 mm, 1.7 µm 130 Å, Waters, USA) was used in this study. A linear gradient of acetonitrile in water was used. The time of analysis was 20 min, with a flow rate of 0.5 mL/min. The solvents were supplemented by 0.1 % ammonium formate. After an initial assessment, the samples were analyzed with mass spectrometry (ultra-high resolution, Orbitrap XL, Thermo Fisher Scientific, USA). Mass detection was performed in positive mode with the detection range set to 160–2500. Data were collected and analyzed using Xcalibur software v.4.4. (Thermo Fisher Scientific, USA). The dereplication of natural products and screening for known compounds was performed using the Dictionary of Natural Products database ver.2019 (CRC Press, Boca Raton, USA), and the following search parameters were used: accurate molecular mass, absorption spectra, and biological source of compound isolation (Whittle et al. 2003). Compounds were considered to be similar when the difference in the accurate mass was less than 10 ppm and when the absorption spectrum and biological source of the compound isolation were identical.

2.5 Reactives and chemicals

All chemicals used in this study for analytics and extraction procedures were characterized as "pharmacopeial grade" and manufactured by Sigma Aldrich (Darmstadt, Germany), MP biomedicals (Eschwege, Germany), and BD (Heidelberg, Germany). For mass spectrometry (both LCMS and MALDY), we used ultra-pure chemicals with the grades "for mass spectrometry", "HPLC", and "molecular biology grade". The chemicals used to prepare the nutrient media and for the cultivation of *Penicillium* sp.

were characterized as "microbiological grade", except soy flour. The soy flour was bought at a local market.

2.6 Statistical analysis

The experiments to estimate antimicrobial activity were performed three times to standardize the cultivation parameters and to reduce the risk of research being performed with a wild and nonstable strain. Only qualitative analysis was performed during the current study. For mass spectrometric analysis, we used a combined sample pooled from the above-mentioned three extracts.

3 Results

3.1 Estimation of antibiotic activity

In this study, the strain *Penicillium* sp. LPB2019K3-2 was isolated from endemic species of the amphipod *E. cyaneus*. This strain of fungus has been found in 80% of the amphipod *E. cyaneus* from Listvyanka village. Being detritivorous, phytophagous, and necrophagous, amphipods of this species undoubtedly have associations with microorganisms. The latter may provide defense against various pathogenic agents ingested by amphipods. Due to the disease caused by bacterial infection with *E.cyaneus* being unknown, it can be described by its close association with aquatic fungi. The disease caused by fungal infection associated with *E. cyaneus* has never been mentioned in the literature.

In addition to the studied strain, in this study, we isolated another 14 morphologically different strains of fungi. However, due to the fast sporulation and hazardous experiments carried out with microscopic sporulating fungi, here, we perform an analysis to characterize the biotechnological potential of only one strain—*Penicillium* sp. LPB2019K3-2.

3.2 Estimation of antibiotic activity

The chosen strain is characterized by the presence of activity against Gram-positive and Gram-negative bacteria. According to classical assays of microorganism cultivation (Zhao et al. 2019), the strain of *Penicillium* sp. LPB2019K3-2 was cultivated in liquid nutrient media using an orbital shaker to produce secondary and bioactive metabolites (Nahar and Sarker 2012). The extracted natural products were tested using the model and nonpathogenic test cultures of microorganisms (Surabhi et al. 2018).

Table 1 demonstrates the antibacterial activity of the strain *Penicillium* sp. LPB2019K3-2 cultivated in the liquid nutrient media TSB and HMP. The results of the study revealed that the fungal strain *Penicillium* sp. LPB2019K3-2 growing in TSB liquid medium was able to synthesize natural products extracellularly, and inhibiting the growth of the bacterial test cultures *B. subtilis* and *P. putida*. Moreover, we found that the extracts of cellular biomass of the strain *Penicillium* sp. LPB2019K3-2 cultivated in TSB medium were active only against the Gram-positive bacteria *B. subtilis*. Antibiotic activity against *S. cerevisiae* was not observed. It was revealed that the strain cultivated in HMP nutrient medium also showed similar activity to that of the strain cultivated in the TSB medium. Cultivation of the strain *Penicillium* sp. LPB2019K3-2 in the tested nutrient media did not lead to the synthesis of natural products with activity against *S. cerevisiae*.

3.3 Screening and identification of secondary metabolites

Liquid chromatography and high-resolution mass spectrometry were used to assess the composition of the metabolites produced by the Baikal strain of *Penicillium* sp. Figure 1 presents chromatograms of the cell-free liquid culture and cellular biomass of strain *Penicillium* sp. LPB2019K3-2 cultivated in TSB and HMP liquid nutrient media.

The analysis of the cell-free liquid culture and cellular biomass extracts of the strain *Penicillium* sp. LPB2019K3-2 cultivated in a TSB liquid medium allowed for the identification of 8 out of 88 detected compounds known for the genus *Penicillium*. Cultivation of the strain in the HMP nutrient medium revealed 8 out of 58 detected compounds. Forty-six detected natural products had no hits in the used database and were characterized as having masses from m/z226.1672 to m/z 1305.2320 in the amphiphilic and nonpolar parts of the chromatograms. The identification and a brief description of the secondary metabolites are presented in Table 2.

Table 1 Antibiotic activity of strain *Penicillium* sp. LPB2019K3-2 cultivated in liquid nutrient media TSB and HMP

Medium	Crude extract	*B. subtilis*	*P. putida*	*S. cerevisiae*
TSB	Cell-free liquid culture	+	+	-
	Cellular biomass	+	-	-
HMP	Cell-free liquid culture	+	-	-
	Cellular biomass	+	+	-

"+" and "-"represent the presence or absence of antibiotic activity; TSB and HMP—nutrient media.

Table 2 Natural products identified within the Dictionary of Natural Products (CRC Press) from crude extracts of strain *Penicillium* sp. LPB2019K3-2 cultivated in liquid nutrient media

No	Retention time (min)	Natural products	Detected mass	Accurate mass	Δ ([M] (ppm)	Bioactivity	TSB medium		HMP medium	
							Cellular biomass	Cell-free liquid culture	Cellular biomass	Cell-free liquid culture
1	2.3	Penicolinate B	398.22681	398.220558	15.7	Antimalarial agent. Exhibits antibacterial and antifungal props	-	-	-	+
2	3.0	TryptamineNb-[2-(Methoxycarbonyl)acetyl	260.1154	260.116093	3.0	-	+	+	+	-
3	4.0	Cyclo(leucyltyrosyl) (3S,6S)-form	276.1467	276.147393	2.4	Inhibits biofilm formation of *Staphylococcus epidermidis*	-	+	+	-
4	4.3	Cyclo(4-hydroxyprolylleucyl); (3S,7R,8aR)-form	226.13092	226.131743	3.6	-	-	-	+	-
5	5.4	Cyclo(phenylalanylprolyl) (3S,8aS)-form	244.1202	244.121178	4.1	Shows a broad spectrum of antibacterial and gastrointestinal cell maturation-enhancing activity	-	+	+	-
6	7.1	Meleagrin A	433.1737	433.175005	2.9	Shows structural similarity to tremorgenic mycotoxins. Closely relatedtoNeoxaline	-	+	+	+
7	8.01	Territrem C	512.2044	512.204635	0.5	Strongly inhibits acetyl cholinesterase. Tremorgenic toxin	-	+	-	-
8	9.7	1,3,8-Trihydroxy-6-propylanthraquinone2'S-Hydroxy	314.0819	314.07904	9.1	-	-	+	-	-
9	11.8	Austinoneol A	414.20325	414.20424	2.4	-	+	-	-	-
10	11.9	Andrastin A	486.26044	486.261755	2.7	Protein farnesyltransferase (PFTase) inhibitor Mycotoxin	-	-	+	+
11	13.6	Predecaturin E	477.28498	477.287909	6.1	-	-	-	+	-
12	18.39	Citriquinone A	336.15891	336.15729	4.8	Antibacterialagent	+	-	-	-

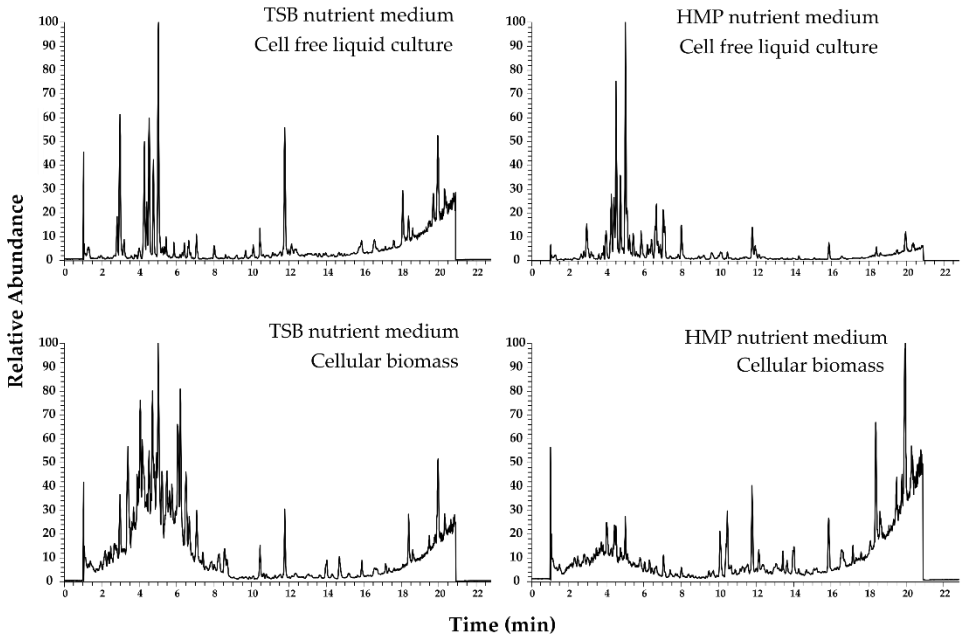

Figure 1 Chromatograms of cell-free liquid culture of strain *Penicillium* sp. LPB2019K3-2 cultivated in the TSB and HMP nutrient media

4 Discussion

The results of the current research describe the first study highlighting the antibiotic potential of the Baikal strain of *Penicillium* sp. isolated from the endemic amphipod *E. cyaneus*. In this study, *Penicillium* sp. was isolated and used, this strain was often (80%) found in the amphipod *E. cyaneus*. The results of the study found that the isolated strain was characterized by its antimicrobial activity. The strain inhibited the growth of Gram-positive *B. subtilis* and Gram-negative *P. putida*. By superposing the data from Tables 1 and 2, it can be assumed that activity against *P. putida* could be induced by the natural product cyclo (phenylalanylprolyl) (3S, 8aS)-form. Based on library data and published biological activity, this natural product demonstrates a broad spectrum of antibacterial and gastrointestinal cell maturation-enhancing activity (Bertinetti et al. 2009; Santos et al. 2019). Additionally, at least two natural products namely tryptamine Nb-[2-(Methoxycarbonyl) acetyl and meleagrin A are responsible for the activity against *B. subtilis*. Meleagrin A, known as a mycotoxin and antimicrobial agent, is produced by marine *Penicillium* sp. (Nielsen et al. 1999). However, the toxicity of meleagrin A does not explain the absence of activity against *S. cerevisiae*, as demonstrated in another study (Scopel 2013; Varga et al. 2015; Hamed et al. 2020). Thus, despite the presence of bioactive natural products in the list of identified metabolites (Table 2), there is a strong possibility that here, we have a low concentration of meleagrin A. Additionally, another (new) molecule could be or its non-active monomer could be responsible for the observed activity. Furthermore, the analysis of natural products revealed a low number of natural products that can be identified with high reliability based on the analyzed parameters, such as the accurate mass, UV spectrum, and biological source.

Accordingly, similar to previous studies of Baikal Lake microorganisms, there is no doubt that further studies on the secondary metabolites and metabolic pathways of this strain have great potential. Such potential is confirmed by the presence of a great number of new nonidentified natural products.

Nowadays, biologically active compounds produced by various microorganisms isolated from unusual habitats are receiving more attention (Gonçalves et al. 2013; Devi 2014; Durvasula and Rao 2018; Kumaravel et al. 2018; Zhang et al. 2018). The ecosystem of Lake Baikal and its inhabitants are no exception. Similarly, previous studies performed on microorganisms demonstrated the extent of antimicrobial and enzymatic activity. Studies performed on actinobacteria isolated from the Baikal-endemic mollusk *Benedictia baicalensis* revealed the new molecules Baikalomycins A–C, which demonstrated varying degrees of anticancer activity (Voitsekhovskaia et al. 2020). Other natural products found in Baikal microorganisms isolated from Baikal amphipods are Perquinolines A–C. Although these natural products did not show any prominent activity in the assays employed, the biosynthetic pathway leading to the formation of these compounds represents an unprecedented assembly of the tetrahydroisoquinoline core structure (Rebets et al. 2019). The findings that have been published to date suggest that there is no doubt that Baikal invertebrates are associated with microorganisms. However, the roles of the above-mentioned microorganisms in the life of amphipods is unknown. We can state with confidence that the

above-mentioned associations demonstrate the highest levels of biological organization and adaptation to aggressive environments.

Thus, representatives of *Penicillium* sp. can adapt to new, sometimes extreme, environments and specific conditions (Shukla et al. 2020; Ibrar et al. 2020), such as the ecosystem of Lake Baikal. The environmental peculiarities of Lake Baikal (low temperature, high water transparency, penetrating UV radiation, and high oxygen content) create specific conditions for speciation, the maintenance of the high level of biodiversity (Timoshkin 2009), and the adaptation of organisms.

Lake Baikal is a home to more than 2500 species of aquatic organisms (Berkin et al. 2009). Studies performed on the microorganisms in Lake Baikal have led to a comprehensive understanding of the role of microorganisms in ecosystems. However, only a few studies describe the biosynthetic potential of Baikal microorganisms. This reveals the importance of using modern molecular biotechnology methods and of creating new ways to study the biosynthetic and biomedical potential of microorganisms.

The extreme conditions mentioned here may help fungi of the genus *Penicillium* to produce specific and bioactive natural products. These compounds can play an ecological role in animals' lives and their symbionts. Moreover, the novel secondary metabolites detected in crude extracts of the studied *Penicillium* fungi can help us in the search for new drug candidates and can be used in the field of biopharmacy.

Conclusion

Thus, the study of microorganisms symbiotic to those that are endemic to Lake Baikal may result in the discovery of a new era of the chemistry of natural products in response to the increase in adaptation potential to different stress factors. Additionally, although the golden age of antibiotics ended many years ago and microscopic fungi are well-studied producers of known antibiotics, the water fungi of the Lake Baikal ecosystem possess great potential in the search for new natural compounds for the development of new drugs to act as therapies for new diseases such as SARS, MERS, H5N1, etc.

Conflicts of Interest

The authors declare no conflicts of interest.

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Synergistic Effect of Plant Growth Regulators on Micropropagation of *Eclipta alba*: A Plant with Diverse Medicinal Properties

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ABSTRACT

Synergism between various plant growth regulators is reported to be a key factor for the development of efficient *in vitro* propagation for any plant. Therefore, the current study examines the synergistic impact of plant growth regulators on *in vitro* propagation of *Eclipta alba*, an important medicinal plant possessing diverse medicinal properties. For the establishment of aseptic cultures, nodal segments were employed as explants on MS medium supplemented with 2.5 µM of 6-benzyle adenine (BA). Varying concentrations of BA and Kinetin (KIN)(0.0-5.0 µM), either alone or in combination with α-naphthalene acetic acid (NAA @ 0.0-5.0 µM) and indole 3-acetic acid (IAA@ 0.0-5.0 µM), were found to be effective for promoting shoot proliferation. Compared to KIN, BA was found to promote shoot proliferation and elongation more effectively. Further, the addition of 0.5µM NAA in the MS medium supplemented with 2.5 µM of BA increased shoot multiplication and elongation frequency from 58 and 17 percent to 65 and 21 percent respectively. The rooting frequency was found to be maximum on 1/2 strength MS medium supplemented with 5.0 µM of indole 3-butyric acid (IBA), which was found to be a superior auxin for inducing roots as compared to the NAA and IAA. With a 75% survival rate, *in vitro* raised plantlets were effectively acclimatized first in a poly house and later under greenhouse conditions. Molecular analysis was carried out using RAPD markers, with results indicating that the micropropagated plants were genetically identical to the mother plant. The developed micropropagation protocol for *E. alba* can be used at the commercial level for the mass multiplication of plants.

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1 Introduction

Eclipta alba (Linn.) Hassk also known as *E. prostrate* belonging to the family Asteraceae, is a well-known plant for its diverse medicinal properties (Timalsina and Devkota 2021). Locally it is known as Bhringraj, Bhringaraja, Kesaraja in Hindi, and false daisy in English. This plant is mainly used to treat jaundice for a long time and incorporated as a major ingredient in several antihepatotoxic drugs in various systems of medicines (John et al. 2014). Active ingredients responsible for anti-hepatoxic activity are wedelolactone and desmethylwedelolactone (Han et al. 2013). It is an important constituent of the dasapuspam, a combination of ten promising herbs used as a general tonic for good health and prosperity (Uthaman and Nair 2017). This plant is considered as an important herbal medicine in Ayurveda, Unani, and Siddha healthcare systems alike. Besides liver disorders, it is also considered useful for the treatment of hepatitis, spleen enlargements, chronic skin diseases, tetanus, and elephantiasis. Its scalp hair growth-promoting activities are also widely documented (Xiong et al. 2021).

It's widespread and has diverse medicinal uses that resulted in an increased rate of plant extraction from natural habitats. Therefore, populations of high-value medicinal plant species are decreasing at an alarming rate (Das and Mishra 2021). Continuous, haphazard, and indiscriminate harvesting of medicinal plants in large quantities from many ecosystems, together with habitat damage, is causing irreparable genetic diversity and planting material loss (Aggarwal et al. 2020). Moreover, *E. alba* is emerging as an economically valuable medicinal crop plant due to its utilization in the preparation of a wide array of medical products. Although the plant can grow under diverse environmental conditions, the growth is more profusely in moist or wet areas usually in the surroundings of rice fields, damp areas around wastelands, on the banks of wastewater channels contaminated with residues of toxic materials including chemicals and metals (Singh et al. 2010). Formulations prepared from such low-quality raw materials often results in poor-quality products and have quality control clearance issue also. Furthermore, traditionally *E. alba* is raised through seeds and stem cuttings in moist and wet places, growing plants in moist and wet conditions makes them vulnerable to attack by pests and various diseases, which leads to major crop loss (Singh et al. 2010). This arising the need for high-quality planting material in bulk to meet ever-increasing industrial demand of the plant with a uniform composition of active ingredients.

To overcome these problems, during the past few years, *in vitro* culture techniques have been extensively developed and applied to conserve high-value medicinal plants (Aggarwal et al. 2022). Moreover selection of cell lines with high secondary metabolites of therapeutic value will be significant in improving the pharmaceutical worth of this plant. A potential method of producing enough material for commercial planting is *in vitro* multiplications (Aggarwal et al. 2019). An exogenous supply of phytohormones is reported to be one of the most important variables regulating any plant's *in vitro* propagation, and convincing data suggest that synergism between auxins and cytokinins is one of the primary determinants driving cell differentiation and organogenesis (Alwakil et al. 2022). The effects of synergism on the establishment of effective *in vitro* propagation protocols for diverse plants have been examined in previous research (Rasool et al. 2013; Gupta et al. 2020). Different hormones have different signaling pathways and the ratio of endogenous to exogenous hormone levels can be linked to this behavior of hormones (Gupta et al. 2020). Hence the development of a simple and reproducible micropropagation protocol is essential to ensure the bulk supply of quality planting material for *E. alba* and the present study was carried out to develop an efficient micropropagation protocol for selected plants of *E. alba* by investigating the role of synergism between auxins and cytokinins.

2 Materials and methods

2.1 Plant material, chemicals, glassware

Selected plants of *E. alba* (young, free of any symptoms of diseases) growing at the herbal garden of M.M. college of pharmacy, university campus. Mullana, Ambala (Haryana, India) were selected for the study (Figure 1A). Nodal explants (Fig. 1B) were collected from selected plants and were brought to the laboratory for further processing and culture establishment. Throughout the study, MS medium (Murashige and Skoog 1962) containing 58 mM sucrose, gelled with 0.7% (w/v) agar (MS medium) was used as a basal medium. Before the medium was autoclaved at 121°C for 20 minutes, its pH was adjusted to 5.8. Aseptic cultures (Fig. 1C) were established using the protocol described by Aggarwal et al. (2012). Briefly, explants were inoculated on MS medium supplemented with 2.5 µM benzyl adenine (BA) alone at 25±1^{o}C under cool white fluorescent lamps (Philips India Ltd, Mumbai) with the light intensity of 42 µmolm^{-2}s^{-1} inside the culture vessel in a 16 h light. Nodal explants were surface sterilized with 0.1 % (w/v) mercuric chloride ($HgCl_2$) for 5-7 minutes and rinsed with sterile water for 3-4 times before inoculation into the culture vessel.

2.2 Effect plant growth regulators on shoot multiplication and elongation

To examine how plant growth regulators (auxins and cytokinins) affect shoot multiplication and shoot elongation, newly regenerated shoots were collected from parent explant and inoculated on MS medium that was differentially supplemented with BA and kinetin (KIN) (0.0-5.0 µM), either alone or in combination with NAA and IAA (0.0-5.0 µM). Different combinations and concentrations of cytokinins and auxins used in the study were given in Tables 1 and 2.

Table 1 The effect of different concentrations of BA and KIN on shoot proliferation and elongation of *E. alba* on MS medium

Plant growth regulators (μM)		*Morphogenic Responses*							
		% Explants responded		Average no. of shoots multiplied		Average no. of shoots elongated		Average shoot (cm)	
BA	KIN	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI
0	0	10.7±0.15^{k}	10.22, 11.10	1.1±0.3^{k}	0.76, 1.43	0±0^{j}	0.25, .25	1.5±0.30^{i}	1.14, 1.92
0.1	0	15.7±0.17^{i}	15.25, 16.14	5.5±0.20^{i}	5.23, 5.89	5.8±0.11^{c}	5.60, 6.12	4.5±0.20^{d}	4.14, 4.92
0.5	0	35.4±0.43^{f}	34.95, 35.84	10.4±0.30^{f}	10.10, 10.76	7.3±0.26^{a}	7.04, 7.55	6.4±0.43^{a}	6.01, 6.78
1.5	0	47.2±0.41^{c}	46.82, 47.70	14.2±0.25^{b}	13.90, 14.56	6.1±0.30^{b}	5.91, 6.42	5.1±0.23^{b}	4.74, 5.52
2.5	0	58.2±0.49^{a}	57.82, 58.70	17.4±0.25^{a}	17.10, 17.76	4.7±0.15^{ef}	4.50, 5.023	4.9±0.15^{c}	4.54, 5.32
5	0	52.4±0.404^{b}	52.02, 52.90	13.1±0.25^{c}	12.83, 13.49	4.3±0.20^{g}	4.11, 4.62	4.2±0.15^{ef}	3.84, 4.62
0	0.1	12.1±0.32^{j}	11.72, 12.60	4.3±0.25^{j}	4.00, 4.66	5.2±0.20^{d}	5.01, 5.52	4.4±0.40^{d}	4.07, 4.85
0	0.5	27.3±0.45^{h}	26.92, 27.80	8.5±0.35^{h}	8.20, 8.86	4.8±0.25^{e}	4.6, 5.12	4.2±0.35^{de}	3.84, 4.62
0	1.5	36.6±0.25^{e}	36.22, 37.10	11.6±0.26^{e}	11.26, 11.93	4.6±0.25^{f}	4.41, 4.92	3.8±0.36^{g}	3.41, 4.18
0	2.5	39.1±0.35^{d}	38.72, 39.60	12.3±0.15^{d}	12.03, 12.69	4.1±0.25^{h}	3.91, 4.42	4.1±0.49^{fg}	3.74, 4.52
0	5	32.2±0.41^{g}	31.82, 32.70	10.3±0.37^{g}	10.002, 10.66	3.9±0.17^{i}	3.64, 4.15	4.0±0.26^{h}	3.61, 4.38

Means± SD with the same letter within a column are not significantly different according to Duncan's multiple range test (P< 0.05), 95% CI- represents confidence intervals i.e. minimum and maximum values of mean at 95 % confidence level.

2.3 *In vitro* rooting of microshoots and plantlet acclimatization

For induction of *in vitro* roots, elongated shoots were cultured on full strength, 1/2 strength, and 1/4 strength of MS medium supplemented with varying concentrations of NAA, IAA, or IBA, ranging from (0–5 μM) (table 3). After successful root induction, plantlets were acclimatized in a poly house under regulated conditions of temperature (25–28^{o}C) and humidity (90–95 %) using a mixture of soil and agro peat (3:1 ratio) contained in polythene bags. A minimum humidity of 90% was maintained throughout the early periods and then gradually decreased to 40%. Later, plants were moved to conditions with a 50% light reduction (green net).

2.4 Testing of clonal fidelity

After two weeks of hardening, genomic DNA was isolated from the leaves of randomly chosen plants using Doyle and Doyle's (1990) CTAB technique. By loading an aliquot of samples, the quality of the DNA was examined on a 0.7% (w/v) agarose gel (Figure 2A), and the concentration was calculated using a standard spectrophotometer method (Sambrook and Russel 2001). For this, 10 RAPD decamer primers (OPD1-OPD10; Operon Technologies, Alameda, CA) were used for the PCR amplification and 20 ng of genomic DNA, 1.0 U of Taq DNA polymerase (Larova, Teltow, Germany), 100 μm of dNTP mixture, 2.0 μl of reaction buffer (10X), and 10 nmol of primer made up the reaction mixture was used. Mill-Q water was then added to bring the total volume to 20 μl. Amplifications were carried out using the Gene Amp 2700 thermal cycler (Applied Biosystem, San Francisco, USA). Initial denaturation at 94^{o}C for 5 min was followed by 41 cycles of 94 ^{o}C for 1 min, 45 ^{o}C for 45 sec, and 72 ^{o}C for 1.5 min, with a final extinction at 72 ^{o}C for 5 min. Following ethidium bromide staining, the amplified products were separated on a 1.5% (w/v) agarose gel and examined under a UV transilluminator (VilberLoumart, France).

2.5 Statistical evaluation

All studies were carried out using three replicates, two explants in each culture vessel, and were then repeated three times. GraphPad Prism 4 software was used for the analysis of variance in the data and to compare the means ± standard deviation using the Duncan Multiple Range Test (DMRT). Confidence intervals analysis, predicting minimum and maximum fluctuation in mean value is also carried out.

3 Results

3.1 Effect of Plant growth regulators on shoot multiplication and shoot elongation

Aseptic cultures were established utilizing mercuric chloride as a surface sterilizing agent as it was found satisfactory for the establishment of aseptic cultures. In the first experiment, the effects of various concentrations of BA and KIN cytokinins (ranging from 0.0 to 5.0 μM) on the proliferation and elongation of *E. alba* microshoots on MS media were examined. Among the selected two cytokinins, BA was found better as compared with

KIN (Table 1). The highest proportion of responded explants (58.2) was reported in MS medium supplemented with 2.5 µM of BA (Table1). Similarly, the maximum number of shoots also proliferated on the same medium (17.4 shoots per culture vessel). Whereas the highest amount of elongated shoots (7.3 per culture vessel) and highest shoot length (6.4 cm) were found on MS medium supplemented with 0.5 µM of BA (Table 1).

The second set of experiments looked at how varying concentrations of auxins i.e. NAA and IAA (0.0-5.0 µM) affected the growth and elongation of micro-shoots of *E. alba* on MS media supplemented with 2.5 µM of BA (Table 2). The addition of auxins increased the shoot multiplication and shoot elongation potential of the *E. alba* microshoots (Figure 1D, E). The percent response of explants increased to 65.3% with the addition of 0.5 µM NAA in

Figure 1 Micropropagation of *Eclipta alba,* A. Selected plants of *E. alba* growing at MMDU Mullana campus, B. Nodal segments of *E. alba* used for initiation of aseptic cultures, C. Establishment of aseptic cultures on MS medium supplemented with 2.5 µM BA, D. Shoot multiplication on MS medium supplemented with 0.5µM NAA in combination with 2.5 µM BA, E. Shoot elongation on MS medium supplemented with 0.1µM NAA in combination with 2.5 µM BA, F., In *vitro* rooting of micro shoots on ½ MS in combination with 5.0 µM IBA, f. Acclimatized *E. alba* plants

combination with 2.5 µM BA. A significant increase was also observed in the shoot multiplication frequency of microshoots of *E. alba*, whereas not much variation was observed in shoot elongation frequency and number of elongated shoots (Table 2).

Table 2 The effect of different concentrations of NAA and IAA on proliferation and elongation of *E. alba* on MS medium supplemented with 2.5 µM BA.

Plant growth regulators (µM)		*Morphogenic Responses*							
		% Explants responded		Average no. of shoots multiplied		Average no. of shoots elongated		Average shoot (cm)	
NAA	IAA	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI
0	0	11.1±0.25^{k}	10.84, 11.42	1.5±0.15^{k}	1.27, 1.78	0±0^{h}	0.21, 0.21	1.9±0.15^{i}	1.70, 2.15
0.1	0	28.3±0.25^{i}	28.07, 28.65	17.6±0.25^{i}	17.38, 17.88	7.8±0.20^{a}	7.65, 8.07	6.76±0.15^{a}	6.54, 6.99
0.5	0	65.3±0.25^{a}	65.04, 65.6	21.2±0.28^{f}	21.01, 21.52	6.8±0.05^{b}	6.65, 7.07	6.2±0.28c	6.00, 6.45
1.5	0	47.33±0.11^{b}	47.04, 47.62	18.2±0.15^{b}	18.01, 18.52	4.8±0.11^{e}	4.62, 5.04	5.4±0.15^{d}	5.20, 5.65
2.5	0	44.23±0.15^{d}	43.94, 44.52	14.4±0.26a	14.14, 14.65	4.1±0.26^{f}	3.88, 4.31	5.1±0.23^{de}	4.94, 5.39
5	0	34.7±0.30^{g}	34.47, 35.05	13.7±0.15^{c}	13.47, 13.98	3.9±0.15^{f}	3.72, 4.14	4.4±0.20^{g}	4.24, 4.69
0	0.1	26.4±0.28^{j}	26.17, 26.75	15.2±0.15^{j}	15.01, 15.52	6.3±0.1^{c}	6.08, 6.51	6.4±0.20^{b}	6.20, 6.65
0	0.5	45.5±0.20^{c}	45.24, 45.82	16.4±0.21^{h}	16.14, 16.65	5.6±0.22^{d}	5.38, 5.81	5.1±0.15^{e}	4.94, 5.39
0	1.5	41.4±0.20^{e}	41.14, 41.72	15.8±0.26^{e}	15.54, 16.05	4.6±0.20^{e}	4.45, 4.87	4.8±0.1^{f}	4.57, 5.02
0	2.5	36.4±0.35^{f}	36.14, 36.72	14.6±0.17^{d}	14.34, 14.85	4.3±0.27^{f}	4.13, 4.56	4.5±0.1^{f}	4.34, 4.79
0	5	32.1±0.15 ^{h}	31.87, 32.45	12.7±0.20^{g}	12.51, 13.02	3.8±0.15^{g}	3.65, 4.07	4.1±0.2^{h}	3.87, 4.32

Means± SD with the same letter within a column are not significantly different according to Duncan's multiple range test (P< 0.05), 95% CI- represents confidence intervals i.e. minimum and maximum values of mean at 95 % confidence level.

Table 3 Effect of different auxins and MS media strength on rooting of *E. Alba* micro-shoots

MS medium strength	Auxin (µM)	Percentage of microshoots showing rooting		Average no. of roots per shoot		Average root length (cm)	
		Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	95% CI
Full strength	0	0±0^{i}	0.23,0.23	0±0^{i}	0.17, 0.17	0±0^{f}	0.21, 0.21
Full strength	1.0 NAA	55.7±0.15^{k}	55.53, 55.99	2.4±0.2^{de}	2.22, 2.57	1.1±0.15^{de}	0.95,1.38
Full strength	2.5 NAA	61.3±0.15^{h}	61.10, 61.56	2.1±0.0 5^{f}	1.96, 2.30	1.4±0.15^{c}	1.21,1.64
Full strength	5.0 NAA	63.4±0.35^{f}	63.20,63.66	2.1±0.05^{ef}	1.99, 2.33	1.3±0.20^{cd}	1.11,1.54
Full strength	1.0 IBA	64.5±0.05^{e}	64.33, 64.79	2.5±0.15^{cd}	2.39, 2.73	1.16±0.25^{c}	0.951,1.38
Full strength	2.5 IBA	67.5±0.3^{d}	67.26,67.73	2.6±0.1^{c}	2.42, 2.77	1.2±0.15^{cd}	1.05,1.48
Full strength	5.0 IBA	71.3±0.2^{c}	71.06,71.53	3.2±0.15^{b}	3.06, 3.40	2.4±0.05^{b}	2.21,2.64
Full strength	1.0 IAA	58.4±0.11^{j}	58.23, 58.69	2.1±0.25^{f}	1.99, 2.33	1.13±0.25^{e}	0.91,1.34
Full strength	2.5 IAA	60.4±0.20^{l}	60.23,60.69	1.7±0.15^{g}	1.59, 1.93	1.3±0.20^{cd}	1.11,1.54
Full strength	5.0 IAA	62.7±0.11^{g}	62.53, 62.99	1.5±0.15^{h}	1.36, 1.70	1.1±0.11^{de}	0.95,1.38
½ MS	5.0 IBA	78.5±0.11^{a}	78.33, 78.79	3.5±0.15^{a}	3.36, 3.70	2.9±0.15^{a}	2.71,3.14
¼ MS	5.0 IBA	72.3±0.25^{b}	72.13,72.59	3.3±0.1^{b}	3.12, 3.47	2.4±0.26^{b}	2.18,2.61

Means± SD with the same letter within a column are not significantly different according to Duncan's multiple range test (P< 0.05), 95% CI- represents confidence intervals i.e. minimum and maximum values of mean at 95 % confidence level.

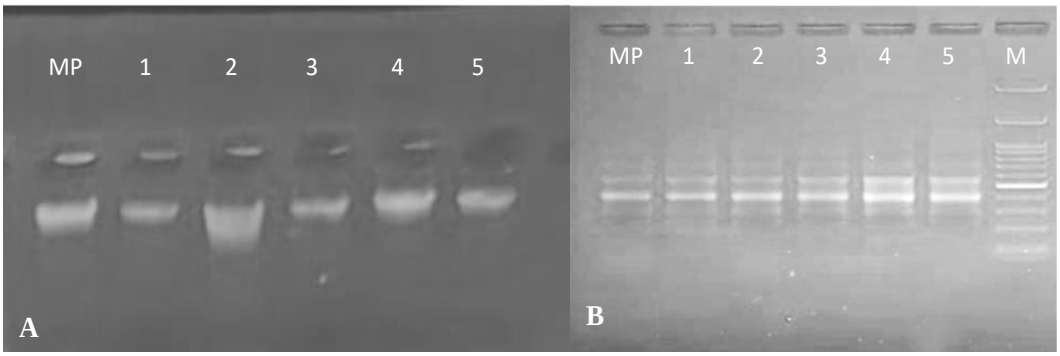

Figure 2, DNA quality check and RAPD profiles of micropropagated plantlets and mother plant of *E. alba*plants. A.Lane MP: Genomic DNA from Mother plant, Lane 1-5: Genomic DNA from micropropagated plant, B. RAPD profile with primer no 3, Lane MP: Mother Plant; Lane 1-5: micropropagated plants; Lane M: 1 kb molecular weight markers

3.2 Influence of auxins and medium strength on rooting of microshoots

On the rooting of microshoots, the impact of various auxins such as NAA, IBA, and IAA as well as medium strength (MS, 1/2 MS, and 1/4 MS) was also investigated. Among the tested auxins, IBA was found superior as compared to the NAA and IAA in the induction of *E. alba* microshoots root (Table 3). Maximum rooting frequency of microshoots i.e.78.5 % was observed on ½ strength MS medium containing 5.0µM IBA (Fig. 1F). Further, the maximum number of roots per shoot (3.5) and maximum root length (2.90 cm) was also observed on same medium (Table 3).

3.3 Acclimatization of plantlets

First, rooted *E. alba* microshoots were successfully acclimatized in polyhouse conditions with a survival rate of 80 %. After 15 days of exposure in the polyhouse, the plants were moved to a net house with a 50% light reduction, here the microshoots have a survival percentage of 75 % (Figure 1 G). After successful acclimatization plants were cultivated under field conditions. A total of 75 rooted microshoots from ½ strength MS medium containing 5.0 µM IBA were used in the study (3 experiments with 25 rooted micro- shoots). A potting mix containing soil, vermicompost, and sand in a ratio of 2:1:1 was used for the acclimatization of plantlets.

3.4 Assessment of clonal fidelity using RAPD analysis

Among the used ten RAPD primers, six were produced scoreable bands with sizes ranging from 250 to 2000 bp. With an average of 4 bands per RAPD primer, the number of bands for each primer ranged from a minimum of 3 to a maximum of 6. Bands had a monomorphic nature. The clonal nature of these shoots was demonstrated by the similarity between the RAPD banding profiles in plants generated from micropropagation and the mother plant (Figure 2B).

4 Discussion

The major problem faced by industries engaged in the production of plant-based medicinal formulations is the uninterrupted and uniform supply of quality planting material throughout the year. The problem can be overcome with the development of rapid, efficient, simple, and most importantly reproducible micropropagation protocol for the selected plants (Moraes et al. 2021). A similar issue arose with *E. alba* as well, while there are reports on its *in vitro* multiplication, they may be genotype-dependent and their techniques lack consistency (Dhaka and Kothari 2005; Singh et al. 2010; Ragavendra et al. 2014; Yesmin et al. 2015). Thus arises the need for the development of a genotype-specific micropropagation protocol for *E. alba* to meet ever increasing demand of the industry.

One of the foremost difficulties with the development of micropropagation protocol for any plant is the establishment of aseptic cultures from mature explants. Due to the problem of infestation by various microbes and enhanced frequency of contamination in explants selected from plants growing under field conditions as compared with explants chosen from plants growing under protected areas like a nursery, it is difficult to establish aseptic cultures (Bhadaneand Patil 2016). Field-grown plants are found to be more infected with bacteria and fungi both exogenously and endogenously. So it becomes important to properly disinfect explants for the establishment of aseptic cultures. In the present study mercuric chloride ($HgCl_2$) at the concentration of 0.1% (w/v) was used as a surface sterilizing agent and was seen to be satisfactory for the establishment of aseptic cultures. Mercuric chloride was the choice of surface disinfecting agent for the establishment of aseptic cultures in various micropropagation studies (Kajla et al. 2018, Samala et al. 2022).

In the present investigation both BA and KIN increased the frequency of explant regeneration as well as the number of shoots that proliferated and elongated, in comparison to the MS medium

without any PGR (Table 1). These results are in agreement with earlier studies where BA was found to be beneficial for *in vitro* shoot multiplication of various plant species including *E. alba* (Bhaskaran and Jayabalan 2005; Choudhary et al. 2021; Iiyama and Cardoso 2021).

Auxins are a group of naturally occurring and synthetically produced plant hormones. They assume a significant role in plant development and are known as master growth regulators in terms of various plant physiological activities like morphogenesis including cell division, differentiation, and elongation (Bishopp et al. 2011). Furthermore, other plant hormones, such as cytokinins, abscisic acid, gibberellins, and polyamines cooperate synergistically or antagonistically with auxin to trigger cascades of events prompting morphogenesis and other development processes in the plant (Hu et al. 2019). Therefore in the present study, the effect of NAA and IAA was investigated in combination with BA on various plant development processes (Table 2). The addition of NAA led to enhanced explant regeneration frequency including enhancement in the number of shoots proliferated and elongated per explant in comparison to MS medium with BA only (Table 2). NAA is reported to play an important role in cytokinin metabolism and stability (Saini et al. 2013) which may help in obtaining higher shoot regeneration frequency in explants including the number of shoots proliferated and elongated.

The success of any *in vitro* propagation protocol depends upon the induction of efficient *in vitro* rooting. Keeping in mind the importance of *in vitro* rooting, ample work has been carried out to develop efficient rooting protocols for various plants, and for this, auxins are the first choice as plant growth regulators (Aggarwal et al. 2020; Oanh et al. 2022). Therefore in the present study, also the effect of auxins i.e. NAA, IBA, and IAA (0-5.0 µM) was examined for efficient in vitro rooting of *E. alba* microshoots (Table 3). Furthermore, it has been shown that plants grown *in vitro* conditions occasionally experience nutritional stress and exhibit restricted root system growth to maximize nutrient absorption. Therefore, it's important to maximize nutrient concentrations since they frequently affect root growth by activating local and systemic signaling pathways (Elmaataoui et al. 2020). Therefore the impact of medium strength (full strength MS, ½ MS, and ¼ MS) on the rooting efficiency of *E. alba* microshoots was also investigated (Table 3). Lowering of basal medium composition in combination with IBA resulted in enhanced rooting of microshoots (Table 3). The rooted plantlets were finally acclimatized under polyhouse and greenhouse conditions and showed healthy growth and survival upon transfer to the potting mixture.

Clonal fidelity is one of the most important aspects of the micropropagation industry. Molecular markers (like RAPD) are found to be suitable for generating DNA profiles and have proved to be an effective tool in assessing the genetic stability of plants propagated through *in vitro* methods (Williams et al. 1990). The use of RAPD and ISSR markers to identify genetic similarities or differences in micropropagated material from distinct plants has proved successful (Aggarwal et al 2012, Singh et al. 2012). The remarkable degree of genetic homogeneity displayed by the micropropagated plants in the current study (Figure 2), which attributable to the genome's resistance to aseptic manipulations and cultural stresses throughout the various stages of micropropagation.

Conclusion

Conclusively, the present study presents a simple, efficient, and reproducible micropropagation protocol for selected plants of *E. alba.* The interaction among auxins and cytokinins is found to be critical for the optimization of micropropagation protocol. Lowering of basal medium composition was found suitable for efficient rooting of microshoots. The developed protocol can be successfully used for large-scale production of *E. alba* plants for a uniform and stable supply of quality plantlets for the pharmaceutical industry.

Conflict of Interest

There are no conflicts of interest among the authors.

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Elucidation of the morpho-physiological traits of maize (*Zea mays* L.) under salt stress

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ABSTRACT

Agriculture is an essential sector for the increasing world population, hence the need for more food production. However, the aim of increasing food crop production is mostly suppressed by abiotic stresses such as drought and salinity. Salinity is a major limiting factor that inhibits the potential of plant growth and productivity worldwide. Hence, understanding the mechanisms behind plant stress response is important for developing new biomarker approaches that will increase salt tolerance in crops. To survive, plants exhibit various morphological, physiological, and biochemical processes when faced with saline conditions. This study was carried out to explore and evaluate the morphological and physiological effects of salinity on maize grown in the absence/presence of NaCl, followed by measurement of the various growth parameters at the end of a treatment cycle. Results of the study revealed that salt stress significantly decreased growth parameters such as plant height, leaf number, leaf width, leaf area, leaf length, and shoot (weight and length). On the other hand, salinity decreased physiological traits such as stomatal count, stomatal density, transpiration, and respiration rates. This study has shown the negative effects of salt stress on the morphology and physiology of maize. These findings can be used as a reference tool in stress response studies focusing on salt stress pathways in maize and other related crops.

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1 Introduction

Like any other living organism, plants also experience several abiotic and biotic stresses. For these reasons, plants are the most significant organisms to study as they are more vulnerable to morphological, physiological, and molecular changes when subjected to variable environmental conditions. Major environmental conditions that impact the growth, development, and survival of plants include extreme temperatures, drought, and salinity (Aslam et al. 2015; Alkahtani 2018). These stresses can cause damage either individually or in combination, however, extensive damage is mostly experienced when the stresses are in combination since different stress factors are considered additive or cumulatively interactive (Rafique et al. 2020).

Salinity is among the foremost problems in crop production since it strongly inhibits plants from exhibiting their genetic potential globally (Kaushal and Wani 2016; Singh et al. 2018; Singhal et al. 2021). Lack of adequate rainfall, poor irrigation, and secondary soil salinization mostly causes and intensify salinity stress (Alkahtani 2018). Consequently, this salt stress inhibits plant growth through osmotic and ionic effects; however, different plant species have established mechanisms to survive these effects (Khosravinejad et al. 2008). Under severe states, salinity decreases the average yields of major crops such as wheat, rice, cotton, and maize worldwide (Bartels and Sunkar 2005). Notably, various morphological, physiological, and biochemical processes allow certain crops to resist or adapt to these severe conditions. These resistance mechanisms may typically be in a form of escape, avoidance, or tolerance (Muhammad et al. 2015).

The interference of uptake and transport of essential nutrients caused by ion toxicity or low water potential results in high osmotic stress, which is a potential effect of salt stress on crop growth. Physiological processes such as photosynthesis, respiration, starch metabolism, and nitrogen fixation are also highly affected by saline conditions, leading to quantifiable loss of crop productivity (Farooq et al. 2015). Plant physiological and biochemical responses to salt stress and its tolerance mechanisms have been a major focus for plant scientists (Zhu et al. 2012). To this point, current knowledge about the key driver processes involved in plant adaptations to abiotic stress conditions, particularly salinity, is still very limited. Therefore, there is a need to understand the mechanisms of maize response and tolerance/adaptation strategies to salinity. On that point, information on the effects of salt stress in maize from germination to harvest stages has so far been partially presented, hence this study then focused on the morphological and physiological responses of this crop plant, particularly its photosynthetic, transpiration and respiratory processes, using some laboratory cultured maize (*Zea mays*) plants under salt conditions.

Maize is one of the third most important cereal crops after rice and wheat, which is grown in mild, sub-tropical, and tropical regions worldwide (Shekhar and Singh 2021). It belongs to the Poaceae family, which is moderately sensitive to salt stress (Maas and Hoffman 1977; Maas et al. 1983; Chinnusamy et al. 2005; Farooq et al. 2015). It is generally grown in numerous countries and acts as a key crop with multipurpose roles including human consumption, animal feed, and bioenergy production (Muhammad et al. 2015). In addition, maize is considered as a great crop model used for multiple investigations such as the determination of genetic components of certain crop plants and their stress adaptation mechanisms - this is due to its significant metabolism adapted for survival in extreme environmental conditions (Rafique et al. 2020). Apparently, maize responds negatively to salt stress as demonstrated by a decreased germination rate, stunted growth, reduced photosynthesis, and less productivity (Farooq et al. 2015; EL Sabagh et al. 2021).

Salt stress causes a serious financial strain on the agricultural farming sector due to reduced crop yield (Munns and Gilliham 2015). Moreover, it is a major obstacle to global food security considering that maize is a dominant crop plant used as a staple diet, animal feed, and energy source. Consequent to population growth and high limiting standards in several areas in arid and semi-arid regions, increasing crop yield has become a controversial topic concerning food security, specifically in Africa (Hussein et al. 2007; Pholo 2009).

Several studies have investigated salt stress effects on the developmental growth stages of maize, however, there is no comprehensive study that has evaluated the physiological responses of this crop to salinity, specifically concerning its respiratory and transpiration rates (Farooq et al. 2015). To the best of our knowledge, there is limited information on the evaluation aspects of these physiological parameters to salinity, more specifically in the QN701 cultivar. It is, therefore, imperative to understand the salinity concentration that triggers/modulates respiration and transpiration rates in maize. This study may perhaps assist to clarify the mechanisms of salt stress on transpiration and respiration in maize. This study focused on the assessment of salt stress responses and/or adaptation systems in a drought-tolerant maize cultivar (QN701) at the morphological (e.g., root morphology and plant growth) and physiological (stomatal quantity, leaf respiration, and transpiration rates) levels when irrigated with saline or non-saline solutions. The acquisition of morphological and physiological responses of crops to salinity is valuable data that can be utilized for breeding programs and advisory purposes by plant breeders.

2 Materials and methods

2.1 Plant material and seed sterilization

This study was conducted at the Plant Biotechnology Research Laboratory, Department of Botany, North-West University, South

Africa. The *Zea mays* (QN701) seed cultivar utilized in this study was acquired from Quality Seed (Dalton, KwaZulu-Natal, RSA). This cultivar is regarded as a white single cross hybrid that is non-GMO, suitable for irrigation and dry land conditions and is normally used for grain and silage production. The selected cultivar has been reported to be of excellent yield potential and disease-resistant (Quality Seed). A total of 30 seeds were selected for uniformity in terms of size and physical appearance, and 5 seeds per plant pot during experimentation. The seeds were surface sterilized following the procedure described by Dikobe et al. (2021), where they were collected in a 50 ml falcon tube with 70% (v/v) ethanol and vortexed for a minute, followed by further decontamination with 1.25% (v/v) sodium hypochlorite solution (bleach) for 10 minutes. Immediately after surface sterilization and decontamination, the seeds were washed three times with 3 ml of sterile distilled water. The washed seeds were allowed to imbibe in sterile distilled water at room temperature for 20 minutes, to promote rapid germination.

2.2 Germination of seedlings and treatment growth conditions

Five seeds were sown in each of the 6 plastic plant pots (16 cm diameter), filled with a 3:2 (v/v) mixture of sterile organic soil (Culterra, Muldershift, South Africa) and vermiculite (serial# SMC-9001, Rajasthan, India). The intended maize plants were randomly grown under greenhouse conditions of long days (16-hour days) and short nights (8-hour nights) at a temperature of 25/22°C day/night. The sown seeds were irrigated at every 2 days intervals with 200 ml of sterile tap water until germination was initiated on day 7. Plant treatment commenced on the 8th day after germination, whereby the plants were separated into two groups i.e. T_C= Control, and T_E= 100 mM NaCl.

The non-salt stressed plants (control) were irrigated at every 2 days intervals with 200 ml sterile tap water while the salt-stressed plants (experiment) were irrigated with 200 ml of 100 mM NaCl solution, for 28 days. After 28 days (treatment period), plants were harvested to measure the morphological growth traits and physiological parameters. Each treatment group was conducted in three independent biological replicates (n = 3).

2.3 Measurement of shoot and leaf functional traits

The protocols as described by Huang et al. (2019) were used for measurements, wherein leaf length was defined as the distance between the leaf base and leaf apex, which is at the junction of the petiole and leaf blade. Leaf width was defined as the maximum distance between the edges of the blade that is perpendicular to the straight line through the leaf apex and leaf base. The number of leaves per plant was manually counted. Similarly, a ruler measured the plant heights, leaf widths, and shoot lengths in cm. Leaf area was calculated using the formula: leaf area= leaf length × leaf width × K, whereby K = 0.75 as a coefficient constant (Musa and Usman 2016). The shoot fresh weights were measured with an electrical weighing balance (Radwag, model # PS 750/C/2, Lasec, Midrand, South Africa) in grams. All the morphological measurements were taken from three biological replicates of each treatment (n = 3).

2.4 Measurement of the physiological parameters

2.4.1 Leaf stomatal density

The leaf stomatal density was determined following the Xu and Zhou (2008) method, which expresses the number of stomata per unit leaf area from smooth leaves with little to no leaf hairs. The leaf epidermal structures of both the abaxial and adaxial surfaces of fleshy expanded maize leaves from the control and treatment plants were identified following the method described by Volenikova and Ticha (2001). A thin layer of clear nail polish was spread on each surface (abaxial and adaxial) and allowed to dry. A strip of clear sticky tape (approximately 12 mm x 20 mm) was placed over the dried leaf for both the abaxial and adaxial sides and pressed down to form a leaf impression. The sticky tape was peeled off and placed on a microscope slide and immediately viewed under 400x magnification using a Primo Star light microscope (Carl Zeiss Microscopy, Germany), and images were captured with a digital camera coupled to the microscope (Axiocam 208 color, Zeiss, Germany). The stomatal density of each leaf was recorded per unit area, as the number per square mm. The microscope diameter of the field of view (FOV) was 0.05 mm and its area was calculated using the formulae as described by Volenikova and Ticha (2001):

$$\text{FOV} = \frac{\text{field number}}{\text{magnification number}}$$

$$\text{Area of FOV} = \pi r^2$$

where π = 3.14 and r^2= radius of the field of view

Therefore, to determine the stomatal density, the below formula was used:

$$\text{stomatal density} = \frac{\text{number of stomata in entire FOV}}{\text{Area (mm}^2\text{)}}$$

2.4.2 Transpiration and respiration rates

Various physiological traits such as photosynthesis, transpiration, and respiration were measured by following the method described by Dikobe et al. (2021), using an LCpro-SD infra-red gas analyzer (IRGA) (ADC BioScientific, Hertfordshire, UK). The physiological readings were taken under randomized design by clamping a leaf into the leaf chamber of the IRGA. Measurements were then taken from the leaf adaxial

surface on three independent biological replicates for each treatment group (T_C and T_E). The resultant readings, displayed on the device's screen, were taken for 3 minutes at 10-second intervals. The following parameters were measured: photosynthetic rate (A) ($\mu mol.m^{-2}.s^{-1}$) and transpiration rate (E) ($mol.m^{-2}.s^{-1}$) under ambient temperature (25-27°C). Photosynthesis and transpiration graphs were then constructed by plotting the obtained response values against time. To measure the respiration rate, negative values from the photosynthetic responses were recorded and used to plot a graph against time.

2.5 Statistical data analysis

Analysis for all the morphological and physiological parameters was based on the means of three independent replicates, where corresponding responses for each process were subjected to one-way analysis of variance (ANOVA) (Super-Anova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). To verify the significance of variations between treatments, the means (n = 3) were separated using *post hoc* Student Newman Kuehls (SNK), multiple range test ($p \leq 0.05$).

3 Results

3.1 Morphological impact of salinity stress on maize

The effects of salinity on maize plants were initially analyzed on the appearance of T_C (non salt stressed plants) and T_E (salt-stressed plants) (Figure 1). Various morphological growth changes between the T_C and T_E were observed and recorded after 28 days of treatment, wherein T_E phenotypically showed some noticeable changes in plant growth attributes compared to T_C. Numerous morphological traits were induced by salt stress as indicated in Table 1.

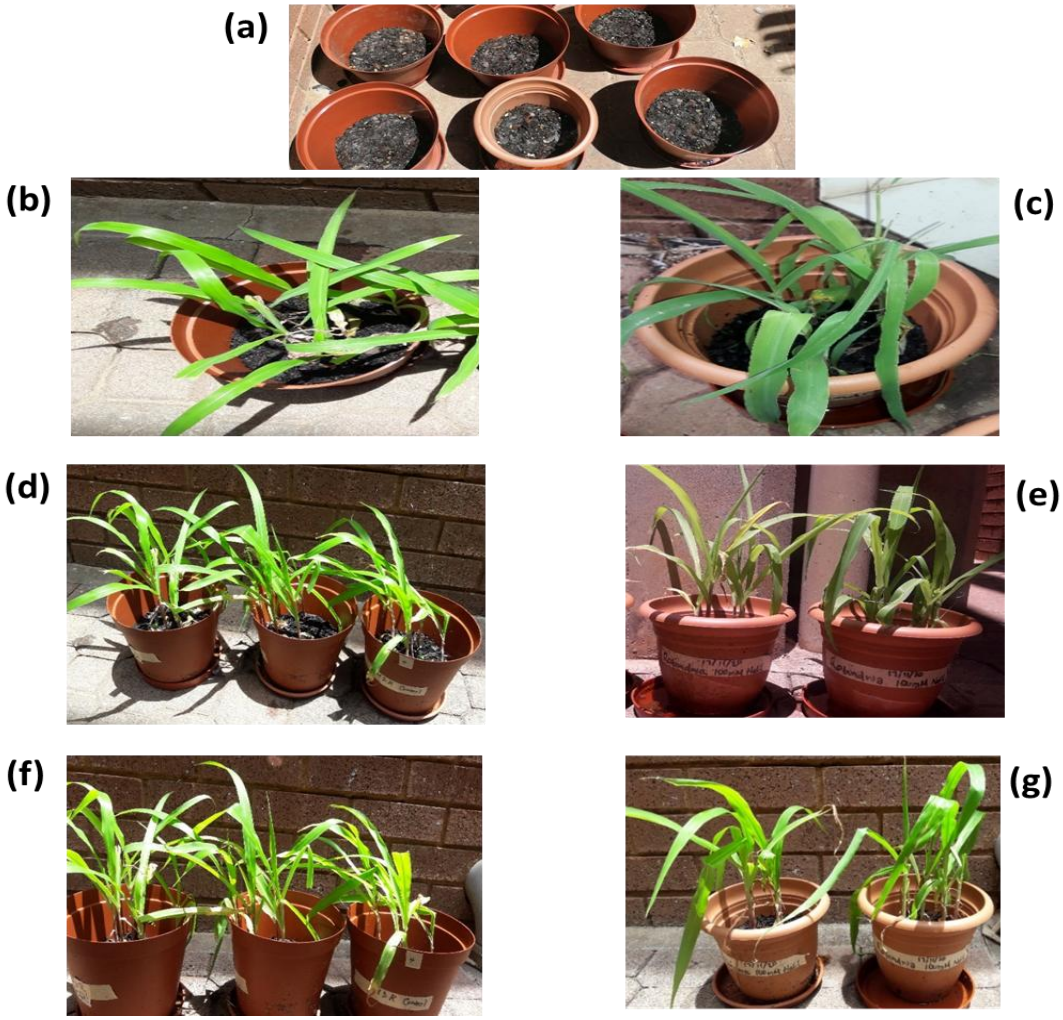

Figure 1 Morphological responses of Zea mays to salt stress; the phenotypic appearance of maize plants in response to salt stress (a) planting pots and soil used to sow the seeds, (b) non-salt stressed plants on day 8, (c) salt stressed plants on day 8, (d) non-salt stressed plants on day 18, (e) stressed plants on day 18, (f) non-salt stressed plants on day 28, and (g) salt stressed plants on day 28

Table 1 The effects of salt stress on the morphological parameters of maize plants exposed to 0 mM and 100 mM NaCl treatment for 28 days.

Morphological Parameters	Control (0 mM NaCl)					Experiment (100 mM NaCl)				
	T_C	T_C	T_C	Mean ± SD	SEM	T_E	T_E	T_E	Mean ± SD	SEM
Plant height (cm)	53	45	36	40.50 ± 6.36	3.67	38	30	35	34.33 ± 4.04	2.33
Leaf length (cm)	36.5	30	28	31.50 ± 4.44	2.57	32	29	30	30.33 ± 1.53	0.88
Leaf area (cm^2)	76.5	81	75	77.55 ± 3.10	1.79	34.56	31.61	25.2	30.46 ± 4.79	2.76
Leaf width (cm)	2.1	2.7	2.2	2.33 ± 0.32	0.19	1.08	1.09	1.05	1.07 ± 0.02	0.01
Leaf number	15	17	16	16.00 ± 1.00	0.58	15	13	11	13.00 ± 2.00	1.15
Shoot length (cm)	14	11.5	13.6	13.03 ± 1.34	0.78	10	12.5	8.5	10.33 ± 2.02	1.17
Shoot fresh weight (g)	1.72	1.02	1.27	1.34 ± 0.35	0.2	1	1.4	0.9	1.20 ± 0.28	0.16
Leaf color	Bright green	Bright green	Bright green			Dark green with brown apex	Dark green with brown apex	Dark green with brown apex		

T_C: Treatment control; T_E: Treatment experiment; means ± SE of three independent experiments (n=3); SD: standard deviation; SEM: standard error of the mean ($p \leq 0.005$).

3.2 Effects of salinity on maize growth

The plant height, total number of leaves, leaf length, width, and area; shoot fresh weight and shoot length were measured to assess plant growth after 28 days of salt stress exposure (Figure 2). Plant height, leaf number, and leaf length did not show much variation (Figure 2a-c), whilst leaf width and leaf area significantly decreased for the treated seedlings (Figure 2d-e). Furthermore, shoot length and shoot fresh weight were measured between the control and experiment. A non-significant reduction in shoot length and shoot biomass was observed in salt-stressed plants as compared to the non salt stressed group (Figures 3a and b).

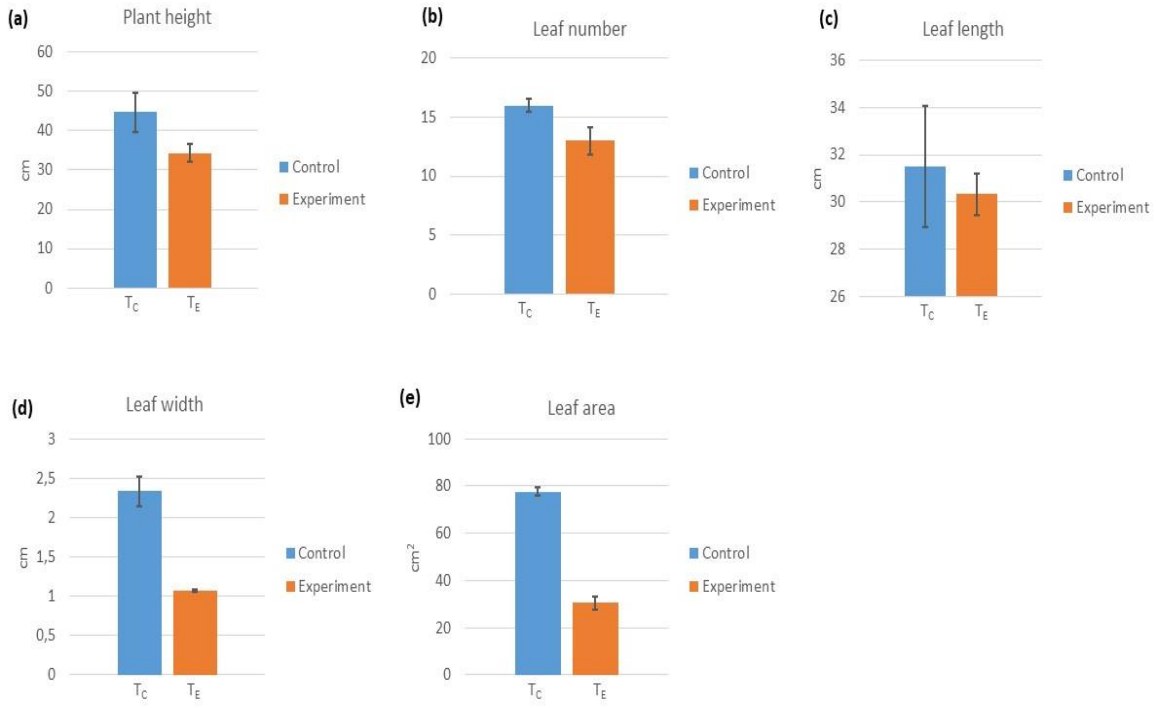

Figure 2 Effects of salt stress on growth parameters of maize; Growth traits of maize plants exposed to salt stress for 28 days at T_C (0 mM NaCl) and T_E (100 mM NaCl); Salinity decreased plant growth parameters including (a) plant height, (b) leaf number, (c) leaf length, (d) leaf width and (e) leaf area. Error bars represent the mean values of the standard error of three independent treatments (n = 3; $p \leq 0.005$)

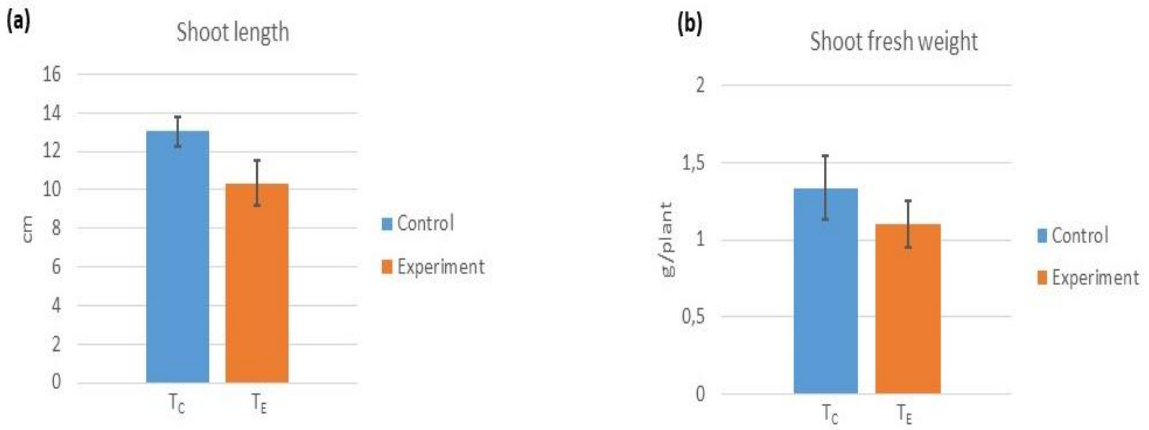

Figure 3 Effect of salt stress on growth attributes (a) Shoot length and (b) shoot fresh weight of maize plants grown under non-salt treatment T_C (0 mM NaCl) and salt treatment T_E (100 mM NaCl). Error bars indicate the mean values of the standard error of three independent seedling treatments ($n = 3$; $p \leq 0.005$)

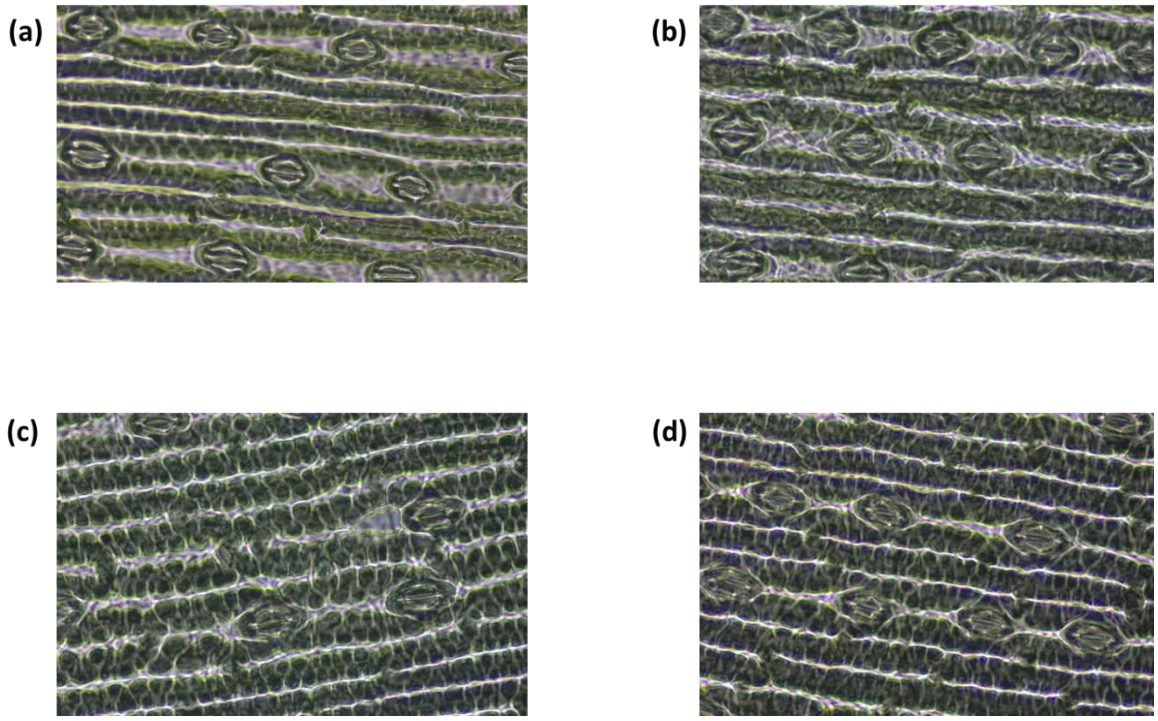

Figure 4 Microscopic images of the abaxial and adaxial surfaces of non-salt stressed (T_C) and salt stressed (T_E) maize leaves; the images were taken from leaf impressions of the (a) abaxial (lower surface) for T_C, (b) adaxial (upper surface) for T_C, (c) abaxial (lower surface) for T_E and (d) adaxial (upper surface) for T_E

3.3 Evaluation of the physiological parameters

3.3.1 Effects of salinity on the stomatal count

Following the successful assessment of the effect of salt stress on maize plant morphology, further physiological investigations were carried out to determine the stomatal count and density. Stomata located on both the abaxial and adaxial leaf surfaces displayed a substantial difference in T_C and T_E values, whereby T_C showed more stomata on both surfaces than T_E (Figure 4). Stomatal density was recorded from 2632 to 10000 mm^2 in the non-salt stressed leaves while ranging from 4 736 to 10 000 mm^2 in the salt stressed leaves (Table 2). In leaves of both plant groups (T_C and T_E), the adaxial (lower) surface displayed a higher number of stomata as compared to the abaxial (upper) surface (Figure 5a). Furthermore, salt stressed maize leaves (T_E) showed nonsignificant difference in the stomatal

Table 2 Morphological modifications of stomatal number and density for the non-stressed and salt stressed maize plants

Leaf sample	Magnification (ocular x objective)	Surface (upper/ lower)	FOV #	Number of stomata in entire FOV (mm^2)	Stomatal density stomata/mm^2
T_C	400x	Upper	1	16	8 421
	400x	Lower	1	19	10 000
T_C	400x	Upper	1	09	4 736.8
	400x	Lower	1	17	8 947.3
T_C	400x	Upper	1	05	2 632.0
	400x	Lower	1	07	3 684.2
T_E	400x	Upper	1	12	6 315.7
	400x	Lower	1	19	10 000
T_E	400x	Upper	1	13	6 842.1
	400x	Lower	1	14	7 368.4
T_E	400x	Upper	1	09	4 736.84
	400x	Lower	1	10	5 263.15

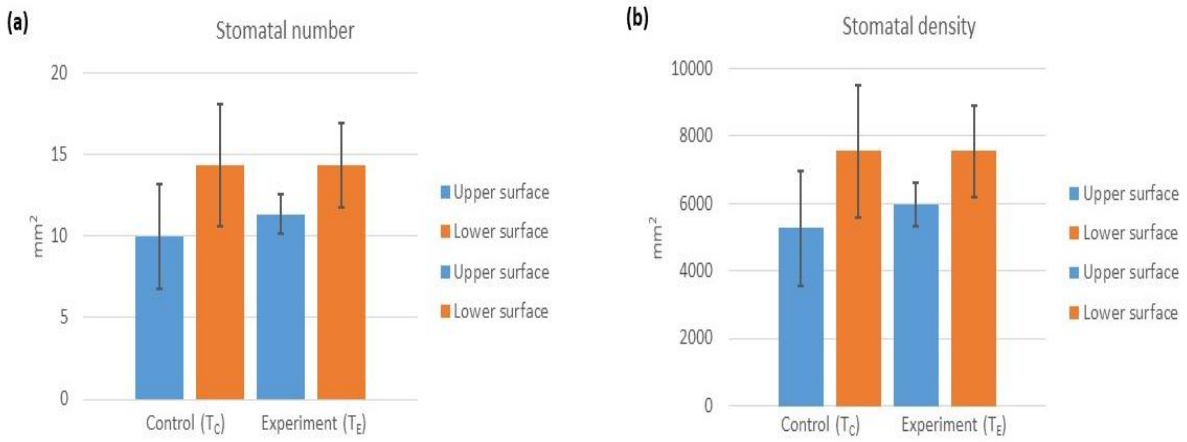

Figure 5 Assessed impact of salt stress on the (a) stomatal number and (b) stomatal density for the adaxial (upper) and abaxial (lower) surfaces of non-salt stressed (T_C) and salt stressed (T_E) maize leaves; Error bars indicate the mean values of the standard error of three independent seedling treatments ($n = 3$; $p \leq 0.005$).

number and density for both surfaces when compared to the leaves of non-salt stressed (T_C) plants (Figures 5a and b).

3.3.2 Effects of salt stress on transpiration rate

The effects of salt stress on transpiration rate were also analyzed in maize leaves. Salt stress significantly decreased the transpiration rate (Figure 6). Salt stressed leaves resulted in a moderate increase in the first 90 seconds, followed by a constant transpiratory response that was lower than those of the control (non-salt stressed) until the 150th second (Figure 6). Furthermore, salt stressed leaves displayed a moderate incline until the end of the reaction rate. Responses for the non-salt stressed maize leaves maintained a higher transpiration rate as compared to the salt stressed leaves (Figure 6).

3.3.3 Effects of salt stress on the respiration rate of maize

Salt stress significantly inhibited the respiration rate. Results presented in Figure 7 show a constant respiratory response in salt stressed plants that were lower than that of the control (non-salt treatment) in the first 60 seconds, followed by an unstable trend, where there was a steady increase and fell off for the next 120 seconds. Furthermore, responses for the non-salt stressed maize leaves maintained a higher respiration rate as compared to the salt stressed leaves (Figure 7).

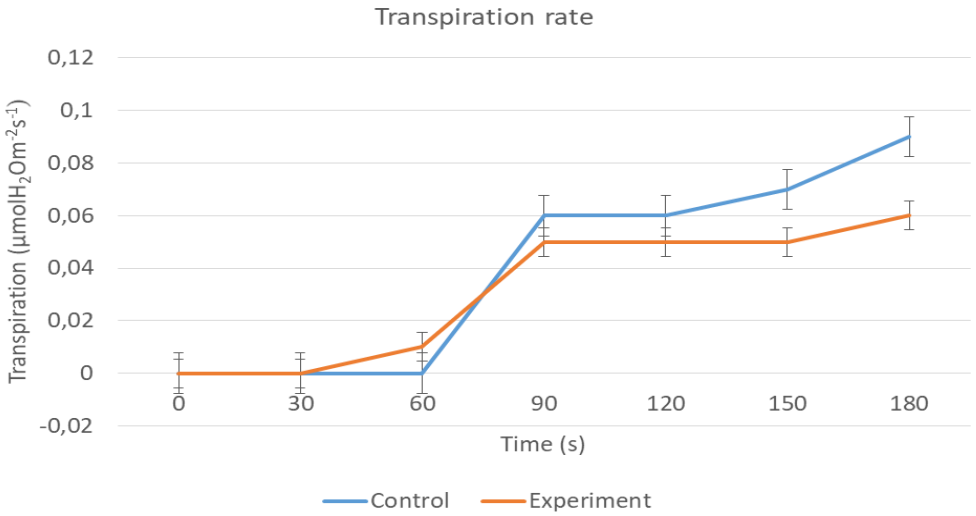

Figure 6 Comparative representations of the effects of salt stress on transpiration in maize; transpiration rates of *Z. mays* leaves treated with 0 mM NaCl (control) and100 mM NaCl (experiment) observed during the assaying process; Error bars indicate the standard error means (SEM) of three biological replicates (n = 3) for various response values ($p \leq 0.005$)

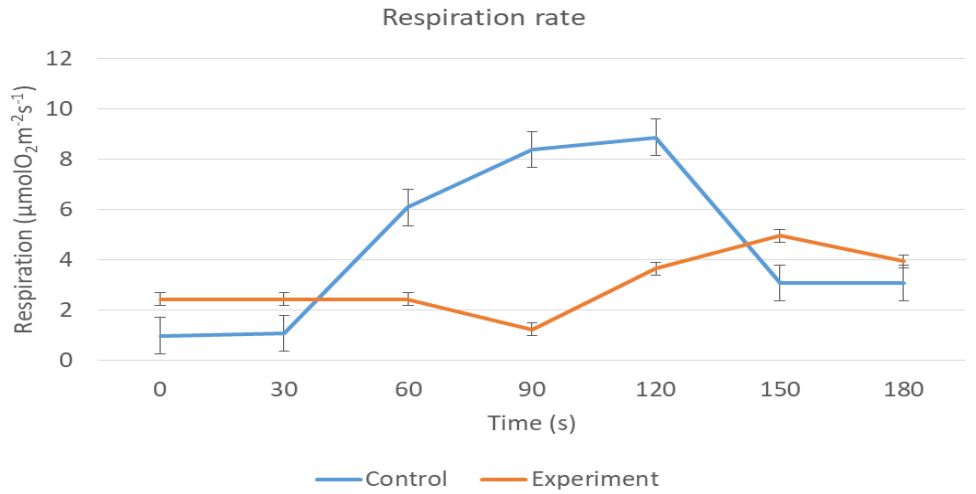

Figure 7 Comparative representation of the effects of salt stress on respiration in maize; respiration levels of *Z. mays* leaves treated with 0 mM NaCl (control) and 100 mM NaCl (experiment) observed during the assaying process. Error bars indicate the standard error means (SEM) of three biological replicates (n = 3) for various response values ($p \leq 0.005$)

4 Discussion

Salinity is an indispensable environmental challenge that restricts plants from reaching their complete genetic potential; therefore, salt-stress induces enormous growth restrictions on the morphological, physiological, and biochemical processes of plant species (Nazar et al. 2011; Hala et al. 2020). Since plants respond differently to environmental stresses, researchers have focused on various effects of salt-stress, in different plant species to understand their pathways that lead to tolerance mechanisms. Furthermore, salt stress causes water deficiency and imposes drought in plants. On that note, it was, therefore, necessary for this study to focus on the morphological and physiological processes of *Z. mays*, particularly the transpiration and respiration rates of plants exposed to salt stress over 28 days under greenhouse conditions. Elevated soil salinity affects agricultural production, thus having an impact on the economy (Zadeh and Naeini 2007).

This study has shown how salt stress significantly decreased the growth of the QN701 maize plants. Under salt stress, numerous morphological alterations were observed, whereby a negative relationship between salt stress and vegetative growth parameters such as leaf number, leaf area, and leaf weight were exhibited. Similar changes have been reported by various researchers in previous studies related to salt stress (El Sayed and El Sayed 2011; Ramezani et al. 2011; Alam et al. 2016; Dikobe et al. 2021).

Among the morphological traits, leaf color varied between the control and experiment (Table 1), wherein leaves of the salt-stressed plants (T_E) appeared dark green with brown apex whilst those of the non-salt stressed plants (T_C) were bright green (Figure 1b-e). Furthermore, various studies have supported the fact that salt stress induces a reduction in the number of leaves as indicated in *Brassica napus* L. and maize (Zadeh and Naeini 2007; El Sayed and El Sayed 2011). Whereas in this current study, the number of leaves in T_C were higher than those in T_E (Table 1), the reduction of leaf numbers in salt-stressed seedlings may be as a result of the deficiency in the uptake of water from the roots (Alam et al. 2016). Besides the reduction in the number of leaves that were observed (Figure 2b), the widths and lengths of the leaves showed a difference, whereby T_C was having larger wide leaves than T_E (Figure 2) while at the same time, shoot lengths (cm) were longer than those of T_C (Figure 3a). Hala et al. (2020) previously reported similar findings in pea plants under salinity.

According to Khodarahmpour et al. (2012), the reduction of seedling height is a common phenomenon in many crops grown under saline conditions, mostly as a result of osmotic effects even in salt-resistant plants (Wakeel et al. 2011). Sodium chloride reduced the growth rate of various morphological parameters including plant height, leaf length, width, and shoot weight whilst increasing shoot length. The reduction in plant height was highly evident for the salt stressed maize plants as compared to the control (Figure 2a). However, Mansour et al. (2005) suggested that the reduction in plant growth could be a salt stress coping mechanism that may assist plants with tolerance and energy-saving processes. Takemura et al. (2000) also confirmed that higher NaCl (mM) concentration lowers plant height (cm).

Negrao et al. (2017) suggested that the reduction in shoot growth is a familiar signal of salinity. The present study supports the above assertion, with a decrease in shoot length in experimental plants as a response signal to salt stress (Figure 3a). An increase in shoot growth mostly occurred due to turgor potential, which is reduced by water deficit caused by the accumulation of salts (high concentrations) in the soil. Salt-stress stimulates an increase in growth inhibitors and a decrease in growth promoters, hence water disturbance in salt-stressed plants limits the uptake of water or absorption of the required nutrients (Khatoon et al. 2010; Hala et al. 2020). The reduction in fresh weight due to salt stress has been studied by numerous researchers and appeared as a common phenomenon in most trees and crop plants (Gurbanov and Molazem 2009; Alam et al. 2016). In this study, a drastic reduction in shoot fresh weight (Figure 3b) was observed, which may be due to the disturbances in physiological activities under salt stress.

It was previously noted that salinity affected the expansion of leaves, which in turn limits the leaf area (Negrao et al. 2017). The decline in leaf area under salinity might be due to salt stress-induced reduction in plant fresh weights as leaves are the units of an assimilatory system (Hussain et al. 2013). The reduction in leaf area (Figure 2e) was observed and this may be associated with elongation and impaired cell division caused by the salt induced osmotic stress. Salt stress brings many changes in the physiological and biochemical processes in almost all the growth stages, one of the changes being reduced production of biomass (Alkahtani 2018). For instance, overall maize growth and plant development, including physiological parameters such as photosynthesis (P_n), transpiration rate (E), stomatal conductance (gs), and were heavily affected by salinity stress, which lead to crop yield losses (AbdElgawad et al. 2016). The transpiration rate is determined by the stomatal conductance (gs), which depends on the stomatal density (Camargo and Marenco 2011). In this study, leaf stomatal density declined with salt stress (Table 2). In a broader sense, the decrease in stomata density resulted in a decrease in transpiration rate, consequently reducing CO_2, which causes a reduction in the photosynthesis rate. However, Xu and Zhou (2008) suggested that although the stomatal density is closely related to the development of a leaf, the reaction ranges of cell number and size to stress depends on the time of leaf development. Although the stomatal density is mostly related to leaf area, it could be that increasing leaf thickness provides additional protective cells and plasticity for a given leaf area under drought conditions (Galme´s et al. 2007). The pattern of number of stomata was interesting, similar in the adaxial (upper) surfaces of both the control and experimental plants, whereas there was a decrease on the abaxial (lower) surfaces of the experimental plants (Table 2). Gill and Dutt (1982) associated low stomatal frequency with a high photosynthetic rate in beans (*Phaseolus vulgaris* L.), they further emphasized that surfaces with low stomatal frequency transpired less and had higher stomatal resistance than those with a higher stomatal frequency. The anatomy of the stomata for both the adaxial and abaxial surfaces was closed in response to salinity (Figure 4). Furthermore, the adaxial (upper) surfaces of the control and experimental plants displayed a low frequency of stomata as compared to the abaxial (lower) surfaces of the control and experimental plants (Figure 5).

The transpiration results suggest that the balance of water was improved with salt stress for the first 90 seconds (Figure 6) before the stress reduced the hydraulic conductivity, which then resulted in a decrease in water flow. The decline in water flow resulted in stomatal closure as a way of preserving water status in the leaves. The first 90 seconds of this study results corresponded to those of Negrao et al. (2017), which revealed that under salt stress, plants can tolerate salt by maintaining normal transpiration rates. The data presented in Figure 7 indicate that salt stress harmed respiration because respiration rates for the experimental (salt-stressed) plants were lower than those of the control (non-salt

stressed). Under salt stress levels, the seedlings unrestrainedly respired at low rates for the first 60 seconds, and then transpiration rates increased moderately for the last 90 seconds. The results may suggest that for seconds, respiration rates were increasing while the oxygen demand was high, and plant growth may have increased. According to Moud and Maghsoud (2008), respiration rates for plants under stress conditions are expected to be high. However, in our study, values were considerably low for respiration. This indicates that maize seedlings used great quantities of stored carbohydrates to maintain the development of organs under salinity stress. The decline in respiration rates in response to salt stress appears to be a systematic metabolic response that prevails under conditions, where salt severely restricts the availability of CO_2 inside leaf cells, therefore, creating the risk of secondary oxidative stress (Khosravinejad et al. 2008; Iqbal et al. 2020).

Conclusion

The salinity tolerance mechanism involves several complex responses at morphological, cellular, physiological, biochemical, and molecular levels. In conclusion, the present study indicated that salinity significantly affected maize's morphological and physiological traits. It induced a decline in the morphological parameters of the *Z. Mays* cultivar QN701, including plant height, leaf width, leaf height, leaf area, leaf numbers, shoot length, and shoot weight. Moreover, salt stress has shown a great effect on various physiological attributes including stomatal appearance, stomatal count, stomatal density, transpiration, and respiration rates, therefore, causing a significant decline in seedling growth and thus all growth attributes. Data presented in this study adds extra knowledge on the morphological and physiological responses of maize to salinity and could be utilized in the development of relevant biomarker strategies that can improve salt stress tolerance in maize as a major food crop.

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Influence of foliar application with *Moringa oleifera* residue fertilizer on growth, and yield quality of leafy vegetables

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KEYWORDS

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Moringa oleifera

Lettuce

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Organic farming

ABSTRACT

Biofertilizers produced from organic materials help to promote the growth, and yield quality of crops and is more environmentally friendly than chemical fertilizers. *Moringa oleifera* is a leafy vegetable whose leaves are also used to make biofertilizers. The use of moringa non-edible parts in biofertilizer preparation remains under-explored. In this study, a procedure to produce moringa foliar biofertilizer (MFB) from non-edible parts was developed. The effect of composting time (3 to 4 months) on the quality of MFB was investigated, and four-month incubation was found suitable for biofertilizers yield with the highest nitrogen content and optimal pH. Furthermore, the influences of MFB doses (20 to 100 mL per Litre) on the growth of lettuce and mustard spinach were studied. The yield of these leafy vegetables was the highest at 100 mL per Litre of MFB spray. Finally, MFB was compared with other commercial foliar sprays, including chitosan fertilizer and seaweed fertilizer. Each foliar treatment was applied every five days until five days before harvest. Plant height, the number of leaves, canopy diameter, leaf area index, actual yield, ascorbic acid content, and Brix were found to be similar in lettuce sprayed with MFB, chitosan, and seaweed fertilizers. In conclusion, the application of MFB promoted the growth and yield of mustard spinach.

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1 Introduction

Safe and clean vegetables are important for human health and well-being. Biofertilizers (organic fertilizers) are essential for the production of safe leafy vegetables. Furthermore, the use of biofertilizers helps to protect the environment from soil degradation and groundwater pollution. One of the biofertilizers which are widely investigated for their potential of improving plant yield and growth is moringa leaf extract, produced from *Moringa oleifera* (Zulfiqar et al. 2020; Karthiga et al. 2022). Previous studies demonstrated that moringa leaf extracts increase the growth and yield of various plants such as pepper (Matthew 2016), tomatoes (Culver et al. 2012), and maize (Biswas et al. 2016).

Moringa oleifera is a fast-growing softwood species grown in tropical and subtropical regions. Moringa is mainly cultivated for its leaves which are consumed as a vegetable (Price 2007). Recently, aqueous extracts of different parts of moringa (leaves, seeds, and roots) have been used to produce agricultural products. Its aqueous extract reduces the reproduction and galling of root-knot nematodes, and helps to improve plant growth and yield parameters of pea plants (Youssef and El-Nagdi 2021). Moringa leaf and seed extracts are also effective in extending the shelf-life of cut rose flowers (Hassan et al. 2020). Although moringa leaf extract is extensively studied, but the production of moringa biofertilizer and its impact on vegetable growth still remained under-explored. Therefore, this study was carried out to investigate the effect of moringa foliar biofertilizer on the growth and quality of leafy vegetables.

2 Materials and methods

2.1 Experimental site and planting materials

The study was conducted at the experimental field of the Institute of Biotechnology, Hue University (Hue, Vietnam) from October 2019 to March 2021. This region has a humid tropical climate with average temperatures varying from 26 to 35 °C. In the study, a yellow lettuce (*Lactuca sativa* L.) variety obtained from Phu Nong Seeds company and a mustard spinach (*Brassica juncea*) variety obtained from Ha Noi Xanh company were used.

2.2 Moringa foliar biofertilizer (MFB) preparation

Moringa foliar biofertilizer was prepared following the non-aerated process. Briefly, 70 kg of moringa residues (including stems, branches, and leaf petioles) were washed with water to remove dust particles before being chopped into small parts. In a 100-liter container, the chopped moringa residues were spread to form a 20-cm layer. Second, molasses (5 L) and effective microorganism (EM) products (0.2 kg) were subsequently added to the top of the layer. The container was filled with chopped materials and water was added to 2/3 of the container. The container was then tightly covered. The mixture in the container was stirred once every month until the end of the composting period (three to four months). The extract was collected and filtered. The obtained fertilizer was kept in an airtight container.

2.3 Effect of composting time on the quality of MFB

To evaluate the effect of composting time on the quality of MFB, the residue was incubated for either 3, 3.5, or 4 months. Physicochemical properties of the extract including the percentages of nitrogen (N), phosphorus (P), phosphorus pentoxide (P_2O_5), potassium (K), potassium oxide (K_2O), and organic matter (OM) were determined.

2.4 Effect of different doses of MFB on growth, yield, and quality of leafy vegetables

Three to four leaf plants in a 10 m^2 plot were sprayed with either 100 mL, 50 mL, 33.3 mL, 25 mL, or 20 mL of MFB diluted in 1 L of water (Nwokeji et al. 2022). MFB has sprayed every five days intervals until five days before harvest. The experiment was designed in a Completely Randomized Design (CRD) with five fertilizer doses and three replicates per treatment.

2.5 Effect of different foliar fertilizers on growth, yield, and quality of leafy vegetables

Three to four leave plants in a 10 m^2 plot were sprayed with MFB (100 mL per Litre), commercial chitosan fertilizer, seaweed fertilizer, and water (control). MFB has sprayed every five days intervals until five days before harvest. Commercial fertilizers were diluted with water at a ratio of 1.25:1 (volume: volume). The experiment was designed in a Completely Randomized Design (CRD) with five fertilizer doses and three replicates per treatment.

2.6 Statistical analysis

Growth time (day) was the time taken from sowing to harvest. Growth parameters including plant height (cm), canopy diameter (cm), the number of leaves, and leaf area index (leaf area/ground area) were determined for five plants in each treatment. The plant height (cm) was measured from the ground to the highest point of the leaves. The leaf area index is the multiplication of the number of plants/ground area (m^2) and the leaf area (m^2)/plant. The yield components included (i) fresh mass/plant (g/plant) (combined weight of stem, leaves, and roots); (ii) estimated yield (ton/ha) (average fresh mass/plant × plant density); (iii) actual yield (ton/ha). Statistical analysis was performed using one ways analysis of variance (ANOVA) followed by Turkey's test in IBM SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, USA). Data represented significant differences as $p < 0.05$.

3 Results

3.1 Effect of composting time on the quality of moringa foliar fertilizer (MFB)

Results of the study revealed that the chemical properties of MFB depended on the composting time (Table 1). Results presented in table 1 showed that the nitrogen content and pH increased with composting time. These parameters peaked after composting for four months (nitrogen content of 11.9% and pH of 5.04). On the other hand, the contents of P and P_2O_5 were similar between 3.5 and 4 months, which were higher than those of 3 months. However, the contents of K and K_2O at 3 months were higher than those of 3.5 and 4 months. OM varied between 29% and 38% after 4 months of composting.

3.2 MFB doses influence growth, yield and quality of leafy vegetables

Lettuce was grown from 35 days to 37 days in the first planting, and from 32 days to 34 days in the second planting (Table 2). Plant height, number of leaves, canopy diameter, and leaf area index were found to be the highest when MFB was applied at 100 mL per litre (Table 2). Foliar application of MFB at 100 mL per litre significantly increased the fresh mass and estimated yield compared to the lower doses (Table 3). The actual yields were comparable between 100 and 50 mL per litre treatments and were significantly higher than those of other treatments. Higher ascorbic acid content and Brix were observed in the first planting with 100 and 50 mL per Litre treatments, however, these observations were not reproducible in the second planting.

Mustard spinach also has a similar grown period to lettuce and it was recorded from 33 to 36 days in the first planting, and from 28 to 32 days in the second planting (Table 4). Plant height, number of leaves, canopy diameter, and leaf area index slightly changed and tended to decrease with decreasing amounts of MFB. Similarly, fresh mass, estimated yield, and actual yield of mustard spinach also decreased when fewer MFB was applied (Table 5). The highest dose of MFB (100 mL per Litre) correlated with the freshest weight and highest yield of mustard

Table 1 Effect of composting time on the physicochemical properties of moringa foliar biofertilizer (MFB)

Composting time	N (%)	P (%)	P_2O_5 (%)	K (%)	K_2O (%)	OM (%)	pH
Three months	4.20^{c}	2.21^{b}	5.06^{b}	7.20^{a}	8.68^{a}	37.73^{a}	3.37^{b}
Three and a half months	8.52^{b}	3.04^{a}	6.97^{a}	5.39^{b}	6.49^{b}	29.13^{a}	4.82^{a}
Four months	11.90^{a}	2.63^{ab}	5.89^{ab}	5.07^{b}	6.11^{b}	32.77^{a}	5.04^{a}

Values given in table are mean of three replicates; mean value followed by the different letter in same column are significantly different at $P<0.05$

Table 2 Effect of different doses of moringa foliar biofertilizer (MFB) on the growth of lettuce

Dose (mL/Litre)	Growth time (day)	Plant height (cm)	Number of leaves/plant	Canopy diameter (cm)	Leaf area index
First planting					
100	36	$22.9^{a} \pm 1.10$	$12.1^{a} \pm 0.51$	$30.9^{a} \pm 1.68$	$57.65^{a} \pm 2.94$
50	37	$20.3^{ab} \pm 1.22$	$11.2^{ab} \pm 1.40$	$30.8^{a} \pm 1.59$	$55.36^{ab} \pm 3.61$
33.3	36	$20.9^{bc} \pm 0.56$	$10.8^{ab} \pm 0.40$	$30.7^{a} \pm 2.31$	$44.67^{c} \pm 3.42$
25	35	$19.4^{c} \pm 0.57$	$10.5^{b} \pm 0.42$	$27.3^{b} \pm 0.98$	$45.57^{c} \pm 3.12$
20	36	$22.0^{ab} \pm 1.26$	$11.0^{ab} \pm 0.81$	$29.4^{ab} \pm 1.83$	$49.43^{bc} \pm 3.17$
$LSD_{0.05}$		1.98	1.42	3.34	5.59
Second planting					
100	32	$23.0^{a} \pm 1.35$	$12.2^{a} \pm 1.41$	$29.6^{a} \pm 0.87$	$51.30^{a} \pm 2.23$
50	33	$20.9^{ab} \pm 0.75$	$10.7^{ab} \pm 1.05$	$27.3^{b} \pm 1.36$	$48.42^{ab} \pm 2.85$
33.3	34	$19.7^{b} \pm 1.06$	$10.5^{b} \pm 0.62$	$26.7^{bc} \pm 0.45$	$45.71^{b} \pm 1.89$
25	33	$19.5^{b} \pm 1.26$	$11.5^{ab} \pm 0.53$	$26.5^{bc} \pm 0.72$	$45.40^{b} \pm 3.07$
20	34	$19.7^{b} \pm 1.14$	$10.2^{b} \pm 0.91$	$25.7^{c} \pm 1.03$	$45.92^{b} \pm 1.52$
$LSD_{0.05}$		2.02	1.54	1.51	3.41

Values given in table are mean of three replicates; mean value followed by the different letter in same column are significantly different at $P<0.05$

spinach at both times of planting. The ascorbic acid content remained relatively constant across a range of MFB doses. On the other hand, the data for Brix were not reproducible and it decreased from 8.07 (100 mL per Litre) to 5.26 (20 mL per Litre) in the first planting but it did not significantly change in the second planting.

Table 3 Effect of different doses of moringa foliar biofertilizer (MFB) on the yield and quality of lettuce

Dose (mL/Litre)	Fresh weight (g /plant)	Estimated yield (ton/ha)	Actual yield (ton/ha)	Ascorbic acid (%)	Brix (%)
First planting					
100	127.3 ^{a}± 9.02	33.7^{a} ± 2.40	21.3^{a} ± 0.60	2.67^{a} ± 0.12	5.53^{a} ± 0.25
50	108.6^{b} ± 6.43	29.0^{b} ± 1.07	19.7^{ab} ± 0.95	2.57^{ab} ± 0.15	5.10^{a} ± 0.15
33.3	106.0^{bc} ± 4.01	28.0^{bc} ± 1.71	18.3^{bc} ± 1.03	2.34^{bc} ± 0.21	4.53^{b} ± 0.11
25	96.0^{c} ± 6.24	26.7^{bc} ± 0.53	18.2^{bc} ± 0.67	2.19^{c} ± 0.07	4.47^{b} ± 0.18
20	100.0^{bc} ± 2.18	25.6^{c} ± 1.66	17.7^{c} ± 0.43	2.16^{c} ± 0.16	4.43^{b} ± 0.24
$LSD_{0.05}$	10.88	2.95	1.68	0.28	0.43
Second planting					
100	140.2^{a} ± 8.26	34.4^{a} ± 1.83	21.7^{a} ± 1.26	3.45^{a} ± 0.38	5.45^{a} ± 0.15
50	117.0^{b} ± 6.15	28.7^{b} ± 1.91	20.0^{ab} ± 0.95	2.94^{a} ± 0.27	4.94^{a} ± 0.26
33.3	107.3^{bc} ± 5.23	27.0^{bc} ± 1.34	19.0^{bc} ± 0.78	3.01^{a} ± 0.41	5.01^{a} ± 0.68
25	101.6^{c} ± 2.55	26.3^{bc} ± 0.95	18.0^{bc} ± 1.14	3.07^{a} ± 0.06	5.07^{a} ± 0.22
20	99.3^{c} ± 4.79	25.8^{c} ± 1.06	17.3^{c} ± 0.87	3.04^{a} ± 0.09	5.04^{a} ± 0.17
$LSD_{0.05}$	10.85	2.54	2.36	0.72	0.71

Values given in table are mean of three replicates; mean value followed by the different letter in same column are significantly different at P<0.05

Table 4 Effect of different doses of MFB on the growth of mustard spinach

Dose (mL/Litre)	Growth time (day)	Plant height (cm)	Number of leaves/plant	Canopy diameter (cm)	Leaf area index
First planting					
100	34	35.1^{a} ± 2.97	11.4^{a} ± 0.31	31.9^{a} ± 2.07	46.30^{a} ± 3.71
50	33	27.2^{b} ± 3.23	11.3^{a} ± 0.35	30.9^{ab} ± 1.58	43.55^{ab} ± 2.96
33.3	33	31.7^{ab} ± 4.15	10.2^{bc} ± 0.50	28.8^{bc} ± 2.00	40.06^{b} ± 2.28
25	34	30.7^{ab} ± 2.24	9.5^{c} ± 0.45	26.7^{cd} ± 1.68	39.53^{b} ± 4.33
20	36	26.8^{b} ± 3.56	10.3^{b} ± 0.37	25.8^{d} ± 1.45	39.09^{b} ± 2.57
$LSD_{0.05}$		5.76	0.68	2.39	5.22
Second planting					
100	31	29.7^{a} ± 1.15	11.5^{a} ± 1.01	31.2^{a} ± 3.07	44.52^{a} ± 3.12
50	29	27.1^{ab} ± 2.24	10.7^{ab} ± 0.75	29.9^{a} ± 3.21	40.19^{ab} ± 1.14
33.3	29	27.8^{ab} ± 1.63	10.5^{ab} ± 0.31	31.4^{a} ± 2.87	39.43^{ab} ± 2.41
25	28	25.5^{b} ± 2.41	10.3^{b} ± 0.54	28.8^{a} ± 2.12	37.50^{b} ± 3.97
20	32	24.9^{b} ± 3.01	10.0^{b} ± 0.16	29.8^{a} ± 1.93	37.21^{b} ± 2.71
$LSD_{0.05}$		3.99	1.17	3.61	5.31

Values given in table are mean of three replicates; mean value followed by the different letter in same column are significantly different at P<0.05

Table 5 Effect of different doses of moringa foliar biofertilizer (MFB) on the yield and quality of mustard spinach

Dose (mL/Litre)	Fresh weight (g /plant)	Estimated yield (ton/ha)	Actual yield (ton/ha)	Ascorbic acid (%)	Brix (%)
First planting					
100	133.0^{a} ± 8.47	35.3^{a} ± 1.47	28.0^{a} ± 1.17	5.76^{a} ± 0.12	8.07^{a} ± 0.09
50	115.7^{b} ± 5.32	30.7^{b} ± 2.21	24.3^{b} ± 1.35	5.54^{a} ± 0.07	7.13^{b} ± 0.11
33.3	113.0^{bc} ± 2.19	30.3^{bc} ± 1.05	24.6^{b} ± 0.98	5.69^{a} ± 0.05	7.01^{b} ± 0.10
25	112.0^{bc} ± 6.20	29.6^{bc} ± 2.14	23.7^{b} ± 1.61	5.68^{a} ± 0.10	6.77^{b} ± 0.07
20	101.7^{c} ± 7.56	27.0^{c} ± 3.02	22.3^{b} ± 2.21	5.62^{a} ± 0.09	5.26^{c} ± 0.13
$LSD_{0.05}$	11.67	3.41	3.14	0.23	0.48
Second planting					
100	137.7^{a} ± 4.41	37.0^{a} ± 1.92	29.7^{a} ± 0.66	5.52^{a} ± 0.21	4.80^{a} ± 0.24
50	126.0^{b} ± 6.92	33.7^{b} ± 2.04	27.3^{b} ± 1.05	5.02^{a} ± 0.34	4.20^{a} ± 0.19
33.3	119.3^{bc} ± 4.65	31.6^{bc} ± 1.99	25.3^{c} ± 1.24	4.73^{a} ± 0.08	4.53^{a} ± 0.20
25	114.7^{c} ± 8.07	30.7^{c} ± 2.31	24.0^{c} ± 0.68	5.28^{a} ± 0.17	4.43^{a} ± 0.16
20	102.3^{d} ± 5.42	27.3^{c} ± 2.11	21.7^{d} ± 0.41	5.20^{a} ± 0.09	4.40^{a} ± 0.32
$LSD_{0.05}$	9.53	2.50	1.91	0.86	0.62

Values given in table are mean of three replicates; mean value followed by the different letter in same column are significantly different at P<0.05

3.3 Effect of various foliar fertilizers on growth, yield, and quality of leafy vegetables

The results suggested that the application of MFB promoted the growth of lettuce (Table 6). Furthermore, the growth time, the number of leaves, canopy diameter, and leaf area index of lettuce plants applied with MFB was comparable to those sprayed with commercial biofertilizers. The plant height of lettuce slightly changed among foliar treatments in the second planting and peaked at 24.3 cm in plants treated with MFB. The yield of lettuce was enhanced by spraying foliar fertilizers at both plantings. The treatment of MFB increased the fresh weight of lettuce. Estimated yields ranged from 33.8 tons per ha to 37.5 tons per ha and actual yields ranged from 21.3 tons per ha to 23.9 tons per ha across foliar treatments. On the other hand, the ascorbic acid content was not influenced by foliar treatments. Lettuce treated with MFB and chitosan fertilizer had higher Brix in the first planting but these results were not reproducible in the second planting seasons.

Table 6 Effect of various foliar fertilizers on the growth of lettuce

Treatment	Growth time (day)	Plant height (cm)	Number of leaves/Plant	Canopy diameter (cm)	Leaf area index
First planting					
MFB	34	25.4^{a} ± 1.21	12.8^{a} ± 1.02	23.6^{ab} ± 1.33	41.9^{a} ± 2.57
Chitosan fertilizer	33	23.8^{a} ± 1.83	11.5^{ab} ± 1.00	24.9^{a} ± 1.65	38.6^{ab} ± 4.98
Seaweed fertilizer	35	24.6^{a} ± 0.92	11.6^{ab} ± 0.25	24.4^{a} ± 0.61	38.8^{ab} ± 2.81
Control	35	18.4^{b} ± 2.97	10.2^{b} ± 0.82	21.1^{b} ± 1.51	34.0^{b} ± 3.24
$LSD_{0.05}$		3.18	1.48	2.96	5.68
Second planting					
MFB	35	24.3^{a} ± 0.69	12.1^{a} ± 0.52	23.9^{a} ± 1.76	42.2^{a} ± 3.04
Chitosan fertilizer	36	21.5^{bc} ± 1.14	11.2^{ab} ± 0.31	24.9^{a} ± 0.55	39.0^{a} ± 2.56
Seaweed fertilizer	35	22.9^{ab} ± 0.76	11.8^{a} ± 0.67	25.4^{a} ± 1.15	40.1^{a} ± 2.18
Control	35	20.5^{c} ± 1.41	10.3^{b} ± 0.71	21.8^{b} ± 1.37	34.8^{b} ± 1.19
$LSD_{0.05}$		1.74	0.96	1.84	3.61

Values given in table are mean of three replicates; mean value followed by the different letter in same column are significantly different at P<0.05

Table 7 Effect of various foliar fertilizers on the yield and quality of lettuce

Treatment	Fresh weight (g /plant)	Estimated yield (ton/ha)	Actual yield (ton/ha)	Ascorbic acid (%)	Brix (%)
First planting					
MFB	$146.7^{a} \pm 12.12$	$37.5^{a} \pm 3.23$	$23.9^{a} \pm 1.07$	$4.59^{a} \pm 0.37$	$5.13^{a} \pm 0.27$
Chitosan fertilizer	$132.3^{ab} \pm 11.46$	$35.3^{a} \pm 2.39$	$21.9^{ab} \pm 1.92$	$4.77^{a} \pm 0.29$	$5.10^{a} \pm 0.13$
Seaweed fertilizer	$127.3^{b} \pm 4.16$	$33.9^{a} \pm 2.67$	$21.4^{b} \pm 1.06$	$4.87^{a} \pm 0.55$	$4.53^{b} \pm 0.15$
Control	$105.3^{c} \pm 5.04$	$28.0^{b} \pm 1.81$	$17.7^{c} \pm 0.84$	$3.96^{a} \pm 0.77$	$4.27^{b} \pm 0.19$
$LSD_{0.05}$	15.17	3.66	2.10	1.92	0.33
Second planting					
MFB	$137.7^{a} \pm 3.05$	$34.7^{a} \pm 1.55$	$23.5^{a} \pm 1.42$	$4.77^{a} \pm 0.27$	$5.34^{a} \pm 0.34$
Chitosan fertilizer	$129.6^{b} \pm 4.14$	$34.6^{a} \pm 2.01$	$21.8^{ab} \pm 1.15$	$4.68^{a} \pm 0.13$	$4.93^{a} \pm 0.15$
Seaweed fertilizer	$123.0^{c} \pm 2.39$	$33.8^{a} \pm 1.79$	$21.3^{b} \pm 1.08$	$4.72^{a} \pm 0.56$	$5.00^{a} \pm 0.09$
Control	$101.7^{d} \pm 1.81$	$27.1^{b} \pm 1.43$	$17.8^{c} \pm 1.41$	$3.63^{b} \pm 0.48$	$4.96^{a} \pm 0.47$
$LSD_{0.05}$	4.92	2.29	1.87	0.88	0.72

Values given in table are mean of three replicates; mean value followed by the different letter in same column are significantly different at $P<0.05$

Table 8 Effect of various foliar fertilizers on the growth of mustard spinach

Treatment	Growth time (day)	Plant height (cm)	Number of leaves/Plant	Canopy diameter (cm)	Leaf area index
First planting					
MFB	30	$26.0^{a} \pm 1.21$	$12.3^{a} \pm 0.31$	$32.9^{a} \pm 2.52$	$41.3^{a} \pm 4.44$
Chitosan fertilizer	30	$24.4^{a} \pm 2.00$	$11.1^{b} \pm 0.36$	$27.7^{b} \pm 2.30$	$37.6^{a} \pm 3.08$
Seaweed fertilizer	32	$25.3^{a} \pm 2.24$	$12.1^{a} \pm 0.62$	$33.5^{a} \pm 1.88$	$38.2^{a} \pm 5.42$
Control	33	$21.2^{b} \pm 2.08$	$10.3^{b} \pm 0.57$	$26.5^{b} \pm 0.97$	$28.9^{b} \pm 1.90$
$LSD_{0.05}$		3.19	0.84	3.36	6.74
Second planting					
MFB	29	$27.0^{a} \pm 1.17$	$12.3^{a} \pm 0.41$	$29.9^{b} \pm 1.51$	$41.2^{a} \pm 5.05$
Chitosan fertilizer	28	$25.9^{a} \pm 1.54$	$11.8^{a} \pm 0.61$	$27.2^{c} \pm 1.69$	$36.9^{ab} \pm 4.87$
Seaweed fertilizer	28	$26.0^{a} \pm 1.13$	$12.6^{a} \pm 0.52$	$31.7^{a} \pm 0.94$	$42.7^{a} \pm 4.51$
Control	30	$21.9^{b} \pm 1.36$	$10.7^{b} \pm 0.66$	$25.4^{d} \pm 1.07$	$31.2^{b} \pm 3.01$
$LSD_{0.05}$		1.81	0.87	1.77	6.02

Values given in table are mean of three replicates; mean value followed by the different letter in same column are significantly different at $P<0.05$

Like lettuce, mustard spinach growth was also affected by foliar treatments. In the first planting, plant height and leaf area index did not vary between different treatments, however, the number of leaves and canopy diameter was found to be higher in plants treated with MFB and seaweed fertilizer. In the second planting, plant height, the number of leaves, and leaf area index were similar among foliar treatments and higher than those of the control. Canopy diameter ranged from 27.2 cm (chitosan fertilizer) to 31.7 cm (seaweed fertilizer), compared to 25.4 cm of the control. The highest fresh weight and estimated yield of mustard spinach grown in the first planting were found in those treated with MFB but these results were not reproducible in the second planting. Actual yields of plants treated with MFB were comparable to those treated with seaweed fertilizer and higher than those treated with chitosan fertilizer and the control plants. The ascorbic acid of plants grown in the first planting varied from 3.31% (control) to 5.21% (seaweed fertilizer treated), however, the changes were not significant in the second planting. The Brix of mustard spinach across treatments remained constant (larger than 6.0).

Table 9 Effect of various foliar fertilizers on the yield and quality of mustard spinach

Treatment	Fresh weight (g /plant)	Estimated yield (ton/ha)	Actual yield (ton/ha)	Ascorbic acid (%)	Brix (%)
First planting					
MFB	$158.0^{a} \pm 5.55$	$37.1^{a} \pm 1.06$	$26.7^{a} \pm 1.29$	$3.92^{b} \pm 0.61$	$6.47^{a} \pm 0.49$
Chitosan fertilizer	$140.2^{b} \pm 3.60$	$32.9^{b} \pm 1.60$	$24.4^{b} \pm 0.76$	$4.06^{b} \pm 0.78$	$6.60^{a} \pm 0.08$
Seaweed fertilizer	$136.7^{b} \pm 6.01$	$32.1^{b} \pm 1.42$	$25.6^{ab} \pm 1.22$	$5.21^{a} \pm 0.30$	$6.67^{a} \pm 0.34$
Control	$116.0^{c} \pm 5.78$	$27.3^{c} \pm 0.95$	$19.2^{c} \pm 0.87$	$3.31^{b} \pm 0.54$	$6.33^{a} \pm 0.44$
$LSD_{0.05}$	7.89	1.85	1.75	0.88	1.73
Second planting					
MFB	$157.3^{a} \pm 10.78$	$37.1^{a} \pm 2.05$	$25.4^{a} \pm 1.75$	$5.22^{a} \pm 0.06$	$6.73^{a} \pm 0.49$
Chitosan fertilizer	$146.7^{a} \pm 12.24$	$32.9^{b} \pm 3.32$	$23.0^{b} \pm 0.99$	$5.12^{a} \pm 0.14$	$6.82^{a} \pm 0.35$
Seaweed fertilizer	$155.6^{a} \pm 13.42$	$36.6^{a} \pm 2.69$	$25.2^{ab} \pm 1.42$	$5.73^{a} \pm 0.45$	$6.98^{a} \pm 0.10$
Control	$117.3^{b} \pm 9.97$	$27.5^{c} \pm 3.02$	$18.6^{c} \pm 1.86$	$5.08^{a} \pm 0.58$	$6.07^{a} \pm 0.38$
$LSD_{0.05}$	17.07	3.61	2.33	0.87	1.05

Values given in table are mean of three replicates; mean value followed by the different letter in same column are significantly different at $P<0.05$

4 Discussion

In this study, the effects of moringa foliar biofertilizer (MFB), prepared from non-edible parts, on the growth and yield of leafy vegetables was investigated. Results of the study revealed that the composting time impacted the quality of MFB (Table 1) and a four-month composting time yielded biofertilizer with the highest nitrogen content. Further, phosphorus content also slightly increased when the composting time was longer than three months, while the organic matter remained unchanged. Furthermore, the pH of the composite biofertilizer increased from 3.37 to 5.04 with increasing composting time.

In this study, high nitrogen content in moringa foliar biofertilizer was prioritized as nitrogen is one of the most essential elements to enable fast growth and optimal production of vegetables (Tam and Cong 2018; Hoa and Thanh 2020). Hence, these results suggested that a four-month composting period was suitable to produce biofertilizer from non-edible moringa plant parts. Apart from macronutrients, moringa plant extracts also contain various antioxidant compounds like zeatin, ascorbic acid, phenolic, flavonoids, vitamin E, minerals, and many other growth hormones such as indole-3-acetic acid (IAA), and gibberellins (GAs) (Isman 1997; Rady and Mohamed 2015; Latif and Mohamed 2016). The previous study also indicated that the stem of moringa was found to enrich nutrients such as vanillin, β-sitostanol, 4-hydroxymellin, β-sitosterol, and octacosanoic acid (Faizi et al. 1994).

During the application of MFB to the leafy plants, the higher the dose of MFB enhanced fresh mass and yields (Tables 3 and 5). In both planting seasons, the dose of 100 mL per Litre MFB produced the highest fresh mass and yields in both lettuce and mustard spinach. It had been reported that the concentration of moringa leaf extract at 200 mg per Litre was sufficient to enhance the quality of baby leaves (Toscano et al. 2021). In this study, the spray of MFB at 25 mL per Litre and 20 mL per Litre did not improve the yields of these vegetables compared to the Control. Similarly, leaf area indices in both lettuce and mustard spinach decreased in these treatments which could be justified by the poor nutrient supply in these treatments (Tables 2 and 4). Previously, it was demonstrated that the extracts derived from moringa stem bark enhanced the leaf area and fruit yield of sweet bell pepper fruit (Nwokeji et al. 2022). Taken together, the application of 100 mL per Litre MFB produced the highest yield and quality vegetables in this study.

Different types of foliar fertilizer used in this study had comparable effects on the growth of lettuce. However, the actual yield was higher when treated with MFB compared to the seaweed fertilizer treatment. Since leaf areas and plant sizes were similar in plants treated with different foliar fertilizers, it is suggested that MFB stimulated root formation in lettuce which resulted in the differences in yield. Consistent with this, previous studies (Culver et al. 2012; Yasmeen et al. 2013) had shown that the application of moringa plant extract increased root dry weight and root length of tomatoes and wheat. Mustard spinach plants grown on the first planting achieved the highest yield when treated with MFB but these results were not reproducible in the second planting. The effects of seaweed fertilizer on the growth and yield of vegetables in this study were similar to those reported by Hoang et al. (2022).

In lettuce, the ascorbic acid content was not significantly influenced by spraying different foliar fertilizers. Yaseen and

Hajos (2022) found no significant difference in the ascorbic acid content between moringa plant extract treated and non–treated lettuce in 2019, but moringa plant extracts were found to improve the ascorbic acid content of lettuce in 2020. This can be explained by the temperature fluctuation in 2020, which caused physical stresses to plants. In this study, lettuce that was grown in the second planting showed a higher percentage of ascorbic acid when treated with foliar fertilizers despite the effect of higher temperatures from February to March (average temperatures at 18.2°C in January, 21.1°C in February and 25.7°C in March, data not shown). Meanwhile, the ascorbic acid content in mustard spinach varied when treated with different foliar fertilizers on the first planting, although no difference was observed in the second planting. Furthermore, MFB did not affect the percentage of ascorbic acid when various doses (20 mL per Litre to 100 mL per Litre) were sprayed on mustard spinach. These results were contradictory to the findings of Cintya et al. (2018) who found an increase in the content of vitamin C with increasing doses of organic fertilizers in spinach (*Amaranthus tricolor* L.), mustard (*Brassicca rapa chinensis*). Brix of lettuce tended to decrease when the doses of MFB decreased in the first planting; however, there was no significant difference across doses in the second planting. Similarly, the application of MFB and chitosan fertilizer improved the Brix in lettuce, compared to seaweed fertilizer and control treatments only in the first planting. Meanwhile, in mustard spinach, Brix varied greatly (5.26%–8.07%) across the different doses of MFB in the first planting but remained relatively constant in the second planting. The effects of MFB on the quality of lettuce and mustard spinach were consistent with previous studies on kale and broccoli baby leaves (Toscano et al. 2021).

Conclusion

In this work, moringa residues including stems, branches, and leaf petioles, were fermented using EM product and molasses to produce moringa foliar biofertilizer (MFB). To obtain optimal MFB, the composting should be allowed to continue for four months. MFB application enhanced the growth and yield of both lettuce and mustard spinach grown in January and February but did not affect the ascorbic acid content and Brix consistently. The application of MFB produced similar effects compared to the chitosan and seaweed fertilizers. To the authors' knowledge, this was the first study to investigate the effects of MFB on the growth, yield, and quality of leafy vegetables grown in the tropical.

Author contribution

HD designed the experiment. HD, CQN, TDTN, and BQLN carried out the experiments and performed data analyses. HD, CQN, NHTH and LTD prepared all of the tables, and all authors contributed to data interpretation. TTP and HD wrote the first draft of the manuscript, and HTHT edited the draft. All authors reviewed the manuscript.

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Declaration of Competing Interest

The authors declare no competing interests.

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Effects of Liquid Organic and NPK Fertilizers on the Nutrient composition of Grass Jelly (*Premna oblongifolia* Merr) in Tropical Peat Soil

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ABSTRACT

Peat soil is deficient in nutrients and agricultural development in this type of low in fertility soil is very difficult. Grass jelly (*Premna oblongifolia*) is a dark green shrub-like medicinal plant that has been widely used for its nutritional and medicinal properties. This study aimed to evaluate the effect of foliar application of organic fertilizers and NPK on the growth, nutrient absorption, and flavonoid content of grass jelly plants grown in peat soil. The study was carried out in a completely randomized factorial design with two factors including liquid organic fertilizer and NPK inorganic fertilizers. Three doses of liquid organic fertilizer consisting of P0 (without liquid organic fertilizer), P1 (Agrobost), and P2 (Nasa), and three levels of NPK fertilizer consisting of N0 (0g NPK polybag^{-1}), N1 (1 g NPK polybag^{-1}), and N2 (2 g NPK polybag^{-1}). The results of the study revealed a nonsignificant interaction between liquid organic fertilizer and NPK fertilizer in terms of leaf growth, leaf area, fresh weight, and flavonoid of grass jelly plant growth. Further, in the case of plant nutrient contents, combined application of Nasa liquid organic fertilizer and 2 g polybag^{-1} NPK fertilizer tends to increase the nutrient content of N, P, and K and have the highest impact as compared to other treatments. The results of the study can be concluded that administration of liquid organic fertilizer and NPK did not affect the plant growth characteristics of grass jelly plants while in the case of nutrient content except flavonoids the level of N, P, and K of plant leaves increased.

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1 Introduction

The green grass jelly plant (*Premna oblongifolia* Merr) is a shrub-like plant with dark green stems that can reach up to 5 meters in length. Leaves of this plant have medicinal value and have been traditionally used to produce jelly-like food such as grass jelly ice, which has a chilling effect on the stomach, decreasing fever, and alleviating digestive ailments (Harmayani et al. 2019). According to Hidayat and Napitupulu (2015), grass jelly is also ideal for diet programs due to its low caloric and high fiber content which is also beneficial for removing cancer-causing carcinogens from the digestive system. Further, the leaves of this plant are a rich source of various active ingredients like saponins, glycosides, flavonoids, alkaloids, tannins, steroids, and triterpenoids, these secondary metabolites are well known for their potential for cancer treatment (Harmayani et al. 2019), Moreover, flavonoids are antioxidants that have the potential to prevent the formation of free radicals and because of this, these can be used to treat tumors (Santoso et al. 2008). According to Rahayu et al. (2013), the principal constituent of green grass jelly gel is pectin polysaccharides and because of this, it has the potential to be utilized as agar.

In Indonesia, grass jelly plants are commonly grown in backyards as a family's medicinal plants and as a refreshing beverage product, therefore it has the potential to be developed as a healthy functional beverage product (Rujito et al. 2020). This plant is well recognized in various parts of Indonesia but less common in Central Kalimantan, Indonesia. Peat soil is one sort of prospective land in Indonesia, especially in terms of cultivable land; however, there are many challenges to growing crops in this type of soil, particularly concerning soil fertility (Salampak et al. 2021). Therefore, it is necessary to enhance the nutritive qualities of peat soil so that it can sustain the growth of plants.

According to Anda et al. (2021), in Indonesia, the total peat area is about 13.43 million hectares, which is distributed over Sumatra (5.5 million hectares), Kalimantan (4.54 million hectares), Papua (3.01 million hectares) and other regions of the country. Further, in West Kalimantan, peat soil covers 1.55 million hectares, of which 1.02 million hectares have a depth of fewer than 3 meters. Moreover central Kalimantan, the peat area covers over 2.55 million hectares, of which 1.86 million hectares have a depth of 3 meters. Based on the nutrient content of central Kalimantan peat soil, Tim Peneliti (1986) categorized it from oligotrophic (low fertility level) to mesotrophic (moderate fertility level). Low nutrient availability, extremely high cation exchange capacity (CEC), high base saturation (BS), and low pH are some major limitations of peat soil that limit plant growth or agricultural development (Salampak et al. 2021). Further, peat soils are typically nutrient deficient and have pH ranges from extremely acidic to acidic. In an environment with high H+ ions concentration, nitrate rapidly accumulates and is not easily available to plants (Munir 1996).

To overcome nutrient deficiency and improve peat soil fertility application of liquid organic and inorganic fertilizers can be used as one strategy. Liquid organic fertilizers are suitable for use in leaf vegetable plants because it does not cause long-term side effects and is more easily absorbed by plants when applied through the leaves. Lusmaniar et al. (2020) suggested that the liquid organic fertilizer Agrobost at 6cc liter^{-1} concentration produced the best plant height and the number of branches in green bean plants. Similarly, except for the number of leaves, the application of organic fertilizer Nasa had a significant influence on various growth parameters including plant height, tuber diameter, root volume, fresh weight, and dry weight of oil palm (Yanto et al. 2016). The application of liquid organic fertilizer Nasa's fulfills the nutrient needs of plants because it contains 4.15% N, 4.45% P_2O_5, 5.66% K_2O, 9.69% organic C, 505.5 ppm Fe, 1931.1% Mn, 1179.8% Cu, 1986.1% Zn, 806.6% B, 8.4 ppm Co, 2.3 ppm Mo and has pH of 5.61 (Sangadji, 2018). Like organic fertilizers, the addition of inorganic fertilizers to peat soil also increases the nutritional status and quality of peat soil. According to Butar-Butar research's (2020), the application of NPK fertilizer at an optimal dose of 318 kg ha^{-1} influences the wet and dry weight of green grass jelly plants.

Information regarding the influence of liquid organic fertilizer and NPK inorganic fertilizer on the growth of green grass jelly in peat soil is scanty. Hence, therefore current study has been carried out to evaluate the effect of NPK and organic fertilizer interaction on the cultivation, phytochemical constitution, and yields of grass jelly in peat soil. Also, the effect of these two types of fertilizer interaction on the improvement of peat soil fertility was also evaluated in this study.

2 Materials and Methods

The study was carried out in a completely randomized factorial design with two factors including liquid organic fertilizer and NPK inorganic fertilizers The liquid organic fertilizer (P) comprised three doses i.e. P0 (no liquid organic fertilizer), P1 (Agrobost), and P2 (NASA) similarly in the case of inorganic NPK fertilizer (N), the imposed levels are N0 (no NPK), N1 (NPK 1g polybag^{-1}), and N2 (NPK 2g polybag^{-1}).

The study was carried out in 20 x 30 cm polybags having a 6 kg filling capacity, for this, peat soil was collected from the Kalampangan Sub District, Central Kalimantan, Indonesia, and the collected peat soil was air-dried. The air-dried peat soil was mixed with 5 tons ha^{-1} of chicken dung and 1 ton ha^{-1} of lime and incubated for two weeks and filled in the plastic polybags.

Being supporting data, the original soil nutrient content was calculated. The standard methodology proposed by Page et al. (1982) was used for the estimation of pH H_2O 1:2.5 (Glass Electrode method), available P, and exchangeable K (ammonium acetate pH 4) while the estimation of total N (Persulfate digestion method) was carried out by the Purcell and Andy King (1996) method with some modification.

About 20 to 25 cm in length (consisting of 2-3 nodal segments), robust development, green hue, and consistent diameter grass jelly stem cuttings were collected for this study. After this, these cuttings were submerged in a solution of growth regulator to encourage root development. After 30 minutes of soaking, the stem cuttings were transferred to the prepared polybags media (peat soil mixed with chicken dung and lime). Fertilization with NPK fertilizer was performed in two halves, where the first half of the recommended dose of NPK fertilizer i.e. 0.5 and 1 gram/polybag was administered one week after the first shoots developed (3 WAP) while the remaining half of the dose was administered seven weeks later (7 WAP). Selected fertilizers were applied around the 5 cm from stem cuttings. The utilized liquid organic fertilizer (LOF) Agrobost and Nasa (each with a solution concentration of 3 cc $liter^{-1}$, as much as 50 ml), sprayed every two weeks on the upper and lower surfaces of the leaves till the plants are three months. The plants were watered once each day or according to weather circumstances, up to 250 ml. Intensive weeding is performed by eliminating the visible weeds from the polybags. Grasshoppers, pests, and caterpillars that damage plants were eliminated manually by pests and disease management programs. Meanwhile, flea-borne disorders are controlled with insecticides including garlic extract. When the stem cuttings have grown for 12 WAP, they were harvested by taking old grass jelly leaves, which have a dark green leaf color, typical leaf size, and new leaves. At the time of harvesting (12 WAP), observations related to the leaf area (measured in cm^2plant^{-1} by multiplying the length X width X correction factor technique), leaf length (cm), and leaf widths (cm) were recorded. Fresh leaf weight (g) per plant was determined by weighing the total leaf weight at 12 WAP. At the age of 12 WAP, an analysis of leaf nutrient absorption including Total N (Purcell and King, 1996), Total P (Lambert, 1992), and K (Jones and Case, 1990) was conducted at the Chemistry Laboratory of Lambung, Mangkurat University, Banjarmasin, Indonesia. Further, the estimation of total flavonoid content (Azizah et al. 2014) was carried out at the Laboratory of Pharmacy, Lambung Mangkurat University, Banjarmasin, South Kalimantan, Indonesia.

2.1 Data Analysis

To determine the effect of treatment, data were analyzed using the Ficher test (F test) at the level of $\alpha = 5\%$ and $\alpha = 1\%$. If there is a real or very significant effect, it will be continued with the BNJ Test at the level of $\alpha = 5\%$.

3 Results and Discussion

3.1 Leaf surface area and leaf weight of grass jelly seedlings

The analysis of variance revealed a nonsignificant influence of the liquid organic and inorganic fertilizers interaction on the leaf area and leaf weight of grass jelly stem cuttings grown in peat soil (table 1). Further, the number of leaves was significantly affected by the interaction of organic and inorganic fertilizers.

Results presented in table 1 revealed that the application of liquid organic fertilizer Agrobost (P1) did not differ substantially from NASA (P2) but these two are significantly different from the control. Further, at the time of harvesting (12 WAP), grass jelly plants grown in peat soil polybags treated with liquid organic fertilizer have shown higher leaf area and leaf weight. Results of the study also suggested that the application of liquid organic fertilizer to grass jelly stem cuttings grown in peat soil can improve their leaf area and leaf weight. This might be due to the higher concentration of macro and micronutrients in NASA's organic fertilizer which is essential for leaf production. The total concentration of available macro and micronutrients in 500 cc Nasa are 0.18% N, P_2O_5, K_2O, 4.6% C-organic, 41.04 ppm Zn, 8.43 ppm Cu, 2.42 ppm Mn, 0.29% Cl, 0.15% Na, 60.84 ppm B, 0.01% Si, 6.38 ppm Al, 0.98% NaCl, 0.11 ppm Se, and 0.05 ppm Cr (Kardinan 2011). The biological fertilizer Agrobost is a rich

Table 1 Effect of liquid organic and inorganic fertilizer application on leaf surface area and leaf weight at the age of 12 WAP of green grass jelly stem cuttings grown in peat soil

Treatments	Leaf area (cm^2 $plant^{-1}$)				Leaf weight (g $plant^{-1}$)			
	N0 (0g $polybag^{-1}$)	N1 (1g $polybag^{-1}$)	N2 (2g $polybag^{-1}$)	Average P	N0 (0 g $plant^{-1}$)	N1 (1g $plants^{-1}$)	N2 (2 g $plant^{-1}$)	Average P
P0 (withoutLOF)	574.64 ±27.84	2,113.14±119.39	1,099.29±184.04	1,262.36±120.79[a]	14.75±5.56	39.21±6.44	32.80±14.33	28.92±6.04[a]
P1 (Agrobost)	2,166.94±136.83	2,123.12±296.49	2,217.30±230.29	2,169.12±323.04[b]	38.08±2.49	38.88±6.91	46.17±1.35	41.04±2.51[b]
P2 (Nasa)	1,013.78±84.12	2,797.16±113.17	3,159.27±402.68	2,623.40±374.47[b]	31.89±4.15	51.22±3.19	57.96±7.17	47.02±4.67[b]
Average N	1,551.79±317.26[a]	2,344.47±481.49[b]	2,158.62±419.54[b]	2018.29±272.766	28.24±4.09[a]	43.10±3.52[b]	45.64±5.90[b]	38.99±4.40

Data are mean of five replicates; ± Standard Error of mean; Values without common letters in same column are differ significantly according to the HSD 5% test

source of various phosphate solubilizing and cellulosic microbe's like *Azotobacter* sp., *Azospirillum* sp., *Lactobacillus* sp., and *Pseudomonas* sp., which help in phosphate solublization and nitrogen fixation (Rahmi 2014; Lusmaniar et al. 2020). Since Agrobost can increase microbial population and their activity in peat soil, which in turn improves nitrogen fixation and P solubility, in the presence of N elements that may be anchored by bacteria such as *Azotobacter* and *Azospirillum*, chlorophyll production is enhanced. As a result, this will affect the process of photosynthesis in plants. Further, Ikhwan et al. (2015) reported that the application of *Azospirillum* isolates resulted in an increase in the number of leaves, leaf area, and plant biomass in maize plants. Nitrogen is a building block in the synthesis of various important organic substances such as amino acids, proteins, nucleoproteins, and a variety of enzymes; as a result, nitrogen has a significant impact on cell division and growth (Gardner et al. 1991). The presence of the various types of microorganisms in Agropost improved the development of the grass jelly plant leaf surface area and leaf weight. According to Lusmaniar et al. (2020), the application of 6 cc liter^{-1} Agrobost organic fertilizer significantly influences the plant height as well as the number of branches that were produced by each green bean plant.

In contrast to the organic fertilizers, the use of inorganic NPK fertilizer had a substantial influence on leaf area and leaf fresh weight per plant, and various dosages of NPK inorganic fertilizer i.e. N1 (1g plant^{-1}) and N2 (2g plant^{-1}) have resulted in considerably higher leaf area and leaf mass as compared to N0 (control). The plants grown in peat soil without NPK fertilizers generated a leaf area of 1551.79 cm^2 per plant, which was much less than the N1 (1g plant^{-1}) and N2 (2g plant^{-1}) treatments, which produced leaf areas of 2344.47 cm^2 and 2158.62 cm^2, respectively. While in the case of fresh weight, grass jelly plants grown in peat soil without inorganic fertilizer (N0) have shown leaves fresh weight of 28.24 g, which was less than the N1 (43.10 g plant^{-1}) and N2 (45.64 g plant^{-1}) treatments. As compared to the nitrogen treatment (N1 and N2), plants grown in control (N0) treatments have less leaf area and leaf weight. These results suggested that peat soil without nitrogen fertilizers has poor availability of nutrients and the available nutrients also have complex bonding with the peat soil particles due to this, these nutrients are not easily available for plant growth.

The chemical characteristics of the untreated peat soil analysis revealed the presence of 2.65% N, 474.60 ppm P, and 1.89 me 100g^{-1} K. At the early stage of grass jelly stem cuttings growth, the need for nitrogen, phosphorus, and potassium can be met by applying chicken manure (5 tons ha^{-1}) and half the dose of NPK (0.5g and 1g plant^{-1}), but these nutrients have not been able to significantly increase shoot length, number of shoots, number of leaves and leaves weight. All the administered doses of NPK fertilizer had been properly responded to by increasing leaf area and leaf fresh weight of grass jelly stem cuttings at the age of 12 WAP, it reached the optimal development and may be transplanted directly to the field.

This indicates that applying 1-2g of high-quality NPK fertilizer per plant can enhance the leaf area and leaf weight of grass jelly stem cuttings. Phosphorus is essential for the formation of the cell nucleus, lipids, and proteins. Along with this, phosphorus also play important role in root development, blooming, and fruit/seed/grain ripening. The primary role of potassium is to activate enzymes and maintain cellular hydration. With this, potassium is also an important component in the activation of various cellular processes like starch synthesis, ATP generation, photosynthesis, nitrate reduction, and sugar translocation to seeds, fruit, tubers, or roots are active enzymes (Budianta and Ristiani 2013).

3.2 Nutrient content of grass jelly plant leaves

The results of the N, P, and K nutrient uptake by the leaves of grass jelly cuttings showed various trends and it is depending on the type of used liquid organic fertilizer and the dose of NPK fertilizer. The results of the nutrient content analysis of N, P, and K in the leaves of grass jelly cuttings are presented in Table 2.

Results presented in table 2 suggested that the highest N content was reported from the leaves of plants grown in the peat soil treated by liquid organic fertilizer Nasa + N2 (NPK 2 g polybag^{-1}) combination. This is because of the higher N nutrient content in Nasa fertilizer as compared to the Agrobost liquid organic fertilizer

Table 2 Effect of the various fertilizer applications on the NPK content in the leaves of green grass jelly cuttings

Organic Fertilizers	N Content (%)				P Content (PPM)				K Content (me/100 mg)			
	N0	N1	N2	Average	N0	N1	N2	Average	N0	N1	N2	Average
P0 (Without)	0.4626	1.4492	1.0286	0.9801	0.0031	0.0118	0.0135	0.0095	11.3457	35.8066	25.0658	24.0727
P1 (Agrobost)	0.8103	1.1321	1.6030	1.1818	0.0047	0.0116	0.0072	0.0078	29.2911	29.9065	35.5140	31.5705
P2 (Nasa)	1.1429	1.7210	2.2071	1.6903	0.0095	0.0124	0.0119	0.0113	24.5298	46.7741	44.5828	38.6289
Average	0.8053	1.4341	1.6129	1.2841	0.0058	0.0119	0.0109	0.00953	21.7222	37.4957	35.0542	31.4240

Based on composite of all 3 replication samples, here N0 (0 g polybag^{-1}), N1 (1g polybag^{-1}) and N2 (2g polybag^{-1})

and the dose of NPK given is higher (2 grams) compared to treatments N0 and N1. N nutrient uptake is greatly influenced by the nutrient content of the liquid organic fertilizer and the dosage of application because the roots of grass jelly cuttings will absorb this nutrient through the soil and accumulate in the leaf organs. The wider and heavier leaves per plant were reported from the plants treated with higher N contents. Further, the results of measuring the leaf area and leaf weight of grass jelly cuttings showed that treatment P2N2 has the highest leaf area and leaf weight as compared to the other treatments.

Like N contents, the highest P and K nutrient content in the leaves of the grass jelly cuttings grown in peat soil treated with the Nasa organic fertilizer (Table 2). This might be due to the tendency of the liquid organic fertilizer which does not affect the P nutrient uptake in the leaves and phosphorus nutrients are more translocated to food reserve storage organs such as seeds, which causes P nutrient uptake in the leaves to be relatively low. In addition to this, the P nutrient also plays a more significant role in the process of energy transfer within the cell. The application of NPK fertilizer at dosages of 1, and 2 g per polybag resulted in an increase in the phosphorus (P) and potassium nutrient absorption than the administration at 0 g of the fertilizer (N0).

Higher potassium content in the Nasa liquid organic fertilizer enhance the rate of K accumulation in the leaves of the green jelly plant and it was higher than the Agrobost liquid organic fertilizer. Agrobost fertilizer is a biological fertilizer that contains microorganisms that play a role in binding nutrients and reduced the availability of nutrients for plants. These microorganisms could not play a significant role in peat soils due to the acidic nature of peat soil and because of this extremely low nutrient content is available for the plants in peat soil. However, there are no direct shreds of evidence to confirm this.

3.3 Flavanoid content in the leaves of grass jelly plant

Leaves of the green jelly plant have very higher medicinal value and this might be due to the presence of secondary metabolites, especially flavonoid content. Results present in Table 3 revealed the total flavonoid content of green grass jelly leaves following the application of liquid organic fertilizers (Agrobost and Nasa) and NPK inorganic fertilizers.

Following the findings of the analysis of variance, the interaction between liquid organic fertilizer and inorganic fertilizers did not have a significant influence on the amount of flavonoid that was present in green grass jelly cuttings. Further, individual application of liquid organic or NPK inorganic fertilizer did not have any substantial effect on the leaves flavonoid contents. This demonstrates that the application of organic fertilizer Agrobost, and Nasa along with the two doses of NPK fertilizer (1g and 2g) did not significantly alter the flavonoid concentrations. The application of Agrobost liquid organic fertilizer without NPK (P1N0) gave the highest yield of total leaf flavonoid content in green grass jelly stem cuttings (0.19 µg mg^{-1}), while the treatment of Nasa foliar fertilizer with NPK inorganic fertilizer as much as 2g $polybag^{-1}$ gave the lowest yield of total leaf flavonoid content (0.11 µg mg^{-1}). Further, the findings of this research suggested that the total flavonoid content of grass jelly stem cuttings leaves without NPK (0 g $polybag^{-1}$) was greater than that of grass jelly stem cuttings with NPK of 2g $polybag^{-1}$. It is speculated that plants that were not provided with NPK fertilizer experienced nutritional stress, which led to an increase in the production of secondary metabolites of flavonoids. This was also confirmed by the results related to the treatment P0N0 and P2N0 (0.15 µg mg^{-1} and 0.15 µg mg^{-1}, respectively) which resulted in greater amounts of flavonoids as compared to the P0N2 (0.12 µg/mg^{-1}) and P2N2 (0.11 g mg^{-1}) treatments.

Flavonoids are a type of secondary metabolite that is generated by plants and are considered to be one of the naturally occurring antioxidants. When plants are subjected to particular degrees of stress, they create secondary metabolites in specified quantities. The functions of the many types of secondary metabolites are distinct from one another. These compounds are not essential for the continued existence of the plant, but they do offer several benefits, including functioning as a defense mechanism for the plant, both against biotic and abiotic stress, acting as an attractant, and certain compounds having the potential to be utilized by humans as antioxidants or as raw materials for medicinal products. The production of secondary metabolites is controlled by several

Table 3 Effect of organic and inorganic fertilizers application on the flavonoid content of the green grass jelly leaves

Organic Fertilizer	Flavonoid content (µg/mg)			Average
	N0 (0g $polybag^{-1}$)	N1 (1g $polybag^{-1}$)	N2 (2g $polybag^{-1}$)	
P0 (Without)	0.15	0.13	0.12	0.13
P1 (Agrobost)	0.19	0.13	0.14	0.15
P2 (Nasa)	0.15	0.13	0.11	0.13
Average	0.16	0.13	0.12	0.1367

Note: Based on the composite of all 3 replication samples

factors, including nutrition, a reduced growth rate, feedback regulation, enzyme inactivation, and enzyme induction (Setyorini and Yusnawan, 2016).

Conclusion

Results of the study can be concluded that liquid organic and NPK fertilizer did not interact, and the administration of both fertilizers as a single factor significantly increased the leaf area and leaf fresh weight of grass jelly stem cuttings. The concentration of nutrient absorption and levels of flavonoids in the leaves of grass jelly stem cuttings were not significantly affected by the administration of liquid organic fertilizer or NPK fertilizer, either alone or together.

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Optimizing the ratios of standardized ileal digestible (SID) methionine plus SID cystine and SID threonine to SID lysine in low-protein diets for working boars

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Amino acids pattern

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SID Thr to Lys

ABSTRACT

This study aimed to optimize the ratios of standardized ileal digestible (SID) methionine (Met) plus cystine (Cys), and threonine (Thr) to SID lysine (Lys) in low-protein diets for working boars. Forty-eight working Duroc boars were randomly allocated to one of 12 dietary treatments in a 3x4 factorial experimental design in which factor 1 was the ratios of SID Met plus Cys to SID Lys (50, 60, 70%), factor 2 was the ratios of SID Thr to SID Lys (40, 50, 60, 70%). Semen was collected at a 4 days interval for 6 weeks for 10 ejaculates. Semen volume (V), percentage of sperm with progressive motility (A), sperm concentration (C), and the total number of motile sperm per ejaculate (VAC) were measured. The results of the study revealed that the ratios of SID Met plus Cys to SID Lys in the diets affected the C and VAC. Values of C and VAC were highest at the ratios of SID Met plus Cys to SID Lys of 70% and lowest at 50% ($P<0.05$). Similarly, the ratios of SID Thr to SID Lys affected the C and VAC. Further, the values of C and VAC were highest at the ratio of SID Thr to SID Lys of 60% and lowest at 40% ($P<0.05$). There was no interaction effect between the two factors. In conclusion, the ratios of SID Met plus Cys to SID Lys of 70% and SID Thr to SID Lys of 60% in a 13.5% CP diet are optimal for working boars.

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1 Introduction

The semen quality of boars plays an important role in pig breeding, and it depends on various factors. In terms of nutrition, the contents of dietary essential amino acids highly influence boar semen quality. In general, for pigs, lysine (Lys) is normally the first limiting amino acid in practical diets (Ho et al. 2019). According to Louis et al. (1994), the dietary Lys requirement for working boars should be 0.60% or 12.0g of total Lys/day. Further, as per the NRC (1998), the nutrient requirements for boars are 3265 kcal ME/kg, 13% Crude Protein (CP), and 0.6% Lys at 2.0 kg/day feed intake. Rupanova (2006) reported that boars fed a diet containing 1.03% Lys have better semen quality than those fed a 0.86% Lys diet with no changes in ejaculate volume, while Golushko et al. (2010) determined that the total Lys requirement for boars is about 0.92% (0.76% digestible Lys). In addition, several recent studies showed that a Lys:Met:Thr:Trp ratio of 100:60:65:19 in boar diets improved the reproductive performances (Kiefer et al. 2012), and boars fed a low CP diet (13%) with a ratio of Lys:Thr:Trp: Argas 100:76:38:120 resulted in similar or better reproductive performance than a 17% CP diet (Ren et al. 2015). On the other hand, Ho et al. (2019) suggested that it is necessary to estimate Met plus Cys requirement when formulating diets for pigs because the amount of Met needed in the diet depends on the amount of Cys present. From the above-mentioned results, it is believed that the essential amino acid ratios in low-protein diets are very important for the semen quality of boars. To our knowledge, there are few studies regarding the effect of different amino acid ratios of SID Met plus Cys and Thr to SID Lys in low-protein diets on boar semen quality. Therefore, the objective of this study was to optimize the ratios of SID Met plus Cys and Thr to SID Lys in 13.5% CP diets for working boars.

2 Materials & Methods

2.1 Experimental design

Forty-eight 11 months old working Duroc boars, weighing approximately 150 kg each, were randomly allocated to one of 12 dietary treatments in a 3X4 factorial experimental design in which factor 1 (M) was the three ratios of 50, 60, and 70% SID Met plus Cys to 100% SID Lys, and factor 2 (T) was the four ratios of 40, 50, 60 and 70% SID Thr to 100% SID Lys. The treatments are denoted in Table 1.

The boars were trained to mount a dummy sow for one month before experimenting to provide uniform semen production, and they were individually housed. The experiment was conducted at the Binh Thang Pig Research and Development Center, Institute of Animal Sciences for Southern Vietnam.

2.2 Diets

The diet compositions and calculated nutritive values of the twelve experimental diets are shown in Table 2 and Table 3, respectively. The level of SID Lys was kept constant at 0.8% of all diets. All SID Met plus Cys and SID Thr levels in the diets were adjusted by using synthetic Met and Thr. The energy level was set at 3,000 kcal ME/kg, and the CP level was set at 13.5% for all diets. The ratio of SID tryptophan to SID Lys was fixed at 0.22% for all diets. Other amino acids, vitamin, macro, and micro-mineral element concentrations were formulated to equivalency in all diets. The animals were offered 3.0 kg of feed/day and were provided with *ad libitum* access to water. All boars were fed their experimental diets for one week before collecting semen to evaluate semen quality.

Table 1 Experimental design and denoted treatments

Factor 1 (M)	Factor 2 (T)	Treatments
50 M	40T	50M.40T
	50T	50M.50T
	60T	50M.60T
	70T	50M.70T
60M	40T	60M.40T
	50T	60M.50T
	60T	60M.60T
	70T	60M.70T
70M	40T	70M.40T
	50T	70M.50T
	60T	70M.60T
	70T	70M.70T

Table 2 Developed Diet ingredients and different combination

Ratio of SID Met plus Cys to Lys (%)	50				60				70			
Ratio of SID Thr to Lys (%)	40	50	60	70	40	50	60	70	40	50	60	70
Treatment	50M X 40T	50M X 50T	50M X 60T	50M X 70T	60M X 40T	60M X 50T	60M X 60T	60M X 70T	70M X 40T	70M X 50T	70M X 60T	70M X 70T
Corn	428	429	430	431	429	430	431	432	430	431	432	433
Rice bran	250	250	250	250	250	250	250	250	250	250	250	250
Wheat	100	100	100	100	100	100	100	100	100	100	100	100
Wheat bran	86	83	81	78	83	81	78	76	81	78	76	73
Soybean meal	82	83	84	84	83	84	84	85	84	84	85	85
Fish meal 60%	20	20	20	20	20	20	20	20	20	20	20	20
DCP	9	9	9	9	9	9	9	9	9	9	9	9
Limestone	12	12	12	12	12	12	12	12	12	12	12	12
Salt	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.7
Vit-min. premix	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Phytase	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Kenzyme V	1	1	1	1	1	1	1	1	1	1	1	1
Lys 98%	2.97	2.96	2.96	2.95	2.96	2.96	2.95	2.94	2.96	2.95	2.94	2.94
DL-Met	0.09	0.09	0.09	0.09	0.91	0.91	0.91	0.91	1.72	1.72	1.72	1.72
L-Thr 98%	0.22	1.04	1.85	2.66	0.22	1.04	1.85	2.66	0.22	1.03	1.85	2.66
L-Try 98%	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

Table 3 Calculated nutritive values of imposed diets in various combinations

Ratio of SID Met plusCys to Lys (%)	50			60			70					
Ratio of SID Thr to Lys (%)	40	50	60	40	50	60	40	50	60	40	50	60
Treatment	50M X 40T	50M X 50T	50M X 60T	50M X 40T	50M X 50T	50M X 60T	50M X 40T	50M X 50T	50M X 60T	50M X 40T	50M X 50T	50M X 60T
DM (%)	88.41	88.42	88.43	88.44	88.42	88.43	88.44	88.44	88.43	88.44	88.44	88.45
ME (kcal/kg)	3,000	3,000	3,000	3,000	3,000	3,000	3,000	3,000	3,000	3,000	3,000	3,000
CP (%)	13.50	13.50	13.50	13.50	13.50	13.50	13.50	13.50	13.50	13.50	13.50	13.50
EE (%)	5.90	5.90	5.89	5.89	5.90	5.89	5.89	5.89	5.89	5.89	5.89	5.88
CF (%)	4.56	4.54	4.51	4.49	4.54	4.51	4.49	4.46	4.51	4.49	4.46	4.44
Ca (%)	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Total P (%)	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83
Available P (%)	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Salt (%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
SID Lys (%)	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
SID Met (%)	0.22	0.22	0.22	0.22	0.30	0.30	0.30	0.30	0.38	0.38	0.38	0.38
SIDMet plus Cys (%)	0.40	0.40	0.40	0.40	0.48	0.48	0.48	0.48	0.56	0.56	0.56	0.56
SID Thr (%)	0.40	0.48	0.56	0.64	0.40	0.48	0.56	0.64	0.40	0.48	0.56	0.64
SID Trp (%)	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
SID Arg (%)	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77
SID Val (%)	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
SID Iso (%)	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42
SID Leu (%)	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95
SID His (%)	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
SID Phe (%)	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53

2.3 Semen collection and evaluation

Semen collection from the boars was carried out at an interval of 4 days during 6 weeks for a total of 10 ejaculates by the well-trained technician using a dummy sow with an artificial vagina. Samples collected from the boars for one ejaculate throughout the experiments were subjected to semen quality evaluation.

The semen volume (V) was determined immediately after the collection by filtering the ejaculate through gauze to remove the gelatinous fraction, and the gelatinous-free fraction was weighed using an electronic scale and converted to volume as described by Lovercamp et al. (2013). Sperm concentration (C) was determined by hemacytometer counts as described by Melrose (1966) and Almquist (1973).

Percentage of sperm with progressive motility (A) was determined by light microscopy under ×200 magnification on a warm stage immediately after straining the ejaculate based on the scoring method described by Swierstra (1973).

Total number of motile sperm per ejaculate (VAC) was calculated by multiplying the semen volume (V), sperm concentration (C), and percentage of sperm with progressive motility (A) (Louis et al. 1994).

2.4 Statistical analysis

Collected data were analyzed using an ANOVA by Minitab version 16.2.0 (2010). The Tukey-Test was used to compare average values with a 95% confidence interval. Mean values were considered significantly different when P values were less than 0.05. For statistical analysis, the used statistical Model is as follows

$$Y_{ijk} = \mu + M_i + T_j + MxT_{ij} + e_{ijk}$$

Y_{ijk} = the value of the dependent variable of observation k in factor A level i and factor B level j (i = 1,2,3; j = 1, .., 4; k = 4); In which, μ: general mean; M_i: effects of methionine plus cystine; T_j: effects of threonine; MxT_{ij}: effects of interaction M and T; and e_{ijk}: random error.

3 Results

3.1 Effect of different ratios of SID methionine plus cystine to SID lysine on boar semen quality

Results given in Table 4 revealed that except for the semen volume of boars, the other semen characteristics were affected by the ratios of SID Met plus Cys to SID Lys in the diet (P<0.05). The percentage of sperm with progressive motility from boars fed a diet with a ratio of SID Met plus Cys to SID Lys of 50% was higher than those in the diet with the ratios of SID Met plus Cys to SID Lys of 60 and 70% (P<0.05). However, the sperm concentration and VAC were highest in boars fed the diet with a ratio of SID Met plus Cys to SID Lys of 70% and lowest in boars fed the diet with a ratio of SID Met plus Cys to SID Lys of 50% (P<0.05). It can be seen that the ratio of SID Met plus Cys to SID Lys of 70% in the diet improved the semen quality of boars.

3.2 Effect of different ratios of SID threonine to SID lysine on boar semen quality

Results presented in Table 5 show that the ratios of SID Thr to SID Lys in boar diets did not affect semen volume and the percentage of sperm with progressive motility (P>0.05), but they did affect sperm concentration and VAC (P<0.05). The sperm concentration in boars fed the diet with the ratio of SID Thr to SID Lys of 40% was the lowest and was highest in boars fed the diet with the ratio of SID Thr to SID Lys of 60%. However, there were no significant differences in sperm concentration between in boars fed the diets with the ratios of SID Thr to SID Lys of 60 and 70%(P>0.05), as well as in boars fed the diets with the ratio of SID Thr to SID Lys of 40 and 50%. The VAC was lowest in boars fed the diet with a ratio of SID Thr to SID Lys of 40%, followed by 50%, and highest in boars fed the diet with a ratio of SID Thr to SID Lys of 60%. Therefore, it can be concluded that the ratio of SID threonine to SID lysine of 60% in the diet would be optimal for working boars.

Table 4 Effect of different ratios of SID methionine plus cystine to SID lysine on boar semen quality

Item	Ratios of SID Met plusCys to SID Lys (%)			SEM	P-value
	50M	60M	70M		
V, mL	298.0 ± 1.49	297.9 ± 1.25	298.2 ± 5.37	0.92	0.967
A, %	84.5^{a} ± 0.28	83.9^{b} ± 0.43	83.9^{b} ± 0.87	0.12	0.012
C, $\times 10^{6}$ mL	331.6^{c} ± 2.05	338.3^{b} ± 2.69	343.9^{a} ± 4.43	1.17	<0.001
VAC,$\times 10^{9}$/ejaculate	83.10^{c} ± 0.19	84.71^{b} ± 0.61	86.31^{a} ± 1.87	0.295	<0.001

Values given in table are mean of selected replicates; mean value followed by the different letter in same row are significantly different at P<0.05

Table 5 Effect of different ratios of SID threonine to SID lysine on boar semen quality

Parameters	Ratios of SID Thr to SID Lys (%)				SEM	P-value
	40T	50T	60T	70T		
V, mL	296.3	297.2	299.5	299.1	1.06	0.122
A, %	84.2	84.1	84.2	84.1	0.16	0.946
C, $\times 10^6$/mL	331.2^{c}	336.3^{bc}	343.3^{a}	340.9^{ab}	1.35	<0.001
VAC,$\times 10^9$/ejaculate	82.66^{c}	84.19^{b}	86.15^{a}	85.84^{a}	0.34	<0.001

Values given in table are mean of selected replicates; mean value followed by the different letter in same row are significantly different at P<0.05

Table 6 Interaction effect between ratios of SID methionine plus cysteine to SID lysine and SID threonine to SID lysine on boar semen quality

Factor 1	50M				60M				70M				SEM	p-value
Factor 2	40T	50T	60T	70T	40T	50T	60T	70T	40T	50T	60T	70T		
V, mL	294.9	297.9	299.1	300.0	298.6	296.0	299.0	298.0	295.4	297.8	300.3	299.4	1.838	0.709
A, %	84.4	84.5	84.6	84.4	84.2	84.0	84.0	83.7	83.9	83.9	83.9	84.2	3.112	0.879
C, $\times 10^6$/mL	424.9^{f}	328.4^{ef}	338.9^{abcde}	334.1^{cdef}	330.7^{def}	337.3^{bcde}	342.0^{abcd}	343.1^{abcd}	337.9^{abcde}	343.1^{abc}	349.2^{a}	345.6^{ab}	2.332	0.816
VAC, $\times 10^9$/ ejaculate	80.9^{e}	82.6^{de}	84.3^{cd}	84.6^{bcd}	83.1^{cde}	84.0^{cd}	86.0^{abc}	85.7^{abcd}	83.9^{cd}	85.9^{abc}	88.1^{a}	87.2^{ab}	0.589	0.872

Values given in table are mean of selected replicates; mean value followed by the different letter in same row are significantly different at P<0.05

3.3 Interaction effect of ratios of SID methionine plus cystine and threonine to SID lysine

Results presented in Table 6 show that there were no statistically significant differences (P>0.05) in semen volume, the percentage of sperm with progressive motility, sperm concentration, or VAC among dietary treatments. Nor was there an interaction effect between the two factors (ratios of SID Met plus Cys to SID Lys and ratios of SID Thr to SID Lys) in this study.

4 Discussion

The main aim of this study was to optimize the amino acids ratios of SID Met plus Cys and Thr to SID Lys in low-protein diets for working boars by evaluating the effect of different ratios of SID Met plus Cys and Thr to SID Lys in 13.5% CP diets on boar semen quality. The present study found that the semen volume of boars ranged from 294.9 mL to 300.3 mL per ejaculate, and there were no significant differences in semen volume per ejaculate among the twelve treatments. These data suggest that the semen volume of boars was not affected by the different ratios of SID Met plus Cys to SID Lys and/or SID Thr to SID Lys in the diets. These results are consistent with previous reports, that there was no effect on sperm production when boars were fed either 6.8 g Lys/kg or 12 g Lys/kg diets (Kemp and Hartog 1989), and that no significant differences in semen volume between the two dietary treatments composed of total Lys levels of 0.64% and 0.96% with the Lys:Met:Thr:Trp ratio of 100:27:73:69 were found (Dong et al. 2016). Rozeboom (2000) also reported that the semen volume of different boar breeds fluctuated from 100 mL to 500 mL in the ejaculate, so the recorded results of boar semen volume in the current study are quite high.

The percentage of sperm with progressive motility in experimental boars ranged from 83.70% to 84.60%, and they were in the normal range of good-quality semen for working boars. The percentage of sperm with progressive motility in boars that consumed diets with different ratios of SID Thr to SID Lys in this study were consistent at approximately 84%, while this parameter for boars fed diets containing a SID Met plus Cys to SID Lys ratio of 50% was the highest, and higher than those in boars fed two other SID Met plus Cys to SID Lys ratios of 60 and 70%. No difference in the percentage of sperm with progressive motility between the SID Met plus Cys to SID Lys ratios of 60 and 70% was found in this study. Rozeboom (2000) reported that the percentage of sperm with progressive motility in boars fluctuated from 70 to 95%, and Vuong (2010) reported that the percentage of sperm with progressive motility in 24 months old boars was 76%. In this study, the percentage of sperm with progressive motility of working boars was attained at approximately 84%, which is considered very well.

In the present study, the sperm concentration per mL of semen was significantly affected by the ratios of SID Met plus Cys to SID

Lys. Sperm concentration increased from 331.6×10^6 sperms/mL of semen in boars fed a diet with a SID Met plus Cys to SID Lys ratio of 50% to 343.9×10^6 sperms/mL in boars fed the diet with a SID Met plus Cys to SID Lys ratio of 70%. On the other hand, sperm concentration increased gradually in boars fed diets with ratios of SID Thr to SID Lys of 40 to 60%, and kept constant at a ratio of SID Thr to SID Lys of 70%. The highest sperm concentration was attained at a ratio of SID Met plus Cys to SID Lys of 70% (343.9×10^6/mL) and a ratio of SID Thr to SID Lys of 60% (343.3×10^6/mL). These values were higher than those in previous studies (Vuong, 2010; Kommisrud et al. 2002). These results are consistent with previous reports that the semen quality of boars is improved by fed a diet with a Lys:Met:Thr:Trp ratio of 100:60:65:19 (Kiefer et al. 2012), and by feeding a 13% CP diet with a Lys:Thr:Trp:Arg ratio of 100:76:38:120 (Ren et al. 2015). These findings suggest that an optimal essential amino acid ratio in diet can be established for the boar to increase semen quality. Presently, nearly all pig farms have been using artificial insemination to breed, thus the higher sperm concentration and percentage of sperm with progressive motility would be favorable to improve the economic efficiency of production.

The VAC of boars differed among treatments in this study. An increase in the ratio of SID Met plus Cys to SID Lys from 50 to 60 and 70% led to a significant increase in the VAC from 83.10×10^9 to 84.71×10^9 and 86.31×10^9. On the other hand, Increasing ratios of SID Thr to SID Lys from 40 to 60% positively affected the VAC, but not at 70%. The VAC values of boars in this study ranged from 80.9–88.1×10^9 sperms. The highest VAC value of 88.1×10^9 was observed in boars fed the diet with a ratio of SID Met plus Cys to SID Lys of 70% and a ratio of SID Thr to SID Lys of 60%. According to Rozeboom (2000), the VAC of boar fluctuated from 10-100×10^9. Knecht et al. (2014) reported that this value for Poland Duroc boars was 76.4×10^9 sperms. Thus, our recorded results of the VAC of working boars in this study can be considered very well. The results from this study show that the ratio of SID Met plus Cys to SID Lys of 70% and the ratio of SID Thr to SID Lys of 60% in 13.5% CP diets of boars improved semen quality, particularly sperm concentration and VAC. However, no interaction effect between the two factors of ratios of SID Met plus Cys to SID Lys, and ratios of SID Thr to SID Lys was found in this study. The results from the current study suggest that the ratio of SID Met plus Cys to SID Lys of 70% and the ratio of SID Thr to SID Lys of 60% in a 13.5% CP diet are optimal for working boars.

Conclusion

In conclusion, the different ratios of SID methionine plus cystine to SID lysine and SID threonine to SID lysine in 13.5% of CP diets of working boars affected semen quality. The ratios of SID methionine plus cystine to SID lysine of 70% and SID threonine to SID lysine of 60% improved sperm concentration and VAC and were found to be optimal for working boars. However, there was no interaction effect between ratios of SID methionine plus cystine to SID lysine and SID threonine to SID lysine on boar semen quality.

Conflict of Interest

The authors declare that there is no conflict of interest associated with this study.

Authors' contributions

La Van Kinh, La Thi Thanh Huyen, and Nguyen Vu Thuy Hong Loan were responsible for the design and performance of the experiments, and for writing the manuscript. Le DucNgoan and PhungThang Long interpreted the data and edited the manuscript.

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Effects of a combination of herbal oils (rosemary, black cumin, and clove) on quail growth, antioxidant enzymes and health status

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ABSTRACT

The purpose of this trial was to evaluate the potential of herbal oil combinations (rosemary, clove, and black cumin) in quail feeding as a natural growth enhancer. The effects of dietary gradual levels of this blend (RCBC) on growth indices, carcass attributes, and blood biochemical variables were compared to the control group (basal diets). For this, 300 1-week-old developing quails were employed. Birds were kept on the baseline diet with or free of herbal oils blend (RCBC) at three different amounts (0, 1.50, and 3.00 cm3/kg diet) from one to six weeks of age to suit their nutritional needs. There were no variations in live body weight or body weight gain over the entire period or at intervals. Compared to the control, birds fed RCBC-supplemented diets devoured more feed ($P<0.01$). RCBC supplementation in the diet did not affect the feed conversion ratio. Except for heart %, all carcass features were statistically ($P <0.01$) different after RCBC treatment when compared to the control. The amounts of total globulins, total protein, and albumin in quails given RCBC were higher than the control ($P<0.001$). In quails, the hepatic levels of GSH and the activity of SOD, catalase, GR, GPx, and GST all increased ($P<0.001$). MDA concentrations in hepatic homogenate were dramatically reduced by RCBC diets. Finally, RCBC supplementation at a dose of up to 3.0 cm^3/ kg diet is recommended to enhance the growth and general health of quails during growth, which would have a favorable impact on the general health of quail meat consumers.

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1 Introduction

Following the ban on antibiotics in poultry feed, natural molecules, medicinal herbs, and oilseed extracts are in high demand due to their health-promoting and biological antibacterial properties against various infections (Abd El-Hack and Alagawany 2015; Abd El-Hack et al. 2018a,b). Due to their broad beneficial benefits for immunological stimulation, growth promotion, and health care systems, herbs and their bioactive constituents are becoming important for the lifestyle of human, cattle, and poultry industries (Alagawany et al. 2015; Alagawany and Abd El-Hack 2021; Abd El-Hack and Alagawany 2022).

Because of their broad spectrum of beneficial effects on productivity, immune strengthening, and health protection, herbs, cold-pressed oils, and essential oils are becoming increasingly important in poultry production (Abd El-Hack and Alagawany 2015; Abd El-Hack et al. 2015, 2022). Furthermore, therapeutic herbs maintain the natural flora's equilibrium while also playing an important antimicrobial role against pathogens (Ghazalah and Ali 2008).

Modern commercial processes extract cold-pressed oils such as rosemary, clove oils, and black seeds by grinding the seeds of the herb under pressure. To limit the heat created by friction, the operation is carried out under thermoregulation, and the process temperature must not exceed 49°C or even be lower. The flavor, aroma, nutritional content, and medicinal characteristics of the resulting cold-pressed oils are all preserved (Lutterodt et al. 2010; Alagawany et al. 2020). To improve animal performance, phytogenic feed additives (PFA) are routinely employed. Because of their nutritional value, and improved general health and behavior of birds, they are currently regarded for poultry feeding techniques (Alagawany et al. 2015; Arif et al. 2022).

This study aimed to see how beneficial it was to use a herbal oil combination (rosemary, clove, and black cumin) in quail feeding. To do this, gradually different amounts of this mix were supplemented with a normal diet to see how they affected growth, carcass attributes, and blood biochemical traits.

2 Materials and Methods

2.1 Rations, quails, and design

A total of 1-wk-old 300 developing quails were used in this experiment with three groups (the average initial body weight was 24.25 g). Each batch was split into five replicas, each containing 20 unsexed quails. They were fed the baseline diet with or without oils blend (RCBC) from one to six weeks of age to suit their nutritional needs, according to NRC (1994). RCBC supplementation was done at three different amounts (0, 1.50, and 3.00 Cm^3/kg diet). The tested diets were as follows: 1) normal diet,

Table 1 Composition the basal diets

Ingredients	%
Corn	53.03
Soybean meal	38.69
Gluten meal	3.20
Soybean oil	1.67
Di Calcium phosphate	0.81
Vit-min Premix*	0.30
NaCl	0.11
Limestone	0.30
DL Methionine	0.39
L-Lysine HCl	1.50
Calculated analysis**:	
Crude protein %	24.04
Metabolizable energy Kcal/kg	2903
Calcium %	0.85
Available phosphorous %	0.45
Methionine + Cysteine %	0.88
Lysine %	1.60

* Growth vitamin and Mineral premix Each 2 kg consists of Vit A 12000, 000 IU; Vit D3, 2000, 000 IU; Vit. E. 10g; Vit k3 2 g; Vit B_1, 1000 mg ; Vit B_2, 49g ; Vit B_6, 105 g; Vit B_{12}, 10 mg; Pantothenic acid, 10 g; Niacin, 20 g , Folic acid , 1000 mg ; Biotin, 50 g; Choline Chloride, 500 mg, Fe, 30 g; Mn, 40 g; Cu, 3 g; Co, 200 mg; Si, 100 mg and Zn , 45 g. ** Calculated according to NRC (1994).

2) normal diet+1.50Cm^3 RCBC /kg diet, (3) normal diet+3.00 Cm^3 RCBC/kg diet. The feedstuffs and calculated analysis of the normal diet are shown in Table 1.

2.2 Birds, diets, and experimental design

All experimental methods were investigated according to the guidelines established by the local committee of experimental animal care and authorized by the institutional committee of ethics. The birds were kept in standard cages (50×30×50 Cm^3). All of the chicks were subjected to the same management, sanitary, and environmental circumstances. Feed and fresh water were available throughout the experiment. Throughout the trial, the birds were also kept on a 24-hour light cycle.

2.3 Data collection

Weekly, all birds were weighed to determine their LBW (body weight) and gain (BWG). The average BWG was computed by subtracting the initial LBW of a certain period from the final LBW of the same period. The author used these data to compute the average AFI (food intake) and FCR (feed conversion ratio). FCR was calculated as the number of grams of food required to produce one gram of BWG. Daily wastage of feed was recorded to estimate AFI.

Six quails (one from each group) were haphazardly selected for carcass attributes at six wks-old, weighed, and slain at the end of the trial. The liver, thigh, breast, gizzard, and heart weights were measured (g / kg slaughter wt). Dressed and carcass weights were compared to live body weight.

2.4 Blood biochemical analysis

After overnight fasting, blood samples were taken from seven quails from each group into centrifugation tubes, and the sample was centrifuged for 15 min at 5000 rpm. Serum samples were utilized to determine some biochemical parameters like ALT and AST activity using commercially available kits, as described by Breuer (1996). Protein and its fractions were analyzed using a commercial kit (Doumas et al. 1981; Drupt 1974).

The total bilirubin in the serum was determined using the available kit. The rate of production of the creatinine-picrate-colored complex influences the creatinine level in serum (Heinegard and Tiderstrom 1973). Serum lipid profiles such as total cholesterol, triacylglycerol, free fatty acids (FFA), and high-density lipoproteins (HDL) were determined by the methods of Lopes-Virella et al. (1977), Allain et al. (1974), Duncombe (1964); and Fossati and Prencipe (1982). The serum content of low-density lipoproteins (LDL) in quails was determined by the method given by Abd El-Hack et al. (2017). The oxidative status and antioxidant parameters in liver homogenate were measured by the method given by Abd El-Hack et al. (2017).

Briefly, one gram of liver tissues was weighed and homogenized with 1.17 percent chilled potassium chloride to detect the concentrations of GSH (reduced hepatic glutathione) and MDA (malondialdehyde) spectrophotometrically as described by Beutler (1969), and Nair and Turner (1984). The antioxidant enzymes in Japanese quail hepatic tissues were evaluated using Sinha (1972) and Misra and Fridovich (1972), methods. Furthermore, GST (glutathione S transferase) and GR (glutathione reductase) were found using the method of Goldberg and Spooner (1983).

2.5 Statistics

Using SAS's GLM techniques, data were treated to an ANOVA procedure with a completely randomized design (SAS Institute Inc., 2001). The author used Tukey's test to study the differences between means (P<0.05).

3 Results and Discussion

3.1 Growth measurements

Results presented in Table 2 revealed the effect of RCBC in quail diets on growth performance measures (LBW, DBWG, DFI, and

Table 2 Body weight and body weight gain of Japanese quails affected by various levels of RCBC addition

Items	LBW (g)			DBWG (g)		
	1 wk	3 wk	6 wk	1-3 wk	3-6 wk	1-6 wk
RCBC (Cm^3/kg diet)						
0.00	24.05±0.08	97.36±0.96	201.60±2.76	5.23±0.10	7.44±0.13	6.34±0.20
1.50	23.86±0.07	95.02±0.90	197.43±3.50	5.08±0.09	7.32±0.14	6.20±0.15
3.00	23.81±0.10	97.63±099	207.58±3.33	5.27±0.08	7.85±0.10	6.56±0.24
Probabilities	0.086	0.498	0.088	0.488	0.049	0.076

RCBC: herbal oils blend (equal amounts of rosemary, clove and black cumin oils); LBW: live body weight, DBWG: daily body weight gain; Means in the same column with no superscript letters after them or with a common superscript letter following them are not significantly different (P < 0.05).

Table 3 Feed intake and feed conversion ratio of Japanese quails affected by various levels of RCBC addition

Items	DFI (g/day)			FCR (g feed/ g gain)		
	1-3 wk	3-6 wk	1-6 wk	1-3 wk	3-6 wk	1-6 wk
RCBC (Cm^3/kg diet)						
0.00	13.12±0.19[b]	21.41±1.44[b]	17.26±0.50[b]	2.54±0.40	2.91±0.15	2.72±0.52
1.50	13.14±0.14[b]	23.21±1.65[a]	18.17±0.45[a]	2.62±0.38	3.29±0.16	2.95±0.10
3.00	13.94±0.26[a]	22.57±1.36[a]	18.26±48[a]	2.68±0.43	2.91±0.20	2.80±0.15
Probabilities	0.005	0.001	0.001	0.958	0.082	0.062

RCBC: herbal oils blend (equal amounts ofrosemary, clove and black cumin oils); DFI: daily feed intake, FCR: feed conversion ratio; Means in the same column with no superscript letters after them or with a common superscript letter following them are not significantly different ($P < 0.05$).

FCR). Further, data related to the LBW and DBWG showed no significant differences throughout the experiment and at intervals. The addition of a 3.0 Cm^3/kg RCBC diet to growing quail diets numerically raised LBW and DBWG values, albeit not significantly. This increase in LBW and DBWG could be linked to the RCBC's enhanced DFI (Table 3).

Several studies have shown that herbal oils can improve poultry growth performance by increasing nutrient digestibility, allowing them to be used as a growth enhancer (Bampidis et al. 2005). Ghazalah and Ali (2008) found that adding varying doses of rosemary leaves meal to broilers at 49 days of age raised LBW and DBWG ($P<0.01$). In comparison to the control, Abd El-Hack et al. (2015) pointed out that adding an oil mixture containing rosemary (1.5 g/kg diet) enhanced DBWG by 5.27 and 3.85 percent at 3-6 weeks of age and overall period (1-6-old), respectively. Alagawany and Abd El-Hack (2015) reported a non-significant influence on LBW or DBWG of the layer due to varying quantities of the rosemary plant, which is consistent with our findings. Mukhtar (2011) discovered that giving the animals a diet rich in 600 mg/kg clove oil enhanced their body weights. The addition of 600 mg clove oil /kg diet boosted BWG by 2.24 percent as compared to the control. Moreover, various active components in essential black cumin oil, including as*p-Cymence*, dithymoquinone, thymoquinone, carvacrol, and thymol may contribute to increased digestion coefficient of feed and nutrient absorption by stimulating intestinal enzymes (Salam et al. 2013). Nigellone is found in 60-80% of black seed essential oil. This chemical has been demonstrated to have antifungal and antibacterial properties that slow down the rate of growth (Shokri 2016).

The preceding data could explain why the DFI has improved. RCBC-supplemented meals resulted in higher feed consumption ($P<0.01$) than the control diet. The aroma of rosemary and clove extracts is pleasing, making the meal more appealing. DFI elevated ($P<0.05$) in a broiler diet enriched with 0.1 or 0.2 g rosemary oil/kg food relative to the control diets, according to Abd El-Latif et al. (2013). According to Mukhtar (2011) broiler chicks fed by a food supplemented with clove oil 600 mg/ kg had higher DFI than control and other treatment groups. Black cumin has also been shown to stimulate the pituitary gland, which can activate the thyroid gland directly or indirectly (Azeem et al. 2014). Thyroid hormones are necessary for body metabolism; they increase metabolic rate, which improves amino acid use by speeding up metabolism (More et al.1980).

Dietary RCBC supplementation did not affect FCR, as demonstrated in Table 3. Extracts from medicinal plants have been shown to increase the release of intestinal enzymes, improving the digestibility of certain nutrients (Ghazalah and Ali 2008). According to Nikaido (2003), essential oils can increase amylase, trypsin, and jejunal chime secretion and inhibit pathogen attachment to the intestinal wall (Jang et al. 2004).

3.2 Carcass characteristics

Except for heart percentage, all carcass features were statistically (P 0.01) different after RCBC treatment (Table 4). When compared to the control, the high dietary amount of RCBC (3 cm^3 /kg diet) produced the best results for all carcass features (dressing, giblets, liver, and gizzard). Growing a quail-fed diet supplemented with a combination of essential oils, including rosemary oil, had the best giblets and dressing percentages (Abd El-Hack et al. 2015). The results of the study suggested that supplementing the quails' diet with the mixture (1.5 g oil) per kg meal gave the highest percentage of the carcass (71.03%) when compared to the control. Furthermore, Isabel and Santos (2009) discovered that chickens fed 100 ppm cinnamon and clove oils had a higher percentage of carcass and breast ($P>0.001$) than all other treatment groups. In contrast, Ferket et al. (2002) investigated the effect of an essential oil blend on broiler growth and internal organ weights. The experimental treatments did not significantly affect the broiler body weights at 21 and 42 days Furthermore, Mehr et al. (2014) observed no significant change in the percentage of the carcass in broilers fed 450 ppm clove oil /kg feed.

Table 4 Carcass characteristics of Japanese quails affected by various levels of RCBC addition

Items	Carcass traits (% of slaughter weight)				
	Dressing	Giblets	Liver	Gizzard	Heart
RCBC (Cm^3/kg diet)					
0.00	74.90±0.55^c	5.82±0.44^b	2.28±0.60^b	2.55±0.11^b	0.99±0.07
1.50	77.51±0.54^b	5.77±0.34^c	2.32±0.70^b	2.48±0.18^b	0.98±0.04
3.00	78.08±0.49^a	6.39±56^a	2.50±0.69^a	2.94±0.15^a	0.94±0.10
Probabilities	0.00<	0.00<	0.00<	0.00<	0.56

RCBC: herbal oils blend (equal amounts of rosemary, clove and black cumin oils); Means in the same column with no superscript letters after them or with a common superscript letter following them are not significantly different (P < 0.05)

Table 5 The potential role of RCBC addition on some liver serum parameters, total protein and total globulins in Japanese quails

Items	TP (g/dl)	ALB (g/dl)	TG (g/dl)	ALT (U/L)	AST (U/L)	TB (mg/dl)
RCBC (Cm^3/kg diet)						
0.00	2.45±0.55^c	1.90±0.55^c	0.56±0.08^c	23.96±3.01^a	279.31±20.56	0.62±0.01
1.50	3.36±0.60^b	2.01±0.60^b	1.35±0.04^b	17.97±2.33^b	284.71±14.33	1.03±0.02
3.00	4.62±0.61^a	2.37±0.45^a	2.25±0.05^a	14.58±2.55^c	251.47±28.56	1.15±0.06
Probabilities	0.00<	0.005	0.020	0.08	0.120	0.349

RCBC: herbal oils blend (equal amounts of rosemary, clove and black cumin oils), TP: total protein, ALB: albumin, TG: total globulins, AST: aspartate aminotransferase, ALT: alanine aminotransferase, TB: Total bilirubin; Means in the same column with different superscript letter following them are significantly different (P <0.05)

3.3 Blood biochemical parameters

When quails are given RCBC the level of total protein, albumin, and total globulins concentrations are substantially increased (P>0.001) as compared to the control group (Table 5). It's worth noting that RCBC decreased the activity of the ALT liver enzyme substantially (P=0.08). The effect of RCBC addition on liver function confirms black cumin oil's impact on enhancing liver functions and, as a result, the birds' overall health (Averbeck 1992). After feeding *Nigella sativa* to chickens, Al-Beitwai and Al-Ghousein (2008) observed similar data with the analyzed indices.

Table 6 shows the anti-atherogenic and hypolipidemic properties of RCBC. When quails were fed RCBC, blood FFA, creatinine, total cholesterol, triacylglycerol, and LDL values were lower than the control. The content of the beneficial cholesterol (HDL) was higher (P< 0.05) in birds fed diets enriched with RCBC than in control birds. These findings suggest that RCBC improved the quails' fat metabolism and liver functioning, which in turn benefited their overall health. The current findings support those of Polat et al. (2011), who discovered that rosemary oil had a hypocholesterolemic impact on chickens.

Table 6 The potential role of RCBC addition on blood creatinine and lipid profile in Japanese quails

Items	FFA (mg/dl)	CR (mg/dl)	TCH (mg/dl)	TC (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
RCBC (Cm^3/kg diet)						
0.00	7.44±10.01^a	0.81±0.05^a	318.57±0.10^a	333.58±15.23^a	83.75±15.23^c	96.46±13.54^a
1.50	5.40±13.23^b	0.56±0.06^b	215.84±0.11^b	218.49±18.21^b	121.71±17.56^b	59.59±17.25^b
3.00	4.02±8.65^c	0.45±0.09^c	176.08±0.03^c	168.10±9.89^c	146.73±10.67^a	45.70±11.34^c
Probabilities	0.00<	0.00<	0.00<	0.00<	0.00<	0.00<

RCBC: herbal oils blend (equal amounts ofrosemary, clove and black cumin oils), FFA: free fatty acids, CR: creatinine, TCH: total cholesterol, TC: Triacylglycerol, HDL: high density lipoprotein, LDL: low density lipoprotein; Means in the same column with different superscript letter following them are significantly different (P <0.05)

Table 7 The effect of RCBC mixture on antioxidant status in experimental groups' liver homogenates

Items	MDA (µmol/g tissue)	GSH (mg/g tissue)	CAT (µmol H_2O_2 decomposed/g tissue)	SOD (U/g tissue)	GPx (µmol NADPH/g tissue)	GR (U/g tissue)	GST (U/g tissue)
RCBC (Cm^3/kg diet)							
0.00	152.49±13.98^a	10.51±6.55^c	17.87±3.01^c	8.01±7.02^c	8.66±2.05^c	0.79±0.57^c	0.17±0.09^c
1.50	87.26±9.87^b	24.29±9.65^b	37.28±2.88^b	28.73±4.59^b	17.52±1.53^b	2.46±1.10^b	0.45±0.10^b
3.00	66.58±8.65^c	30.84±6.53^a	47.07±2.56^a	36.45±5.96^a	22.04±2.01^a	3.21±0.15^a	0.66±0.13^a
Probabilities	0.00<	0.00<	0.00<	0.00<	0.00<	0.00<	0.00<

RCBC: herbal oils blend (equal amounts ofrosemary, clove and black cumin oils), MDA: malondialdehyde, GSH: reduced glutathione, CAT: catalase, SOD: superoxide dismutase, GPx: glutathione peroxidase, GR: glutathione reductase, GST: glutathione S transferase; Means in the same column with different superscript letter following them are significantly different (P <0.05)

Furthermore, Torki et al. (2018) discovered that the oil of rosemary lowered cholesterol and triglycerides in laying quails and had hypolipidemic effects. The main active ingredient of clove oil is eugenol, which has pharmacological properties such as anti-diabetic, and hypolipidemic properties (Khalil et al. 2017). The anti-hyperlipidemic function of eugenol was demonstrated by reduced LDL, total cholesterol, and triacylglycerol concentrations when compared to lovastatin, a lipid-lowering medicine (Venkadeswaran et al. 2014). In a hyperlipidemic zebrafish model, clove oil also lowered serum cholesterol and triacylglycerol (Jin and Cho 2011). Clove oil's hypolipidemic effect may be owing to its capacity to counteract or quench free radicals produced within the quail's body.

RCBC supplementation had a one-of-a-kind antioxidant action (Table 7). In quails, the levels of liver GSH and the activity of GPx, SOD, catalase, GR, and GST all increased (P>0.001) (Table 7). The MDA in hepatic homogenate receiving RCBC diets, on the other hand, was substantially lower than the control. These findings agree with those of Cetin et al. (2017) who discovered that the oil of rosemary improved markers of oxidative stress in quails and hens, respectively. Bulbul (2012) also found that supplementing rosemary and oregano oils together has anti-oxidant effects in quails.

The antioxidant effect of clove oils may be attributed to eugenol, which has an allyl group in its structure and creates an iron-oxygen chelate complex (Ito et al. 2005). Clove oil helps stop hydroxyl radicals from forming, which are subsequent results of lipid peroxidation (Ito et al., 2005). According to Halc et al. (2012), the oil of black cumin improves the antioxidant system and reduces MDA, DNA, and protein damage, all of which help the defense of birds against oxidative damage and affect general health and overall performance.

Conclusions

The current findings demonstrated that feeding developing Japanese quail with a diet supplemented by RCBC boosted the amount of feed intake, BWG, and carcass features. Furthermore, adding 1.5 and 3.0 Cm^3 RCBC/kg to the diet enhanced liver function by lowering serum creatinine, total cholesterol, LDL, triacylglycerol, and FFA while increasing HDL concentration. RCBC supplementation had anti-oxidant benefits, raising GSH levels and SOD, GR, GPx, and GST activities while decreasing MDA concentration. RCBC supplementation at a dose of up to 3.0 Cm3/ kg diet is indicated to promote the growth performances and health of quails during growth.

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Conflict of interest

None

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Gastroprotective activity of yogurt fortified with purple roselle extract in rats exposed with 2,3,7,8-TCDD

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ABSTRACT

Persistent organic pollutant 2,3,7,8-Tetrachlorodiobenzo-p-dioxin (TCDD), known as harmful congeners of dioxin, has many adverse effects on human or animal health. This TCDD enters the body through inhalation, ingestion, and skin contact. Purple Roselle is a well-known herb-medicinal plant having antioxidant properties. This study aimed to evaluate the antioxidant capacity of purple roselle water extract along yogurt against dioxin exposure. The antioxidant properties of the extract were measured by malondialdehyde (MDA) levels and gastrointestinal histology. For this, 25 white male rats (*Rattus norvegicus*) were used, and these rats were divided into five groups negative control, positive control (TCDD 200 ng/kg BW), and three treatment groups (TCDD 200 ng/kg BW + yogurt fortified with purple roselle water extract concentrations 0.5, 1, and 1.5 percent), all the treatments were given orally. Gastric MDA was determined quantitatively using the Thiobarbituric Acid (TBA) method and the one-way ANOVA test, continued by a Tukey post hoc test with a confidence level of 95% while the gastric histology was observed descriptively. Results of the study revealed that supplementation of fortified yogurt with 1% purple roselle extract could dramatically reduce MDA levels ($p<0,05$) and heal histological damage in the lamina propria mucosa of stomach rats subjected to TCDD. Results of the study can be concluded that consuming yogurt with purple roselle extract can reduce MDA levels and repair histological damage to the gastric mucosa caused by dioxin exposure.

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1 Introduction

The rapidly growing population, industrial developments, and domestic production activities increased the pollution of soil, water, and air (Fatmawati et al., 2020). Dioxin or dioxin-like compounds are a group of toxic substances that are persistent organic pollutants (POPs) in the environment persists. These compounds are generated by the combustion of compounds containing chlorine and carbon elements. World Health Organization (WHO) reported one of the most harmful dioxin congeners is TCDD (Schecter et al. 2019).

TCDD is the most dangerous pollutant and more than 90% of TCDD exposure to humans is through food, including fish, meat, milk, eggs, and other processed goods (Fatmawati et al. 2020). TCDD is very stable and lipophilic and has low metabolism and excretion, and it is accumulating in the body. Exposure to TCDD can cause adverse health effects, including skin damage, endocrine disorders, reproductive disorders, neurotoxicity, and disruption of biochemical processes in the body due to oxidative stress (Schecter et al., 2019). Consumption of antioxidants is one way to prevent oxidative stress due to exposure to harmful chemicals.

Yogurt has very high nutritional content and health benefits and because of this, it is a well-consumed food throughout the world. Lactic acid bacteria of yogurt release active peptides from precursor proteins during milk fermentation, acting as a scavenger and preventing the generation of free radicals (Mohanty et al. 2016). Fortification of Yogurt with natural antioxidant ingredients from plants increased the antioxidant activity of yogurt. Roselle (*Hibiscus sabdariffa* L) is a natural antioxidant having anthocyanins, vitamin C, flavonoids, and phenolic acids (Izquierdo-Vega et al., 2020). Roselle extract can be used for fortifying yogurt and can be improved the quality of yogurt as a therapeutic nutritional ingredient. The purpose of this study was to evaluate the effect of purple roselle-fortified yogurt in improving the damage of digestive tissue caused by TCDD exposure in white male rats (*Rattus norvegicus*) by observing the gastric MDA levels and gastric histopathology.

2 Materials & Methods

2.1 Materials

The materials used in this study are fresh milk (obtained from local dairy farms in Batu, Malang, Indonesia), Yogurt starter Yógourmet (LYO-SAN. INC 500 Aéropare, C.P. 598, Lachute, QC. Canada, J8H 4G4 containing the bacteria *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Lactobacillus acidophilus*), Dioxin compound of 2,3,7,8-TCDD with >99% purity (obtained from Supelco, Cat No: 48599), dehydrated flower petals of purple roselle, corn oil, formaldehyde, ethanol, hematoxylin, and eosin dye, and paraffin.

2.2 Purple Roselle Extract Preparation

The aqueous extract of purple roselle was prepared as per the method given by Suharto et al. (2016). For this, 20 g of roselle powder was mixed with 100 ml of water and this mixture was pasteurized at a temperature of 63-65^{o}C for 30 minutes. Pulverized purple roselle was filtered through a 60-mesh strainer. After 30 minutes, the liquid and the sediment were separated.

2.3 Yogurt Making

The procedure for making a liquid starter (mother culture) was based on Padaga et al. (2015). For this, 100 ml of fresh milk was pasteurized in a 250 ml Erlenmeyer tube at 72^{o}C for 15 minutes. This was followed by the cooling of milk (45^{o}C) and mixing of 0.5 g of starter. The prepared mixture was incubated at 45^{o}C for approximately 4 hours to form a yogurt starter. The procedure for making yogurt from a yogurt starter is as proposed by Padaga et al. (2015).

2.4 Making Purple Roselle Extract Fortified Yogurt

The procedure of yogurt fortification by purple roselle extract was as per Suharto et al. (2016). A total of 100 ml of yogurt was homogenized using a blender and the purple roselle extract was added in a predefined concentration of 0.5%, 1%, and 1.5%. After that, it was homogenized again using a blender. Furthermore, fortified yogurt with purple roselle extract was stored in the refrigerator until used.

2.5 Animals Experimental

The twenty-five white male rats (*Rattus norvegicus*) having about 150-200 grams weight and 6-8 weeks of age were obtained from the integrated research and testing laboratory (LPPT) UGM, Yogyakarta, Indonesia. These experimental animals were acclimatized at 25-26^{o}C temperature and 12-hour dark-light cycle under laboratory conditions for seven days. During the period rats were provided with standard commercial feed and drinking ad-libitum. The institutional ethics committee has approved the study under document number 1123.KEP-UB.

Acclimatized twenty-five male white rats were divided into five groups and each group consists of 5 individuals. The formulated five groups are (A) negative control group (given drinking water with a gastric probe), (B) positive control group (experimental animals were exposed to 200 ng/kg BW TCDD only), (C) treatment group 1 (animals exposed with 200 ng/kg BW TCDD and treated with 0.5% purple roselle extract fortified yogurt), (D) treatment group 2 (animals exposed with 200 ng/kg BW TCDD

and treated with 1.0% purple roselle extract fortified yogurt) and (E) treatment group 3 (animals exposed with 200 ng/kg BW TCDD and treated with 1.5% purple roselle extract fortified yogurt). For dioxin exposure, TCDD was dissolved in corn oil and orally given to the experimental rats. The dose of TCDD was determined according to Xu et al. (2008). The treatment group (C, D, E) was given fortified yogurt with purple roselle extract 2 hours after TCDD exposure. Rats were given 1 ml purple roselle extract fortified yogurt at each concentration (0.5%; 1.0%; 1.5%) using a gastric probe orally every day for 12 days. On the day 13th, euthanasia was performed for gastric organ harvesting. For histopathological observation, the gastric organs were put into a 10% formaldehyde solution, and for the examination of MDA levels, the gastric organs were stored at -80 ^{o}C.

2.6 Gastric MDA Level Assay

Gastric malondialdehyde (MDA) levels analyses were carried out by using the thiobarbituric acid (TBA) method. The gastric organs were cut into small pieces of up to 0.1 g, crushed, and added 0.9% physiological NaCl. The homogenate mixture was conveyed to a microtube tube and centrifuged at 1000 rpm for 10 minutes. The supernatant was used for measuring the absorbance at 532 nm with a spectrophotometer and plotted on a standard curve to calculate the sample concentration.

2.7 Gastric Histopathology

Gastric histopathological samples were selected for the pylorus sections and were sequentially prepared by fixation, dehydration, infiltration, embedding, cutting, sticking to object glass, and HE staining. Collected organs were preserved in a 10% formaldehyde solution. This was followed by the dehydration process by immersing the organs in an ethanol solution with a graded concentration for a 1-hour duration for each. Then the clearing was carried out with xylol (I, II) for a 30-60 minutes time period for each. After this, the infiltration process with xylol paraffin (I, II) was carried out for 30 minutes each at 54-56^{o}C. Prepared organs were embedded in paraffin wax and cooled at room temperature. After that, the paraffin block was sliced to a thickness of 3 m, laid on an object glass, dripped with Canada balsam, and finally overlaid with a cover glass. The object glass was then placed in an incubator for one night at 37 ^{o}C. The samples were then stained with hematoxylin and eosin (HE).

Gastric histopathological observations were taken by using Olympus BX 51 light microscope at 40x and 100x magnification. The images were taken using an Olympus XC10 camera. Gastric mucosal erosions and inflammatory cell infiltration were observed in histopathological observations.

2.8 Data analysis

Gastric MDA levels between different groups were determined statistically using one-way ANOVA with a 95% confidence level continued by Tukey's post hoc test. Gastric histopathology was interpreted descriptively.

3 Results

3.1 Gastric malondialdehyde (MDA) levels

Gastric MDA levels of different groups are presented in table 1. Analyzing MDA levels using the TBA method revealed that exposure to TCDD caused an indicative increment ($p<0.05$) in the positive control group (B) and this effect can be reversed by the treatment of natural antioxidants like fortified yogurt with purple roselle extract.

3.2 Gastric Histopathology

Histopathological examination of all groups' stomach pyloric parts is carried out (Figure 1). Based on observations on the gastric histopathology, rats in Group B which were administered TCDD at a dose of 200ng/kg BW for 12 days, show impairments in the mucosal layer, which is characterized by the erosion of the gastric mucosa and infiltration of inflammatory cells. This effect was fewer in the various treatment groups. Further, the slide prepared from treatment group 1 (c) have shown a reduction in mucosa erosion while in the slide of treatment group 2 (D) simplex columnar epithelial mucosa appeared and it did not show any

Table 1 Effect of yogurt roselle combination of the MDA level of TCDD exposed Rat's gastric

Groups	MDA level (ng/mL)
Negative control (A)	207.75±25.85^{b}
Positive control (B)	250.00±6.82^{c}
Treatment 1 (C)	231.05±10.07^{bc}
Treatment 2 (D)	163.00±35.35^{a}
Treatment 3 (E)	239.55±13.27^{bc}

Data are mean of five replicates; ± Standard Error of mean; Values without common letters differ significantly at LSD $P<0.05$; Positive control (TCDD exposed rats), Treatment 1 (TCDD + yogurt roselle 0.5%), Treatment 2 (TCDD + yogurt roselle 1%), Treatment 3 (TCDD + yogurt roselle 1.5%)

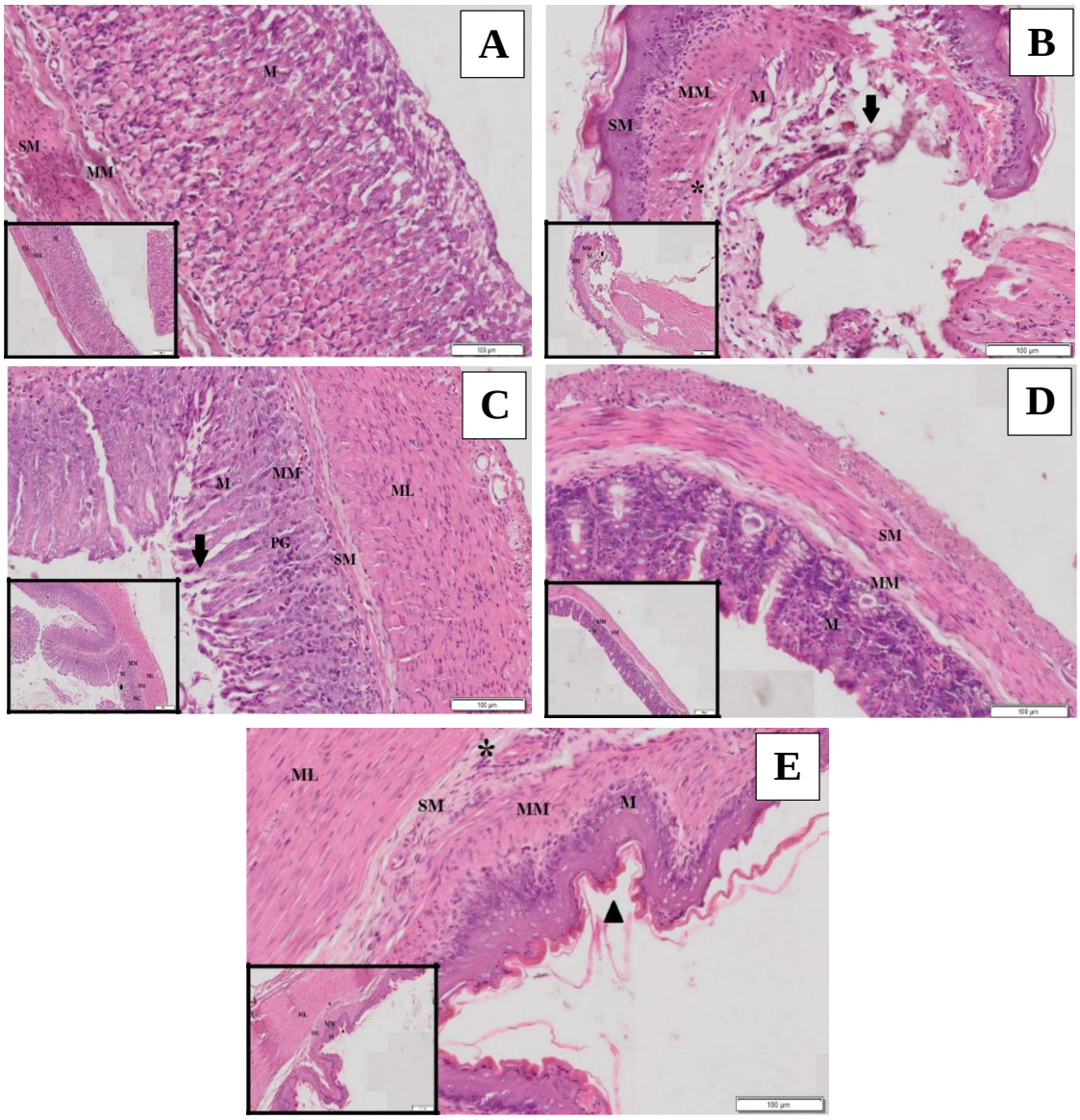

Figure 1 Gastric histopathology of the pylorus of Rat (*Rattus norvegicus*): negative control (A), positive control (B), Treatment 1 (C), Treatment 2 (D), and Treatment 3 (E). HE staining; magnification 400x (inset:100x).M (Mucosa); MM (Muskularis Mucosa); SM (Submucosa)); ML (*Muscle Layer);*PG (*Pyloric Glands*); *(inflamation cell infiltration); ↓(gastric mucosa erotsion); ▲(mucosal desquamation)

gastric mucosal erosion and it delivers results close to the stomach of group A. In the case of group 3 (E), the epithelial mucosa was not intact and showed epithelial desquamation and slight inflammatory cell infiltration.

4 Discussion and Conclusions

Administration of 200 ng/kg BW TCDD for 12 days can cause damage to gastric cells. Exposure to TCDD can cause oxidative stress that started from the entry of TCDD into the body. First of all, TCDD penetrates the cell membrane, binds to Aryl hydrocarbon receptor (AhR) located in the cytosol, and then moves to the cell nucleus. Here in the cell nucleus, the Ah receptor binds with TCDD to form a dimer with Aryl hydrocarbon receptor nuclear translocator (ARNT) protein. Then, the complex AhR-TCDD-ARNT bind with the Dioxin Response Element (DRE), and this binding will increase the expression of cytochrome P450, especially CYP1A1 (Vijaya et al. 2014). Cytochrome P450 was involved in the chain-forming free radicals in the body and the accumulation of a large number of free radicals in the body can trigger oxidative stress. Further, oxidative stress has an impact on damaging several critical cellular components such as fats, proteins, and DNA. Damage due to exposure to free radicals in fats triggers the cell membranes' lipid peroxidation with MDA

metabolite products (Mahdi et al. 2019). Further, induction of TCDD increases the concentration of COX-2 cyclooxygenase enzyme, which affects the synthesis of prostaglandins and increases the inflammatory process in tissues (Mahmoud 2020). Exposure to TCDD also causes impairment in the histopathological appearance of the gastric mucosa. According to Mahmoud (2020), TCDD also damaged the fundus of the stomach in various ways, including irregular fundic glands, desquamation epithelium, inflammatory cells, and dilatation of blood vessels. In addition, hyperplasia and changes in the gastric mucosa, and hyperplasia of enteroendocrine cells were also reported after exposure to TCDD. Similarly, Amer et al. (2013) reported that the toxic effects of TCDD cause glandular degeneration, apoptosis, and gastric ulcers.

Various treatment groups (C-E) have shown recovery from the hepatotoxicity caused by TCDD. Exposure to toxic substances may cause reverse diffusion of H+ from the lumen to the mucosa in the stomach. This mechanism causes mucosal damage followed by pepsin released in large quantities for Na+ ions, and plasma proteins enter the lumen. After that, the body will release histamine and increases gastric acid and capillary permeability. The muscular mucosal tonus will increase, and mucosal erosion occurs.

Based on histopathological observations of the treatment groups 1, 2, and 3 gastric, it was reported that histopathological damage can be prevented by giving yogurt fortified with purple roselle extract. Lactic acid bacteria in yogurt have activity as anti-allergic, anti-inflammatory, anti-cancer, and gastroprotective activities (Gomi et al. 2013). Proteolytic enzymes from LAB can enhance the release of active peptides from precursor proteins in milk. According to Mohanty et al. (2016), biopeptides present in yogurt function as antioxidants and help in free radical scavenging. Further, Rodríguez et al. (2009) also suggested that the content of lactalbumin in dairy products acts as a gastroprotective, and stimulates mucin synthesis and secretion of mucus-producing cells to protect the stomach from damage. In addition, anthocyanins present in purple roselle also have antioxidant effects, and free radical scavenging ability (Khoo et al. 2017).

The daily dose of yogurt-fortified purple roselle extract showed a gastroprotective effect on gastric MDA levels in TCDD-exposed rats and this effect was reported highest in treatment group 2. According to Mohanty et al. (2016), bioactive peptides present in yogurt reduce MDA levels by inhibiting lipid peroxidation and free radical-scavenging activity. In addition, anthocyanins contained in purple roselle can also prevent oxidative stress by stabilizing unpaired free radical electrons through hydrogen atom donors (H) (Apáez-Barrios et al. 2018).

Yogurt fortified with purple roselle extract treatment showed a gastroprotective effect against the toxic effects of TCDD exposure. The synergistic antioxidant activity of yogurt and purple roselle extract can protect the gastric mucosa from damage caused by cell oxidation. In this study, the concentration of fortified purple roselle extracts 1% in yogurt best prevented gastric histopathological damage in rats (Rattus norvegicus) exposed to TCDD. According to the findings, yogurt supplemented with purple roselle extract can reduce stomach MDA levels and gastric histological damage caused by TCDD.

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