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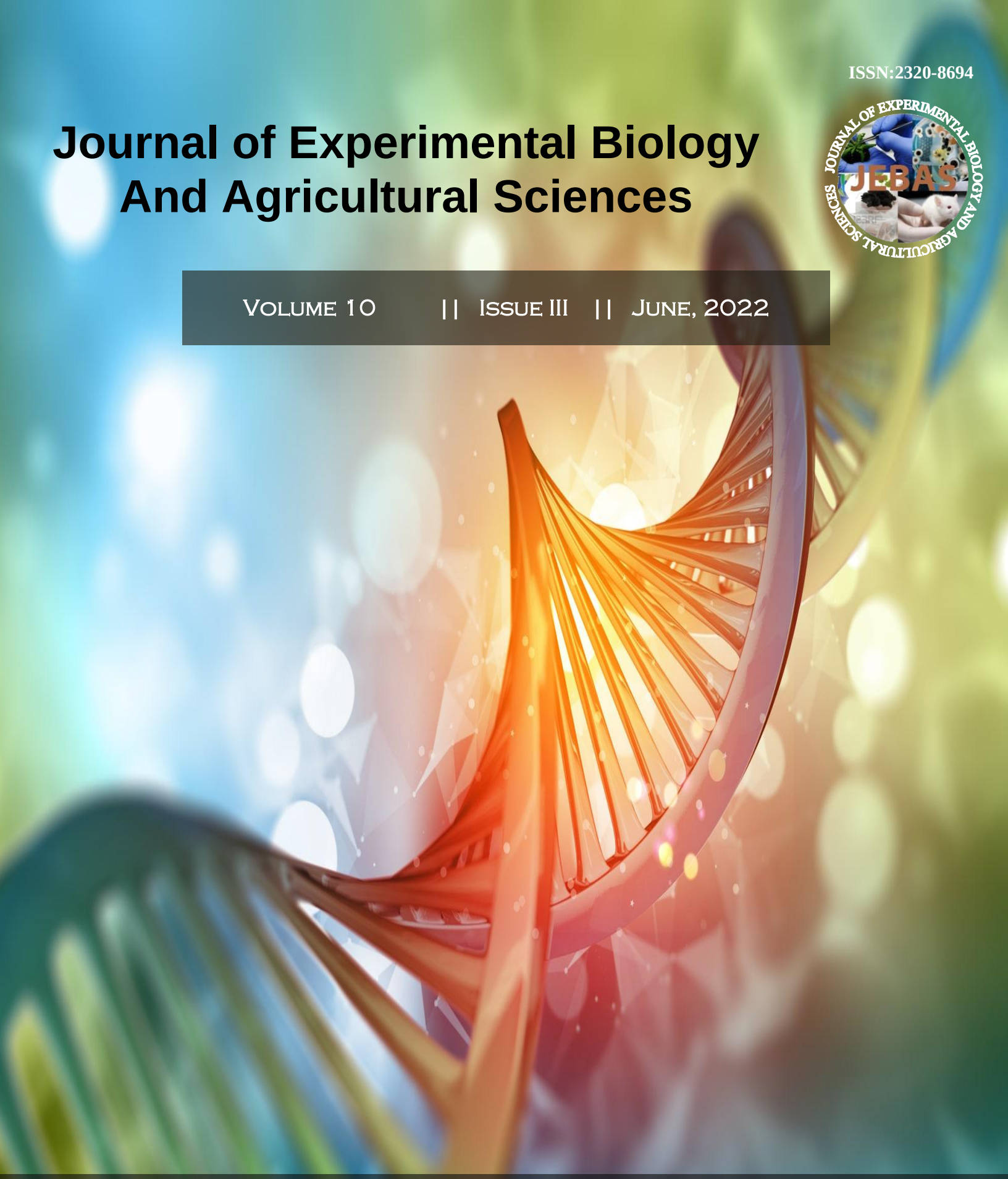

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Dr. Jehangeer Rehman Quereishi
Associate Professor - Botany
Govt. Bangure College, Deedwana.
Email: quereishi.jrq660@gmail.com

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Plastic Waste in India: overview, impact, and measures to mitigate: Review

Sugata Datta[1,3], Abhishek Chauhan[2*], Anuj Ranjan[2,6], Hardeep Singh Tuli[4], Kuldeep Dhama[5], Abul Hasan Sardar[3], Tanu Jindal[2]

[1]Amity Institute of Environmental Sciences, Amity University, Noida 201303, India
[2]Amity Institute of Environmental Toxicology, Safety and Management, Amity University Noida, UP, India
[3]Sarsuna College, University of Calcutta, West Bengal 700073, India
[4]Department of Biotechnology, Maharishi Markandeshwar (Deemed to be University), Mullana, Ambala 133207, India
[5]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Bareilly 243122, India
[6]Academy of Biology and Biotechnology, Southern Federal University, Rostov-on-Don 344006, Russia

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ABSTRACT

India is one of the world's large and fastest-growing economies. With the expanding development, the usage of plastic for anthropogenic activities has expanded many folds and India alone generated around 3.3 million metric tonnes of plastic in the financial year 2019. 79 percent of the plastic generated worldwide enters our land, water, and environment as waste; part of it also enters our bodies through the food chain. The industry in India states that 60 percent of what is generated is recycled and we had assumed that we had solved the problem of plastic waste by recycling, or burying it in landfills. But we were incorrect. Plastic garbage is omnipresent today. It is filling up our oceans and harming marine life and affecting all organisms in the food chain. With the development of economic growth of the country per capita consumption of plastic will only increase in the coming years and we will end up generating more plastic waste The review paper aimed to examine the major impact of plastic waste in India and how to reduce plastic consumption, considering measures such as phasing out or banning multilayered plastics that cannot be recycled, contemplating renewable raw materials, promoting the use of bioplastics, incentivizing the recycling business, and making the rules and guidelines for Extended Producer Responsibility (EPR) simple and enforceable.

* Corresponding author
E-mail: akchauhan@amity.edu (Abhishek Chauhan)

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1 Introduction

As per the annual report of the Central Pollution Control Board (CPCB) for the year 2018-19, India produced over 3.3 million metric tonnes of plastic, an increase of over more than 1 million metric tonnes compared to 2017-18; the major generation came from Maharashtra (12 percent), Tamil Nadu (12 percent), Gujarat (11 percent), West Bengal (9 percent), Uttar Pradesh (8 percent), Karnataka (8 percent) and Delhi (7 percent). The 4,773 registered plastic recycling units, including 7 biodegradable facilities, and 1,084 unregistered plastic recycling enterprises, were employed to manage such a massive amount of plastic rubbish (CPCB 2019). 60 percent of all manufactured plastic is recycled. The remaining 40 percent of plastic becomes waste if it is not cleaned and segregated, and it is either landfilled or pollutes streams or groundwater resources. India, a rapidly rising non-industrial country with a population of 137 billion people, ranks twelfth in the world economy in terms of GDP (Gross Domestic Product). Furthermore, it is ranked third in the world in terms of PPP (purchase power product) (US EPA 2015). Industrialization and urbanization are exploding, resulting in the massive production of plastic waste products (Chauhan et al. 2021). Due to its lightweight, water-resistant, and flexible characteristics (Millet et al., 2018), plastic is now a common component of human life for carrying various types of items. These factors have piqued the public's interest in using plastics. However, people often overlook the fact that it is more toxic and harmful to both humans and the environment because it is non-biodegradable (Phanisankar et al. 2020). Plastics have grown at a rate unparalleled by any other material used in packaging (Schwarz et al. 2019) or building (Mansour and Ali 2015), these are the two crucial areas where plastics are frequently used. The expected increase in future plastic use will be accompanied by an increase in post-consumer plastic trash (Lebreton and Andrady 2019).

Plastics are classified as thermoplastics or thermosetting plastics based on how their physical and chemical properties change before and after heat treatment (Schimpf et al. 2017; Trotta et al. 2019). Thermoplastics are heat-susceptible plastics such as polyethylene (PE), polyvinyl chloride (PVC), polypropylene (PP), polystyrene (PS), polycarbonate (PC), and polytetrafluoroethylene (PTFE) (Dhanumalayan and Joshi 2018; Koomson et al. 2018; Xu et al. 2019; Huang et al. 2019) can be softened or melted into any shape under heating conditions and solidified when cooled, which can be repeatedly deformed with typical plasticity (Ning et al. 2015). Thermosetting plastics such as epoxy resin, phenolic resin, urea-formaldehyde resin, and organo silicon resin do not undergo plastic deformation when heated; instead, they would decompose when the temperature continues to rise between 250-300 ºC (Chen et al. 2019). Thermoplastics account for up to 80 percent of all plastics produced worldwide, while thermosets account for the remaining 20 percent. Thermoplastics are easily recyclable and pose no risk, whereas thermoset plastics such as epoxy resin and polyurethanes are not (Phanisankar et al. 2020). Clear polyethylene terephthalate (PET) bottles and natural high-density polyethylene (HDPE) bottles can be recycled successfully (Velzen et al. 2020), while PVC, PS, and PP are not routinely recycled from post-consumer waste (Joseph et al. 2021). Approximately 50 percent of plastics are used for single-use disposable applications, such as packaging, agricultural films, and disposable consumer items (Fasake et al. 2021), and between 20 and 25 percent for long-term infrastructures, such as pipes, cable coatings, and structural materials, and the remainder for durable consumer applications with intermediate lifespan, such as electronic goods, furniture, vehicles, and so on, and are made of PVC, PS, or PP, which have low recycling rates (Di et al. 2021) and thus are not suitable for reuse; thus contributing significantly to plastic waste (Hopewell et al. 2009).

2 Environmental impact of plastic waste in India

India is a major producer and consumer of numerous types of plastic. However, the waste management situation in India is abysmal, with minimal source segregation, limited recovery, and a large proportion of garbage ending up in dump yards and random littering. India's plastic waste processing capability is only 15 percent of the total waste generated, and because it is a land-scarce country with a high population density, dump yards, and landfill sites are overburdened (Dhanshyam and Srivastava 2021). Even in the most developed metropolitan areas or super urban areas, trash collection, separation, and treatment facilities are inadequate and ineffective (Dharwal et al. 2022). Plastic debris thrown ashore primarily enters civic seepage lines, suffocating them and contributing to floods in major Indian cities. (Mayur et al. 2021). Plastic packets, diapers, tires, and water bottles including building debris have all been blamed for the flooding of several towns in metropolitan India (Pathak and Nichter 2021). Plastic garbage, according to environmental experts, not only clogs drainage lines but also poses a greater risk because it is non-biodegradable and eventually converts to microplastic (tiny microscopic particles less than 5 mm), which clogs pipelines, becomes poisonous, and harms soil layers (Karthik et al. 2018; Vaid et al. 2021). Furthermore, a large number of vertebrates, birds, reptiles, and fish have been identified as being at risk of injury or death as a result of the damage caused by plastics (microplastics). Plastics or microplastics generally have an impact on marine life by trapping or causing them to ingest (Vaid et al. 2021). As a result of these factors, every one of the world's seven turtle species is in jeopardy or under threat as a result of plastic pollution (Chauhan et al. 2021). The toxicity of harmful chemicals absorbed from adjacent water bodies, as well as microplastic contamination, is a growing concern (Yuan et al. 2019). Following ingestion, microplastics may act as vectors for dangerous compounds to

spread to other living things, such as invertebrates, plants, and animals, resulting in physio-chemical impacts and a risk to human health (Wang et al. 2018; Vethaak and Legler 2021). The first report of microplastics in Vembanad Lake in Kerala, India was reported by Sruthy and Ramasamy (2017).

The inability of waste plastic to biodegrade in the atmosphere has created various challenges for both urban and rural India (Adeyanju et al. 2021). Drain clogging, water stagnation, and harmful gas leakage during open incineration are all common concerns linked with waste plastic generation (Gangwar and Tiwari 2021). As plastic is resilient, non-reactive, and mainly non-biodegradable, it stays in landfills for prolonged periods, posing a risk because harmful chemicals are drained out and contaminate underground water bodies (Singh et al. 2008; Devi et al. 2019). Overall, solid waste from most cities is collected and stored in landfills, where it attracts flying creatures, rodents, and bugs, resulting in unsanitary conditions (Alam and Ahmade 2013). The contamination of solid waste results in the release of CO_2, CH_4, and other gases. Furthermore, open dumpsites affect the quality of drinking water and contribute to illnesses such as cholera (Chaturvedi and Singh 2021).

Despite their low cost, plastics are highly engineered materials with exact physical qualities (LaPensee et al. 2010). Rotation, injection, extrusion, compression, blowing, and thermoforming can all be used to mold them into practically any shape. Their material properties are changed before and after synthesis to achieve the appropriate strength, permeability, porosity, opacity, and color (Ali et al. 2020). Plastic shoes (primarily flip-flops) are one such commodity, accounting for up to 6 tonnes of the total waste collected and removed from Aldabra Atoll in Seychelles [in the Southwest Indian Ocean, home to the last remaining population of the Indian Ocean giant tortoises (*Aldabrachelys gigantea*) and the largest nesting sites for endangered green turtles (*Chelonia mydas*) in the western Indian Ocean]. Flip-flops are the most popular shoe among Indians, and they're largely made of plastic foam, and they're stacking up on beaches and in the oceans (Burt et al. 2020). They take decades or centuries to decompose naturally. In 2020, a cleanup initiative resulted in the recovery of almost 60,000 individual flip-flops from a beach in Mumbai, India. (Pathak 2020).

India is confronting a significant waste management crisis as a result of rising urbanization (Srivastav and Kumar 2021). Over 377 million people live in 7,935 cities, producing 62 million tonnes of municipal solid trash each year. Only 43 million tonnes of garbage are collected, 11.9 million tonnes are treated, and 31 million tonnes are thrown in landfills. Solid waste management (SWM) is one of the most important services given by municipal society in the country to maintain metropolitan areas clean. Almost all municipal authorities, on the other hand, indiscriminately discharge solid trash at a dump yard within or outside cities (Kumar et al. 2021). According to experts, India's trash disposal and management system is flawed. Even the most developed metropolitan areas and super urban areas in India lack efficient and effective trash collection, separation, and treatment facilities (Chauhan et al. 2021). The key to effective waste management is to make sure proper waste segregation at the source (Rakib et al. 2021) and that the garbage passes through various recycling and resource recovery channels. Unsegregated municipal solid trash has become a difficult problem for India, as well as other emerging countries (Shukla et al. 2021). The MSWM (municipal solid waste management) system has several issues with waste treatment options such as composting, recycling, and energy generation (Joshi and Ahmed 2016). If the existing MSWM system does not offer the solution to these problems, the entire municipal mixed waste will end up at dumpsites and therefore causing the MSWM system to be dependent upon landfill sites (Ahluwalia and Patel 2018). A huge amount of dumped municipal solid waste (MSW) is becoming the main reason for groundwater pollution, soil contamination, and environmental pollution (Kanhai et al. 2021). The MSW typically includes domestic and commercial wastes generated in municipalities or notified areas either in solid or semi-solid form (Nanda and Berruti 2020). Metro cities are the major contributor to the process of waste generation and due to continuous infrastructure development the production of waste; the amount of waste is also higher than in other regions. About 80 percent of the total generated waste is being collected by various means while the rest 20 percent is again mixed up and lost in the urban environment. About half of the total trash generated is sorted at the source and is amenable to further processing. As a result, approximately 40 percent of the total generated trash is treated in the existing MSWM system, with the remainder deposited into landfill sites (Shukla et al. 2021). The majority of the time, garbage sorting is done by the unorganized sector (Kumar and Agrawal 2020). The segregation and sorting process is carried out in extremely hazardous and unsafe conditions, and the effectiveness of segregation is logically low because the unorganized sector segregates only the most important disposed of constituents from the waste stream, which can offer them a higher monetary return in the reusing market (Agrawal and Anupama 2020; Shukla et al. 2021). Due to a lack of space for inventory, many waste processing industries are utilizing the waste from the dumpsite, which essentially contains mixed waste. This leads to an increase in the cost of waste processing and poor quality of recyclable materials and compost (Rawat et al. 2013).

During the COVID-19 pandemic, a variety of plastic-based personal protective equipment (PPE) played a critical role in keeping people safe (Lockhart et al. 2020). Since the coronavirus pandemic began, there has been an unusual surge of single-use plastics, such as

gloves, protective medical suits, masks, hand sanitizer bottles, takeaway plastics, food, and polymer products packages, and medical test kits. The management of waste generated by single-use plastics is a concerning side effect of the COVID-19 pandemic, which has wreaked havoc on global healthcare systems and damaged national economies (Das et al. 2021; Leal-Filho et al. 2021; Patrício Silva et al. 2021). Since the SARS-CoV-2 outbreak, there has been a significant increase in the number of discarded single-use surgical and face masks, as well as latex gloves, found littering the streets, roads, medical institutions, and parking lots, dumpsites, beaches, gutters, and shopping carts. The COVID-19 pandemic has exacerbated the plastic pollution problem by reviving consumer demand for single-use products and materials for health and safety concerns (Benson et al. 2021; Vanapalli et al. 2021; Parashar and Hait 2021; Nghiem et al. 2021). Inadequate management of bio-medical waste (BMW) leads to many issues, including the spread of infectious diseases and various forms of environmental pollution (Rai et al. 2020). It has been determined that 10–25 percent of BMW is hazardous, and research suggests that the COVID-19 virus is highly contagious and can survive for several days on plastic surfaces. As a result, it has created many challenges for current BMW legislation and management practices around the world (Goswami et al. 2021). During the COVID-19 outbreak in Wuhan, China, healthcare waste generation increased by 600 percent (Lan et al. 2022). To prevent the virus from spreading, recycling restrictions, increased production, and incorrect treatment have created an unsettling situation. Several studies have cited the handling of BMW during this pandemic as a major source of concern since it can enhance the risk of further spread of the corona virus (Goswami et al. 2021).

3 Mitigation Strategies

Plastic is ubiquitous, it's visibly the backbone of globalization. However, like in any other country, plastic waste management is a pressing issue in India, especially with the unceasing growth of consumerism throughout the nation. Only 60 percent of the total plastic generated is recycled, the majority of which escapes into the environment. Eliminating the total of it is quite difficult; however, the majority of the nations are trying to encounter this threat with reasonable and pragmatic solutions (Santhosh and Shrivastav 2019). At this juncture, India needs robust and stringent waste management measures (Figure 1) to substantially improve the situation and for an effective nationwide waste management performance, it is imperative for the government, local bodies, and civilians to achieve a common target of zero plastic waste (Plastic Waste Is India's and the World's Most Formidable Environmental Challenge Today, and the COVID-19 Pandemic Has Made Matters Worse: CSE, 2020).

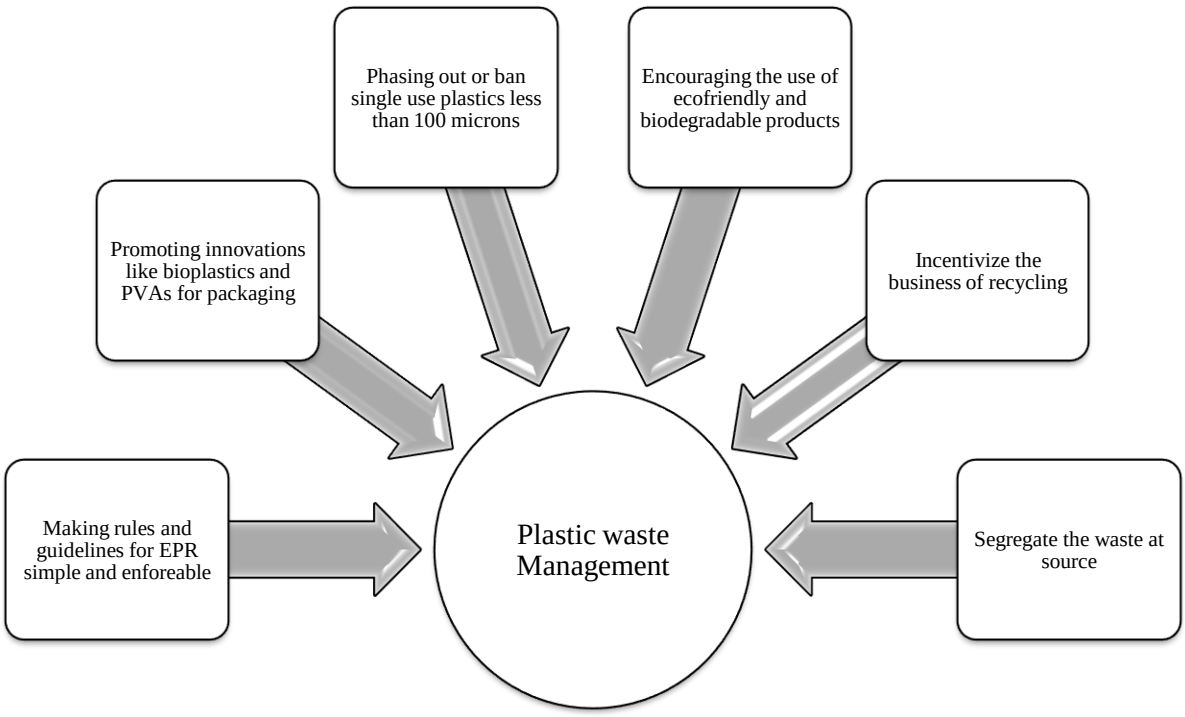

Figure 1 Plastic waste mitigation strategies.

3.1 Reused plastic wastes in construction purposes

In India, the problem of plastic waste is not a recent phenomenon. The entire amount of waste plastic available is enormous. The waste plastic material is either buried or disposed of on land (Jha and Kannan 2021). Plastic waste is combined with municipal solid refuse and spread out across a large area. Both humans and animals have suffered as a result of improper plastic garbage disposal (Bhardwaj et al. 2020). Plastic is harmful and is found in numerous forms (Bahij et al. 2020). The problem of plastic waste can be solved in two ways: first, by substituting other materials for the plastic, and second, by collecting and recycling the plastic garbage (Trimbakwala 2017). A noteworthy technique to exploit or repurpose the plastics is the construction of roads with that plastic waste (Biswas et al. 2020). Plastic is robust and deteriorates extremely slowly. The chemical connections are so strong that they make plastic robust making it resistant to degradation (Trimbakwala 2017; Biswas et al. 2020; Kokare et al. 2021). Road construction from plastic waste isn't new. The director of the Central Road Research Institute (CRRI), in India noted that "bitumen, when blended with rubber or plastic, enhances the life of roads and the quality of roads" although polymers when mixed with bitumen increased the building cost up to six percent but enhanced the longevity of roads manifold (Gunjan et al. 2021). Bitumen is hydrophobic by nature, and plastic is also hydrophobic by nature. When hydrophobic materials are added to bitumen, the strength of the combination rises substantially, and it becomes more water resistant. Plastic carry bags, polyethylene packets, and discarded PET bottles are added to the bitumen mixture at a high temperature to make an aggregate that can be laid like a standard bituminous road (Bhardwaj et al. 2020; Kokare et al. 2021). Mostly shredded plastics are poured over the heated aggregates, thus generating plastic coated aggregates, which are then mixed with hot bitumen to make a plastic coated aggregate bitumen mixture for laying roads (Figure 2). This helps to have better binding of bitumen with the plastic-waste coated aggregate due to higher bonding and greater area of contact between polymer and bitumen, thus preventing moisture absorption and oxidation of bitumen by entrapped air (Yadav et al. 2017).

Since 2001, the plastic man of India, R. Vasudevan, Dean, Thiagarajar College of Engineering, Madurai, and his team at the Centre for Studies on Solid Waste Management (CSSWM), have been researching the possibility of employing plastics in the construction of roadways (Vasudevan 2010). The trend of employing plastics for road building gained momentum in 2015 when the Indian government announced suggestions on plastic use with hot mixes for bitumen roads around urban areas.

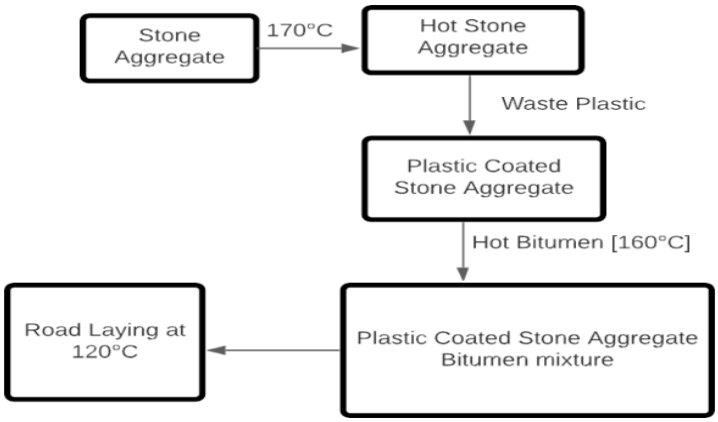

Figure 2 Flow chart for construction of plastic-aggregated coated roads (Yadav et al. 2017).

Figure 3 A road made of waste plastic in Madurai, India (Vasudevan 2010).

Subsequently, India has developed one hundred thousand kilometers of roads in at least 11 states by utilizing waste plastic (Figure 3). The front runners have been the following cities: Chennai, Pune, Surat, and Indore. As of July 2021, 703 kilometers of national highways have been developed using waste plastic in the form of a wearing coat of flexible pavement (Team Prakati 2021). The implementation of such revolutionary technology will not only reinforce road construction but will also increase road life as well as aid to improve the environment by giving a solution to get rid of plastic waste.

3.2 Recycling of PET bottles into textile fibers

PET plastic bottles are considered a major fraction of the plastic waste that is deposited in landfills every year and takes at least 500 years to decompose (Lundell and Thomas 2020). The alarming rate at which the number of used plastic bottles is accumulating in landfills poses considerable damage to the ecosystem. Thus, it seems that recycling PET plastic bottles has a vital function in making the environment friendlier (Jafari 2021). Many companies globally are now coming forward to recycle waste PET bottles to create jobs and to maintain the environmental sustainability. Not just entrepreneurs, but fashion designers are also coming out to join their hands in supporting the environment. With the help of advanced technology, PET bottles are melted, extruded, and spun into polyester yarn, called recycled polyester (rPET). Fabrics can then be woven directly from rPET. This rPET is considered to be eco-friendly, cost-effective, safe and also consumes 30 percent less energy than garments that are made from conventionally manufactured polyester (Mukherjee 2017). With development in recycling technology, plastic PET bottles can now be turned into light, soft and breathable textiles that are not only trendy and fashionable but will help maintain sustainability by minimizing plastic waste generated. Such eco-conscious effort will undoubtedly witness rise in the usage of recycled plastic fibers for daily necessities.

3.3 Biodegradable Leaf plates as an alternative to disposable plastic plates

Plastics such as polyester PE, PP, PET, PS, PC, epoxy resins, polysulfone, PVC, polyvinylidene chloride, and melamine formaldehyde are used to make disposable plates, cups, bowls, tumblers, spoons, bags, covers, sheets, and films (Hanga 2015). During use, these plastics can emit hazardous substances such as bisphenol A, melamine, vinyl chloride, phthalates, and other chemicals into the food they contain (Notardonato et al. 2019). Over the last few decades, an increase in ecological consciousness has encouraged various experts and researchers to work on sustainable and environmentally friendly materials that could partly or substitute synthetic non-biodegradable polymeric materials in all-around applications (Korbelyiova et al. 2021). Currently, substantial interest has been shown in the development of biodegradable products made from non-food materials for usage in various industries (Pandey et al. 2021). The influence of throwaway plastic ware consumption in our day-to-day has led to a quest for alternate renewable resources, i.e., the use of plant leaves as dining plates and food wrappers, a traditional practice in India (Kora 2019). The long-standing tradition has its own cultural, religious, medical, and social relevance in India (Balkrishna et al. 2021). The leaves are one of the non-timber forest products (NTFP) and are harvested from the forests by the tribal people of India. The leaf plates are environment-beneficial, biodegradable, suitable for extended-duration storage, and may be readily thrown off. They are inexpensive and don't require cleaning with phosphate-rich soaps and detergents, a time-consuming and labor-intensive operation. Among the many natural fibers available, areca sheath fibers have enormous potential to be used directly or as a filler material in composites, resulting in the production of bio-degradable, eco-friendly materials (Nayak et al. 2021). The normal procedures involved in leaf plate manufacture include leaf harvesting, leaf drying, hand or machine stitching into plates, and bundling for shipping. Usually, the leaf harvest from neighboring

Figure 4 Compostable and biodegradable machine compressed dining plates made from leaf sheath of Areca palm and Sal leaves (Kora 2019).

forests is done by women and a distant distance by males. But the stitching labor is carried out largely by women at home. The plates are created on a small scale by cottage enterprises. The single-layered hand and sewing machine sewn plates, called Khali, are directly utilized by the consumers for eating purposes or further pressed into thick plates by heat pressing machines, which are procured by the commission agents, dealers, and traders. Among the locally available resources, leaves from sal, areca palm, and palasa are acceptable for commercial leaf plate production (Figure 4) (Kora 2019).

3.4 Bamboo as an alternative to plastic bottles

Molded fiber and its products have garnered considerable attention as a green and sustainable packaging material because of its renewability, recyclability, sustainability, and biodegradability (Didone and Tosello 2019). Molded fiber products (MFPs) are regularly made by some renewable and biodegradable lignocellulosic fibers defined as molded fiber or pulp under a series of processes, such as pulp preparation, forming, pressing, and drying in the mold to form different sorts of three-dimensional fiber products. The MFPs have been commercially used in several packaging markets, like food-related (egg and fruit trays), industrial packing (electronics and car parts), throwaway products (bedpans and urine bottles), and horticulture trays/pots (Didone et al. 2017). This has led to the increase in the popularity of bamboo bottles in various places, in which *Bambusa balcooa Roxb.* (Poaceae: *Bambusoideae*) is utilized (Tewari et al. 2014). *B. balcooa* is a perennial, drought-resistant bamboo species (Kaushal et al. 2021) that is indigenous to north-east India and also known as "poor man's lumber". It is the fastest growing plant and plays a key role in the food and nutritional security of the tribal-dominated northeastern districts of India (Kikon 2021; Behera and Balaji 2021). Eco-friendly bamboo bottles produced from various bamboo varieties (Figure 5) have been found all around the country; this sustainable project has not only restricted the use of plastic but also provided a source of work for many. In 2018, the Indian government revised the National Bamboo Mission to assist the growth of the bamboo sector and help with its marketing (Adil 2021). Lachen, a small hamlet of Sikkim, India, arguably the first to entirely ban plastic drinking water bottles, is proposing bamboo bottles as an alternative. The plastic bottles left behind by tourists spurred the local community to introduce the ban (Senger 2020).

3.5 PVA as an alternative to plastic packaging

Packaging is a vital step in the food industry since it is used to avoid spoilage, improve shelf-life, and offer an appealing presentation of the food product (Soltani-Firouz et al. 2021). Plastic packaging is utilized throughout the world, and its production is expanding year after year. It comes in a range of colors and styles. However, it has generated major environmental difficulties, notably for the ocean, which has become a home for discarded plastic packets (Lombardi et al. 2021; Walker et al. 2021). To solve this issue, biodegradable packaging was designed to replace the use of plastic packaging because it helps to reduce environmental impact and waste management costs (Shaikh et al. 2021). Biodegradable packaging is also known as ecologically friendly packaging since it may be degraded into carbon dioxide, water, inorganic chemicals, and biomass by microbes, algae, fungus, as well as enzyme catalysts. To create biodegradable packaging, biocomposite films like polyvinyl alcohol (PVA) are necessary (Yazik and Tukiran 2021). It is a synthetic water-soluble polymer that is both colorless, odorless, and with a backbone consisting exclusively of carbon atoms and is biodegradable under both aerobic and anaerobic conditions by microorganisms and their PVA degrading enzymes, especially PVA oxidases and hydrolases (Halima 2016). It originally garnered prominence in 1989 (Thyagarajan and Janarthanan 1989) when PVA film was used to package pesticides in unit-dose, water-soluble pouches that protected farmers from accidental chemical exposure (Youssef et al. 2019). This invention then changed the cleaning business, where single-use laundry and dishwasher detergent packs and tablets provided consumers with dramatically better

Figure 5 Eco-friendly and sustainable bamboo bottles found in Indian markets.

convenience, safety, and sustainability (Sultana et al. 2021). PVA is safe and environmentally friendly (Asthana et al. 2021). Beyond the popular laundry and detergent packets, it is used in many households, biomedical (Bialik-Wąs et al. 2021; Kamoun et al. 2021), personal care, and industrial electronic applications (Hashim 2020), including food packaging (Amin et al. 2020), textile yarns (Jhang et al. 2021), paper products (Islam 2020), unit-dose pharmaceuticals (Nemati et al. 2021), water treatment chemicals (Rolsky and Kelkar 2021), "artificial tears" used to treat dry eyes, contact lens lubricants (Yu et al. 2020), transfer printing, agrochemicals, embroidery, and dust abatement (Islam 2020). Food-grade versions of the film are used to deliver pre-measured quantities of grains, pasta, chocolate flavoring, and nutritional supplements (Polyvinyl Alcohol (PVOH, PVA, or PVAl) 2021)

3.6 Role of bioplastics

Bioplastics are polyesters manufactured by a range of microorganisms cultivated under varying nutrient and environmental conditions. These polymers are mainly lipid-based and are collected as storage resources (in the form of mobile, amorphous, liquid granules), facilitating microbial survival in stressful situations (Luengo et al. 2003). Among the many biopolymers, polyhydroxyalkanoates (PHAs) have received interest for industrial-scale manufacturing not only due to their biodegradability and compostable features but also due to their facile conversion to diverse forms, possessing required characteristics as plastic materials (Sirohi et al. 2020). PHA is the only bioplastic that is entirely produced by microorganisms, with more than 30 percent produced by soil-inhabiting bacteria. Many microorganisms in activated sludge (Jayakrishnan et al. 2021), on high seas (Stanley et al. 2021), and in the harsh cold environment are also capable of generating PHA (Prudnikova et al. 2021). In the last 10 years, PHA has been developed fast enough to find applications in numerous fields (Chen 2010). Best researched microorganisms include *Ralstonia, Pseudomonas, Halomonas, Burkholderia, Rhodospirillum,* etc. able to utilize a variety of carbon sources and create different forms of PHA. Even though PHA has various uses and is employed in surgical implants, sutures, artificial blood vessels, tissue engineering, scaffolds, controlled drug administration, etc. (Murab et al. 2021). Despite having the potential to replace conventional plastic (Ganesh Saratale et al. 2021), PHA manufacture is relatively expensive in contrast with petroleum polymers, restricting industrial scale usage. The elevated cost is attributable to the expensive feedstock, synthesis methods, and downstream processes (Bhatia et al. 2021). Consequently, great effort has been directed to minimize the production cost of PHA by enhancing bacterial strain, efficient fermentation, and recovery method (Al-Battashi et al. 2020; Yadav et al. 2020). The stressed environment of salinity off the Gujarat coast, India in which the cyanobacteria *Spirulina subsalsa* thrives nearly overrides the contamination problem and can minimize the sterility requirements of a production facility, thus, decreasing the investment cost. The possible use of these microalgae for PHA generation from sludge, industrial effluent, or other wastewater which has a high salt content can be the way forward (Tharani and Anantha subramanian 2020). The capacity of *Spirulina subsalsa* to flourish in salinity makes them ideal for industrial and bioremediation applications (Shrivastav et al. 2010). The Indian bioplastics industry is improving rapidly and several firms have engaged in the sphere of making bio-based plastic products. In cooperation with Earthsoul, The J & K Agro Industries Development Corporation Ltd. established India's first bioplastics manufacturing facility with an overall production capacity of 960 metric tonnes per year. The key players in bioplastics in India are Truegreen in Ahmedabad, Biotec Bags in Tamil Nadu, Ravi Industries in Maharashtra, and Ecolife in Chennai. Truegreen has an established capability of manufacturing roughly 5,000 tonnes per year of bioplastics products (Rafey and Siddiqui 2021).

4 Plastic recycling targets set by Government of India

As of 2019, India generates approximately 660,787.85 tonnes of plastic garbage per year, 43 percent of which is packaging material, most are single-use plastic, and approximately 60 percent of which is recycled (Wang et al. 2021). The Environment Ministry in India has announced proposed rules that force makers of plastic packaging materials to collect all of their produce by 2024 and ensure that a minimum percentage of it is recycled as well as used in the supply. It has also outlined a system whereby makers and users of plastic packaging can accumulate certifications termed Extended Producer Responsibility (EPR) certificates and trade them in (The Hindu 2021). Only a fraction of plastic that cannot be recycled such as multi-layered will be eligible to be sent for end-of-life disposal, such as road construction, waste to energy, and waste to oil and cement kilns, and here too, only methods prescribed by the CPCB will be permitted for their disposal. Plastic packaging, as per the laws made public on October 6, 2021, falls into three categories i.e. category 1 is "rigid" plastic; category 2 is "flexible plastic" packaging of a single layer or multilayer (more than one layer with different types of plastic), plastic sheets and covers made of plastic, carry bags (including carrying bags made of compostable plastics), plastic sachets or pouches; and category 3 is called "multi-layered plastic packaging", which has at least one layer of plastic and at least one layer of material other than plastic. Producers of plastic will be compelled to submit to the government, via a centralized website, how much plastic they make annually. Companies will be required to collect at least 35 percent of the target in 2021–22, 70 percent by 2022–23, and 100

percent by 2024. In 2024, at least 50 percent of their rigid plastic (category 1) will have to be recycled, as will 30 percent of their category 2 and 3 plastics. Every year, the targets will be raised, and by 2026–27, 80 percent of their category 1 waste and 60 percent of their other two waste categories will have to be recycled. From July 1, 2022, the manufacture of a range of plastic products will be outlawed. These include earbuds with plastic sticks, plastic sticks for balloons, plastic flags, candy sticks, ice-cream sticks, polystyrene for decoration, plates, cups, glasses, cutlery such as forks, spoons, knives, straws, trays, wrapping or packing films around sweet boxes, invitation cards, and cigarette packets, plastic or PVC banners less than 100 microns, and stirrers (Sharma and Mallubhotla 2019; Kapur-Bakshi et al. 2021; The Hindu 2021).

5 Challenges and Future Perspectives

In the recent decade, plastic pollution has emerged as a major global concern in public discourse (Pathak 2020). Packaging currently accounts for the highest consumption of plastics in India, at 24 percent of overall consumption and most of which are single-use plastics. These end up cluttering the environment in part due to reckless individual behavior. Bad waste management systems also play a major role. Governments must recognize the importance of waste management services for a sustainable future. To prevent plastic pollution, measures should be made in line with the waste management hierarchy. The drive to eliminate plastic needs to focus on getting rid of it at the source as the only way to prevent it from getting into the environment (Kim et al. 2022). The adoption of PHA as an alternative to synthetic plastics can be the future way ahead. Not only they are biocompatible and biodegradable, but they can also be made from diverse biowastes such as lignocellulosic biomass, municipal waste, garbage cooking oils, biodiesel industry waste, and syngas. Despite the efforts, PHA manufacturing cost is greater compared to synthetic plastic. In 1998, Bipol a commercially generated PHA was 17 times costlier as compared to the price of synthetic plastic, and with the advancements in science, this price is recently lowered to 5 euro/kg PHA in comparison to 0.8–1.5 euro/kg synthetic plastic. The high cost of PHA is mainly due to the slow development of microorganisms, low conversion of raw material into PHA, high energy requirements in sterilizing and aeration, and expensive downstream processing (Bhatia et al. 2021). Future research activity should be focused on overcoming all these abovementioned challenges.

Despite increasing the efficiency of solid waste management in India, there are large quantities of nonbiodegradable garbage that are left untreated or deposited into landfills. In developing countries like India, as landfill has been the major option for disposing of solid waste (Nanda and Berruti 2020), there would be an ever-increasing landfill in the future, which might put constraints on finding land for further dumping of garbage, unless there is a way to reuse those solid wastes that have significant potential to be utilized again for other purposes. Fortunately, several environmentalists and academics have come up with innovative methods of utilizing these wastes in the construction of buildings (Dadzie et al. 2020) and roads (Vasudevan 2010). Discarded plastic bottles are currently being used for the construction of dwelling units in the form of building blocks, replacing conventional bricks and concrete, decreasing the overall cost of construction considerably, at least 20 to 40 percent as compared to the conventional way of construction (Saji et al. 2019; Obiadi 2020). Roads laid using the plastic waste mix are found to be superior to the regular ones. The binding property of plastic makes the road last longer by supplying increased strength to bear heavier weights. While a standard 'highway quality' road lasts four to five years it is claimed that plastic-bitumen roads can last up to 10 years (Trimbakwala 2017). However, the cost of roads produced out of plastic wastes is slightly higher compared to the usual approach, this should not hinder the adoption of the technology as the benefits are far larger than the cost. Plastic roads would be a benefit for India’s hot and extremely humid climate, where temperatures routinely reach 50°C and torrential rains create chaos, leaving most of the roads with giant potholes (Trimbakwala 2017; Bansal et al. 2017; Mulyono et al. 2021).

SWM has emerged as one of the most enormous development concerns in metropolitan India. Numerous studies demonstrate that the unsafe disposal of trash generates toxic gases and leachates, due to microbial decomposition, climate conditions, refuse characteristics, and land-filling processes (Ray et al. 2021). According to the 12th Schedule of the 74th Constitution Amendment Act of 1992, urban local bodies (ULBs) are responsible for maintaining cities and towns clean. However, most ULBs lack suitable infrastructure and confront several strategic and institutional shortcomings, such as poor institutional capability, financial limits, and a lack of political will (Ghatak 2021). Various legislations have been created for regulating the manner of trash disposal. The Ministry of Environment, Forest and Climate Change (MoEFCC) and the Ministry of Housing and Urban Affairs (MoHUA) have collaboratively carried out policies and programs to address these concerns. However, most of these have failed to fulfill their aims due to a lack of clarity and understanding amongst the stakeholders, and insufficient enforcement by the regulators (Joshi and Ahmed 2016). One crucial feature of efficient SWM is “waste segregation”. It is now necessary for garbage generators to put their rubbish in color-coded bins. This lessens the impact of SWM on ULBs greatly. Segregation can substantially lessen the strain of transportation of trash as well as lower leachate and greenhouse gas (GHG) emissions (Ravichandran and Venkatesan 2021). Currently, the standards for trash segregation and recycling are poorly

administered, and many cities have failed to incorporate door-to-door collection into the informal sector. Further, the restrictions do not address the difficulties generated by the "not-in-my-backyard (NIMBY) syndrome" (Massoud et al. 2021). According to the guidance note on MSWM, compliance with the SWM standards demands that adequate systems and infrastructural facilities be put in place to undertake the scientific collection, management, processing, and disposal of SWM. The 2016 Rules have recommended the formation of a Central Monitoring Committee under the chairmanship of the Secretary of the MoEFCC. This committee will be responsible for overseeing the overall implementation of the 2016 SWM Rules. The COVID-19 pandemic has presented a new set of obstacles in the SWM system in India: preserving social distances at the treatment plants and amongst the collecting workers, and a shortage of PPE gears for conservancy staff (Ganguly and Chakraborty 2021). These concerns weaken the safety of SWM employees, waste treatment regulations, and other operations. There is a lack of effective planning and indigenization of advanced waste process facilities, as well as the provision of regular training to garbage collectors. To boost the effectiveness of SWM in India, citizen participation should be promoted, especially in source segregation and treatment processes (Prajapati et al., 2021). The policy agenda for sustainable SWM must stimulate behavior change amongst individuals, elected representatives, and decision-makers, to limit wastage and littering and boost reuse and recycling. Community awareness and a shift in people's attitudes about solid waste and its disposal can go a long way in enhancing India's SWM system (Singh 2020). Viable decentralized composting plants should be established to minimize the load on ULBs for collection and transportation of MSW, which ultimately culminates in a reduction of the strain, put on the landfills. Characterization of garbage during collection and also at disposal site should be made and be available in the public domain. Government should take initiative to encourage Universities, and technical Institutions to take up waste management in its curriculum. The assistance of academic institutions should be asked in the characterization of waste in their proximity. Thereby most parts of India would be covered and location-specific relevant solutions for waste management can be devised (Joshi and Ahmed 2016). Future studies should focus on eliminating the negative effect of waste-to-energy technology as well as the obstacles that waste-to-energy initiatives confront. Future trends and technologies such as microbial fuel cell technology, an eco-friendly strategy that converts municipal solid waste into high energy yield, and hydrogen gas should be advocated (Prajapati et al. 2021).

The recent Indian EPR framework was built by relying upon successes and failure studies from other nations, previous Indian waste management experience, and opinions from diverse stakeholders. Considering the vastness of the problem, significant socio-economic and cultural diversity, demographic complexity, and the predicted implementation challenges, the Indian EPR framework is substantially different in approach compared to EPR approaches of advanced nations. India being a rising economy and a non-OECD country, its EPR framework has emerged not mainly from international commitment but through the voluntary proactiveness of domestic stakeholders. The Indian EPR method is a break from the typical legislative strategy of 'command and control' (Pani and Pathak 2021). It is non-directive in character and inclusive of a wide range of stakeholders from both the organized and unorganized sectors. It allows many models to co-develop and coexist at the same time, and it will have a huge impact on producers and manufacturers across sectors and industries (MoEFCC 2020). Analyzing the development, application, and ramifications of this EPR strategy, therefore, is of utmost importance to policy makers, environmentalists, industrial stakeholders, and academia.

With its "people first" orientation, Indian government initiatives such as the Swachh Bharat Mission (SBM), formally started on October 2, 2014, have reached all corners of the country and impacted the lives of countless inhabitants. The Mission changed the sanitation paradigm in the country by providing 100 percent access to sanitation facilities in urban India (Ghosh et al. 2021). Now, building on the SBM achievements, the focus of SBM-U 2.0, launched on October 2, 2021, for the next five years will be on sustaining the sanitation and SWM outcomes achieved and accelerating the momentum generated, bringing Urban India to the next level of "Swachhata". The sustainable SWM program will place a stronger emphasis on source segregation with a focus on phasing out single-use plastic, material recovery facilities, and waste processing.

Conclusion

India being a developing country, it is apparent that sustainable development plays a vital role at every step of its development process. One such crucial component that aids in its development sustainably is the efficient handling of plastic garbage in the nation. This review study indicates that dependability and economics are the two driving variables that drive the increase in plastic usage as part of industrialization which eventually also increases the waste output from plastic. Few states in India make more plastic garbage that can be regulated. Hence, novel techniques become important to handle the excessive waste generation from plastic and improve the health and environmental quality of society. People need to be supplied with adequate alternatives that are conveniently available and cheap. It is necessary to encourage and promote the usage of Areca palm and Sal leaves to produce plates and containers. They're not only biodegradable, but they'll also help our fledgling cottage sector develop. Bamboo toothbrushes and bottles, for example, can help

us reduce a lot of plastic waste. Algae-based flip flops which are biodegradable can help us eliminate plastic flip flops which a huge percentage of the Indian people wear, therefore reducing plastic flip flops from accumulating in landfills which ordinarily would take hundreds or thousands of years to biodegrade. Through recent technology advancements, the development of such innovative products employing renewable raw materials would help reduce plastic usage and promote a sustainable environment. As a result, it is up to us to demand biodegradable products from corporations. Companies will not contemplate making such things if we do not demand from them and will continue to supply us with plastic commodities. In this regard, central ministries of the government of India, such as the Ministry of Environment, Forest and Climate Change (MoEFCC), can jointly frame a strategy and action plan to work with the state ministries to build their capacity to implement conventional plastic waste processing and bioplastic production projects. They should collaborate closely to provide the financial and economic incentives required to simultaneously boost the processing of plastic wastes. Such an effort would enhance further research which will lead to new solutions and innovations for better utilization and disposal of plastic garbage.

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Comparative Assessment of Three Fungal Genus in Mycoremediation of Spent Engine Oil: A Brief Review

Selvina Sandiawo, Ong Ghim Hock*, Tan Jia Wen, Cheng Wan Hee, Wong Kok Kee, Wong Rui Rui

Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN, Putra Nilai,71800 Nilai, Negeri Sembilan, Malaysia.

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ABSTRACT

Spent engine oil is composed of various aliphatic hydrocarbons, aromatic hydrocarbons, lubricative additives, and traces of heavy metal. Improper disposal of spent engine oil can lead to deleterious effects on humans due to spent engine oil properties, which can exert toxicity, mutagenicity, and carcinogenicity on cells and organs. The conventional method to remove hydrocarbon in the spent engine oil is not only expensive but unable to degrade the hydrocarbon completely. In comparison, the mycoremediation approach has been reported to be environmentally friendly, efficient, and cost-effective. The main objective of this review article is to identify the fungal isolate which is most efficient to degrade spent engine oil by assessing the biomass production and the percentage of spent engine oil degraded. Based on the comparative information obtained, *Mucor* sp. showed the highest biomass production in the presence of spent engine oil. *Trichoderma* sp. and *Aspergillus niger* were found to have average biomass production and it depending on the strain and incubation period. Both *A. flavus* and *A. nidulans* were found to have the lowest biomass production. In terms of spent engine oil degradation, *Mucor* sp, *Trichoderma* sp. and *A. niger* showed >55% degradation as compared to *A. flavus* and *A. nidulans* which have less than 50% degradation. Therefore, from the results of the study, it can be concluded that *Mucor* sp. has the best potential to degrade spent engine oil within a short period based on the high biomass production and percentage of degradation. The comparative data also suggest that by selecting the right strain and right incubation period, the percentage of spent engine oil degradation by using *Trichoderma* sp. and *A. niger* could also increase.

* Corresponding author

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

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1 Introduction

Engine oil or more correctly, 'engine lubricant' is widely applied in the engine to reduce the frictional force and keep the metals fresh. After the engine oil had been exposed to extremely high temperature and mechanical pressure, it will be referred to as waste oil or spent engine oil due to physical and chemical impurities being formed (Boichenko et al. 2021). Spent engine oil is a mixture of hydrocarbon molecules and organic compounds with some organometallic constituents and encompassed various aliphatic chains and aromatic hydrocarbons (Ossai et al. 2020). According to Singh and Haritash (2019), the hydrocarbon contaminants such as polycyclic aromatic hydrocarbon (PAHs) and metals are hazardous due to their deleterious effect including toxicity, mutagenicity, and carcinogenicity that concerns the public. Furthermore, spent engine oil products can also exert adverse effects on other living organisms (ATSDR 1995; Adeleye et al. 2018), and severely affect agriculture by decreasing the yield of crops (Ismail et al. 2021).

Previous findings by Al-Hawash et al. (2019) have shown that fungal species were capable of reducing a wide range of pollutants as the fungal mycelia structure was able to produce non-specific enzymes and acids to solubilize the insoluble substrates. A recent study showed fungal secreting extracellular enzymes and acids through their mycelia which can effectively degrade the hydrocarbon molecules (Adekunle et al. 2015). However, the main key to successful mycoremediation is to employ the right fungal species in the removal of specific pollutants (Sing, 2006) but this information is scarcely documented. Albeit various hydrocarbon degrading fungal species have been identified, the search for an ideal species for successful mycoremediation of spent engine oil remains challenging due to the complexity of hydrocarbon components found in the spent engine oil (Stanley and Immanuel 2015).

Environmental quality regulations in Malaysia set a maximum content of oil sewage and industrial effluent discharge limit to 10 mg/L (Environmental Quality (Industrial Effluents) Regulations, 2009). Despite the regulation, 121 oil spillage cases were reported from 2009 to 2016 in Malaysia (Fong 2016) and some cases were left unreported due to the occurrence in smaller areas. Industrial manufacturers, disregarding the negative environmental impact, are still disposing of spent engine oil in open sources as a shortcut option to reduce their downstream costs by not processing the hydrocarbon waste. Therefore, there is an urgent need for remediation to reduce the spent engine oil contaminant found in the environment. Hence, this study focuses on the potential of fungal species on hydrocarbon degradation by reviewing their tolerance towards spent engine oil based on the fungal biomass and the percentage reduction of spent engine oil in the growth medium.

Various previous studies have identified five potential fungal species namely, *Mucor* sp., *Aspergillus flavus*, *A. niger*, *A. nidulans*, and *Trichoderma* sp. which have a wider potential for mycoremediation. These fungal species were inoculated on the different growth selective mediums spiked with spent engine oil and the fungal biomass production and the percentage reduction of spent engine oil were recorded by previous researchers.

2 Biomass Production

Based on the previous studies' conclusion (Table 1), each of the tested fungal species possessed different tolerance toward spent engine oil, which influences the biomass production of the fungal species on the culture medium containing spent engine oil. Among the five fungal species, *Mucor* sp was observed to attain the highest biomass production within a comparatively short incubation period of 7 days. This is followed by the *Trichoderma* sp. and *A. niger* these were able to achieve moderate to high

Table 1 The growth of different fungal species cultivated on different growth mediums supplemented with spent engine oil

Species	Fungal biomass production	Composition of the growth medium	Incubation period at room temperature	Reference
Mucor sp.	High	2 mL of spent engine oil + 40 mL of BHB	7 days	Ong et al. 2018
A. niger	Moderate	10 mL of spent engine oil + 40 mL of BHB	7 days	Ong et al. 2018
	High 5th day/OD: 8.000 40thday/OD: 5.250	1 mL of spent engine oil + 100 mL of mineral salt	21 days	Ahmad et al. 2015
	High	2 mL spent engine oil + 10 mL of MSB	40 days	Adekunle and Adebambo 2007
A. nidulans	Moderate	10 mL of spent engine oil + 40 mL of BHB	7 days	Ong et al. 2018
A. flavus	Moderate	10 mL of spent engine oil + 40 mL of BHB	7 days	Ong et al. 2018
	Moderate	1 mL of spent engine oil + 100 mL of mineral salt	21 days	Ahmad et al. 2015
Trichoderma sp.	High	10 mL of spent engine oil + 40 mL of BHB	7 days	Ong et al. 2018
	Moderate- High (+26% to +75%)	BHA complemented with 1% (v/v) of used engine oil	7 days	Daccò et al. 2020

BHB: Bushnell Hass Broth; BHA: Bushnell Hass Agar; MSB: Minimal salt broth

biomass production but needed a relatively long incubation period. Furthermore, both *A. nidulans* and *A. flavus* were able to achieve moderate biomass production but with a longer incubation period relatively. Hence, the sequential order of fungal species tolerance towards spent engine oil based on their observed biomass production is *A. flavus*≈*A. nidulans*<*A. niger*<*Trichoderma* sp. <*Mucor* sp.

Various *Mucor* species can achieve high biomass in BHB containing spent engine oil. According to Marchut-Mikolajczyk et al. (2015), *M. circinelloides* secretes various metabolites that emulsify hydrophobic hydrocarbons and increase hydrocarbon interaction with degradative enzymes such as lipase and alcohol dehydrogenase (ADH) (Durón-Castellanos et al. 2005) and metabolizes the hydrocarbons available in spent engine oil to be utilized for cell biomass production (Balaji et al., 2014; Chimezie Dirisu, 2015).

Biomass production by *Trichoderma* sp. ranged from moderate to high when cultured on the medium containing spent engine oil as a sole carbon source (Daccò et al. 2020). This suggested that *Trichoderma* sp. has moderate to high tolerance toward spent engine oil and can consume spent engine oil as a carbon source to grow (Thenmozhi et al. 2013). Various species in this genus including *T. harzianum*, *T. pseudokoningii*, and *T. viride* (Ravelet et al. 2000; Saraswathy and Hallberg 2002) were reported to degrade pyrene, a recalcitrant hydrocarbon found in spent engine oil, as a carbon source. *Trichoderma* sp. possesses an *N*-acetylation detoxification pathway that enables this fungal species to degrade recalcitrant aromatic hydrocarbons structures to support growth (Cocaign et al. 2013; Kupareva et al. 2013; Zafra et al. 2015). Because of this, *Trichoderma* sp. is the most common fungal species that are isolated from the soil contaminated by petroleum (Makut et al. 2014).

A. niger was observed to have moderate biomass production after 7 days of incubation (Ong et al. 2018), but was able to achieve higher biomass production if the incubation time is extended (Ahmad et al. 2015). Adekunle and Adebambo (2007), also reported a fluctuating growth pattern of *A. niger* for 40 days before reaching the maximum growth peak, which was attributed to the different ratios of spent engine oil added in the experiment. Despite the inconsistent growth pattern observed, *A. niger* can secrete lipase, catalase, and lignin peroxidase to degrade hydrocarbon to support growth (Vatsyayan and Goswami 2016).

At present, information on the biomass production by *A. nidulans* on spent engine oil supplemented media is in scantly. Ong et al. (2018) reported moderate *A. nidulans* biomass production after 7 days of incubation in BHB containing spent engine oil and this might be due to the shorter incubation period. Similarly, moderate biomass production was reported when *A. flavus* was inoculated on the medium spike with hydrocarbons (Ahmad et al. 2015; Ong et al. 2018).

2.1 Reduction of Spent Engine Oil

Table 2 showed all the reported fungal species which were capable to degrade and utilize spent engine oil as the sole carbon source of growth (Thenmozhi et al. 2013). The percentages of spent engine oil being degraded varied according to the present of fungal species, different lengths of the incubation time, type of medium, and the extracellular enzyme secreted by each of the fungal species. As per table 2, the sequential order of the fungal species' effectiveness in reducing spent engine oil in ascending order is *A. nidulans*< *A. flavus* <*Trichoderma* sp. ≈*A. niger*≈*Mucor* sp.

According to Szewczyk and Długoński (2009), *Mucor* sp. can achieve up to 55% reduction in spent engine oil. The species *M. circinelloides* was reported to produce an extracellular emulsifier to emulsify hydrocarbons to increase the bioavailability of hydrocarbons to fungal's degradative enzymes (Marchut-Mikolajczyk et al. 2015). Balaji et al. (2014) reported that *M. racemosus* expresses a relatively higher concentration of lipase enzyme in spent engine oil, compared to other fungal species. These species were also able to produce organic acids that metabolize spent engine oil effectively and then utilize the spent engine oil as a sole carbon source to support growth (Thenmozhi et al. 2013; Paper and Nwinyi 2019). These circumstantial shreds of evidence suggested that *Mucor* sp. is consistent in degrading a high percentage of spent engine oil.

In the case of *Trichoderma* sp, Elshafie et al. (2020) reported that *T. harzianum* strain T22 can degrade a high percentage of spent engine oil, while the finding of Ong et al. (2018) was contradictory and these researchers reported a relatively low percentage of spent engine oil being degraded by *T. harzianum*. This indicated the percentage of spent engine oil degraded by *Trichoderma* sp is highly dependent on the strain and the incubation period. Various species of this genus including *T. hamatum*, *T. harzianum*, *T. koningii*, *T. viride*, *T. virens*, and *T. asperellum* have shown to degrade low-molecular-weight PAHs such as naphthalene and phenanthrene, or more complex PAHs such as anthracenebenzo[a]anthracene, benzo[a]fluoranthene, benzo[a]pyrene, and chrysene (Cerniglia and Sutherland 2010, Lieckfeldt et al. 1999, Zafra et al. 2015). Probable mechanisms for PAHs degradation are hypothesized for *Trichoderma*, including the production of laccases (Cazares-Garcia et al. 2013), peroxidases (Per) (Cristica et al. 2011), and dioxygenases (Hadibarata et al. 2007). This was confirmed by Zafra et al. (2015) and Balcázar-López et al. (2016) for *T. atroviride* who reported that laccase production can cleave the aromatic ring of PAHs, which resulted in the degradation of PAHs compound present in spent engine oil. Even though Table 1 illustrates a moderate to high biomass

Table 2 *In vitro* potential of various fungal species in reducing spent engine oil

Species	Isolated	Reduction (%) of spent engine oil	Composition of medium	Incubation period	References
Mucor sp.	*M. ramosissimus* IM 6203 from Poland	55.5%	5 % of spent engine oil in synthetic medium	10 days	Szewczyk and Długoński 2009
	Ota, Ogun State, Nigeria	OD increased from 0.715 to 1.978	2 mL of spent engine oil + 30 mL of MS	12 days at room temperature	Paper and Nwinyi 2019
A. niger	Shah Alam, Selangor, Malaysia	15.85%	10 mL of spent engine oil + 40 mL of BHB	7 days at room temperature	Ong et al. 2018
	Sokoto Metropolis, Nigeria	61.80%	1 mL of spent engine oil + 100 mL of mineral salt	20 days at room temperature	Ahmad et al. 2015
	Ota, Ogun State, Nigeria	OD increased from 0.292 to 1.731.	2 mL of spent engine oil + 30 mL of MS	12 days at room temperature	Paper and Nwinyi 2019
	Pudukkottai, Tamilnadu, South India	40.5%	Czapeck dox broth with 1 % v/v used motor oil	30 days at room temperature	Thenmozhi et al. 2013
A. nidulans	Shah Alam, Selangor, Malaysia	17.75%	10 mL of spent engine oil + 40 mL of BHB	7 days at room temperature	Ong et al. 2018
A. flavus	Shah Alam, Selangor, Malaysia	11.80%	10 mL of spent engine oil + 40 mL of BHB	7 days at room temperature	Ong et al. 2018
	Sokoto Metropolis, Nigeria	44.60%	1 mL of spent engine oil + 100 mL of mineral salt	20 days at room temperature	Ahmad et al. 2015
	Ota, Ogun State, Nigeria	OD increased from 0.213 to 0.617	2 mL of spent engine oil + 30 mL of MS	12 days at room temperature	Paper and Nwinyi 2019
Trichoderma sp.	*T. harzianum* strain T22 (*Th*-T22)	70.16%	5 mL of BHB and 1% (v/v) of used engine oil	45 days	Elshafie et al. 2020
	Shah Alam, Selangor, Malaysia	15.02%	10 mL of spent engine oil + 40 mL of BHB	7 days at room temperature	Ong et al. 2018

BHB: Bushnell Hass Broth; MSB: Minimal salt broth

production by *Trichoderma* sp., but the percentage of spent engine oil degraded is not so consistent compared to *Mucor* sp, but relatively better than *A. nidulans* and *A. flavus*.

Results of the previous studies suggested that *A. niger* can degrade a mere 15.85% spent engine oil after 7 days of incubation (Ong et al. 2018) to as high as 61.80% after 20 days of incubation (Ahmad et al. 2015). *A. niger* isolated from India was reported to achieve an average of 40.5% spent engine oil degraded (Thenmozhi et al. 2013). The differences observed can be attributed to the different incubation periods and locations. The ability of *A. niger* in reducing spent engine oil was also attributed to the secretion of some specific enzymes such as lipase which hydrolyzes the triglycerides structures (Gupta 2016), manganese peroxidase, and lignin peroxidase which can degrade PAHs compound present in spent engine oil (Ameen et al. 2016). Overall, *A. niger* is a good species that can be used in the remediation of spent engine oil but it needed a longer incubation period to achieve similar results as *Mucor* sp.

Ong et al. (2018) and Paper & Nwinyi (2019) recorded a relatively low amount of spent engine oil degradation with *A. flavus* but the finding of Ahmad et al. (2015) was contradictory and these researchers reported higher percentage degradation (44.6%) by this fungi. Although the fungal strain used by both Paper and Nwinyi (2019) and Ahmad et al. (2015) was obtained from Nigeria but this difference in spent engine oil degradation might be associated with the difference in incubation time. Furthermore, Kota et al. (2014) also had a different opinion and suggested that *A. flavus* is ineffective in degrading crude oil as compared to *Trichoderma* sp., this might be because *A. flavus* could not degrade PAHs compound effectively as compared to other potential fungal species (Chaudhry et al. 2012; Haritash and Kaushik 2016). Moreover, due to poor spore formation *A. flavus* have a relatively slow growth compared to *A. niger* (Marín et al. 1998), which causes lower cell biomass production and enzyme secretion and this might be associated with the low spent engine oil degradation percentage.

There is limited information available regarding the use of *A. nidulans* in remediating spent engine oil which suggests that *A. nidulans* might not be as good as other fungal species. Furthermore, various previous studies showed that the genus *Aspergillus* has the potential of degrading various types of hydrocarbons such as aliphatic and aromatic, but most of the reported studies concentrated on the *A. niger* and *A. flavus* (Olajire and Essien 2014).

Conclusion

Results of the study can be concluded that among the tested various fungal species, based on both biomass production and effectiveness in reducing spent engine oil within a relatively short

incubation period, *Mucor* sp. is the best species. Further, *Trichoderma* sp. and *A. niger* also have the potential of reducing spent engine oil if the right strain and suitable incubation time are selected. Due to limited reports on fungal species oil degradation mechanisms, extensive research is required to be conducted on mycoremediation of the spent engine oil contaminant.

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Effects of Different Substrates on the Growth and Nutritional Composition of *Pleurotus ostreatus*: A Review

M. Karmani, G. Subramaniam, L.A. Sivasamugham, W.H. Cheng, L.S. Wong

Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN, Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia

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ABSTRACT

Mushrooms are a popular food source as they are highly nutritious and flavorful with a high content of proteins, vitamins, and minerals. Mushrooms could be an alternative solution to the world's food crisis as they are inexpensive to grow on different types of substrates including waste materials. *Pleurotus ostreatus*, frequently known as oyster mushrooms, are the second most cultivated mushroom in the world. This species is known for its high protein content and easy cultivation. Oyster mushrooms have the potential to produce protein-rich biomass when grown on various substrates. There is a need to identify substrates that are cost-effective for the commercial production of nutritious oyster mushrooms as the substrates used currently are either costly or inadequate to produce oyster mushrooms in the required quantity or quality. Thus, the effects of 6 different lignocellulosic substrates on the growth and nutritional composition of *P. ostreatus* were reviewed and analyzed in this article. The substrates included in this review were wheat straw, sugarcane bagasse, corncob, softwood sawdust, hardwood sawdust, and general sawdust. Based on the analyzed data, sugarcane bagasse was concluded as the most suitable substrate to grow *P. ostreatus*. These substrates contain a high amount of nutrients and are also likely to produce a significantly high yield of oyster mushrooms in addition to enhancing the nutritional quality of the mushroom. However, these findings must be evaluated and confirmed through further research in this field.

* Corresponding author

E-mail: geetha.subramaniam@newinti.edu.my (Geetha Subramaniam)

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1 Introduction

The process of photosynthesis generates approximately 200 billion tons of organic compounds every year (Philippoussis 2009). This organic bulk can pollute the environment in many cases especially the lignocellulosic components as they are not readily degraded by organisms (Hatakka 1994; Philippoussis 2009). Waste materials from agriculture and industry can be used to grow mushrooms (Abid et al. 2020; Iwuagwu et al. 2020). Mushrooms are macro-fungal fruiting bodies that are extremely rich in flavor and do not have high calories but are rich in vitamins, minerals, and proteins (Mubasshira et al. 2020). Mushrooms are considered to be the world's most untapped resources for nutritious and healthy food (Kakon et al. 2012).

Oyster mushrooms (*P. ostreatus*) are an edible, lignocellulolytic type of mushrooms (Kumla et al. 2020) with therapeutic elements such as phenolic compounds, dietary fibers, minerals, and numerous bioactive compounds. The fruiting body of mushrooms in the *Pleurotus* genus contains minerals like zinc, iron, phosphorus, potassium, copper, etc. (Garuba et al. 2017). Previous research suggests that the consumption of oyster mushrooms can lower the risk of many diseases such as heart disease, impaired immune response, hepatitis B, liver disease, high blood cholesterol levels, gastric cancer, microbial infection, chronic fatigue syndrome, kidney problems, hypertension, and diabetes (Mubasshira et al. 2020; Ba et al. 2021; Krittanawong et al. 2021). Therefore, oyster mushrooms are an alternative rich source of nutrition and an alternative food source that can promote human health (Valverde et al. 2015).

P. ostreatus contain lignocellulosic enzymes that can convert lignocellulose residues into protein-rich biomass (Adebayo and Martinez-Carrera 2014; Mubasshira et al. 2020; Grimm et al. 2021). These mushrooms require a short growth time relative to other mushrooms and are economical to produce commercially (Abid et al. 2020). Multiple lignocellulosic substrates including agricultural waste products such as sugar cane bagasse, corn cobs, and wheat straw, can be used to cultivate *P. ostreatus* (Owaid et al. 2015; Sözbir et al. 2015; Kumla et al. 2020).

Therefore, this paper is aimed at reviewing the effects of different lignocellulose-rich substrates on the growth and nutritional composition of *P. ostreatus* and to identify the most appropriate substrate that might produce the highest yield of nutritious oyster mushrooms.

2 Selection of Literature

This paper involved systematically reviewing and analyzing the currently available research on the effects of different substrates on the growth and nutritional composition of *P. ostreatus*. The search for relevant studies was done using electronic databases like NCBI, Google Scholar, Research Gate, and others. The inclusion and exclusion criteria used to select the papers for this review are stated in Table 1.

3 Cultivation and Analysis of *P. ostreatus* Nutritional Content

Significant differences in the nutritional content, particularly the protein content, have been observed in the cultivation of edible mushrooms. This could be influenced by several factors such as the types of mushroom species, the used substrate, and the level of nitrogen available in the growth substrate (Belletini et al. 2019). Currently, the number of studies that focus on the utilization of lignocellulosic substrates are increasing (Iwuagwu et al. 2020; Nongthomban et al. 2021). Ganash et al. (2021) demonstrated that *Pleurotus* species are very effective in breaking down lignocellulosic residues making it a suitable mushroom to be cultivated on lignocellulosic substrates (Figure 1). This could be due to the type and quantity of enzymes including lignolytic,

Table 1 The inclusion and exclusion criteria that have been conducted for this review paper.

Inclusion Criteria	• Papers for fresh mushrooms belonging to the *Pleurotus* genus • Papers that included information about nutritional content, substrate quality, growth conditions, regional conditions, substrate availability, etc.
Exclusion Criteria	• Papers for fresh mushrooms belonging to other genera of mushrooms except *Pleurotus*. • Papers for dry mushrooms belonging to other genera of mushrooms including *Pleurotus*. • Papers for canned mushrooms or other extracts belonging to other genera of mushrooms including *Pleurotus*.

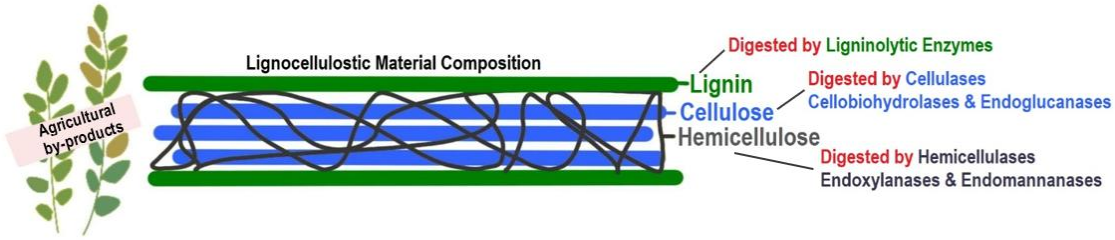

Figure 1 Systemic representation of enzymatic degradation of lignocellulosic agricultural wastes by *P. ostreatus* enzymes

cellulase, and hemicellulase enzymes, that are generated by fungi throughout the vegetative development (Ogundele et al. 2017). The majority of agro-industrial waste consists of lignocellulosic materials; the cultivation of edible mushrooms using these substrates would greatly reduce the environmental waste while providing suitable and economical substrates for mushroom cultivation (Kumla et al. 2020).

Table 2 consists of the proximate size and number of fruiting bodies of *P. ostreatus* that were generated on different lignocellulosic substrates. From the data given in table 2, it can be concluded that the cultivation of wheat straw, corn cob, and sugarcane bagasse produced *P. ostreatus* with the largest cap diameter (84 – 89.3 mm), followed by general sawdust, hardwood sawdust, and softwood sawdust (Upadhyay et al. 2002; Phillipoussis 2009; Fanadzo et al. 2010; Hoa et al. 2015; Girmay et al. 2016; Masevhe et al. 2016; Salama et al. 2016; Ogundele et al. 2017). The cap diameter is an essential growth parameter. The cap or pilieus which contains the spore-bearing surface of the mushroom is supported by the stalk and constitutes the fruiting body. According to Onyango et al., (2011), big fruit bodies, in general, are the desired feature highly valued in mushroom cultivation. It was observed that the cultivation of wheat straw also produced an average of 18 fruiting bodies, the second-highest number compared to sugarcane bagasse and corn cob which produced the lowest number of fruiting bodies (< 10 mm). Among all the substrates, hardwood sawdust seemed to be the least suitable substrate for the cultivation of *P. ostreatus* based on the low number of fruiting bodies (<15 mm) and a comparatively small cap diameter (24 mm). However, it was interesting that cultivation on softwood sawdust had the highest number of fruiting bodies with the smallest cap diameter. Although the size of the fruiting bodies is a definite factor for considering when selecting a suitable substrate for the cultivation of *P. ostreatus*, an equally important factor would be the nutritional content of the mushroom.

Edible mushrooms are rich in proteins, carbohydrates, and minerals while being low in lipid content (Valverde et al. 2015). The substrates on which the mushrooms are cultivated also have an impact on the overall energy of mushrooms and nutritional content (Hoa et al. 2015). Mushrooms, including *P. ostreatus*, are saprophytes; they extract the required nutrients from the substrate they are cultivated on. Based on the data in table 3, *P. ostreatus* grown on corn cob substrate had the highest protein content

Table 2 Proximate size and number of fruiting bodies of *Pleurotus ostreatus* based on cultivation with common lignocellulosic substrates.

Substrate	Cap Diameter (mm)	Stripe Length (mm)	Stripe Thickness (mm)	No. of Effective Fruiting Bodies	References
Corn Cob	86.7	35.3	11.1	7.9	Hoa et al. 2015; Philippoussis 2009
Sawdust (Soft Wood)	17.6	-	-	29.6	Ogundele et al. 2017; Philippoussis 2009
Sawdust (Hardwood)	24.0	-	-	14.6	Hoa et al. 2015; Ogundele et al. 2017; Philippoussis 2009
Sawdust (General)	70.6	38.2	8.5	11.5	Girmay et al. 2016; Hoa et al. 2015
Sugarcane Bagasse	84.8	35.7	10.2	8.1	Hoa et al. 2015; Philippoussis 2009
Wheat Straw	86-89.3	28.1	10.3	18.3	Fanadzo et al. 2010; Girmay et al. 2016; Masevhe et al. 2016; Salama et al. 2016; Upadhyay et al. 2002

Table 3 Proximate nutritional composition and energy of *P. ostreatus* based on cultivation with common lignocellulosic substrates.

Substrate	Moisture	Ash	Fat	Protein	Carbohydrate	Fiber	Energy (Kcal/100g)	References
Corn Cob	90.57%	7.10%	2.67%	29.70%	30.78%	29.75%	265.95	Hoa et al. 2015; Philippoussis 2009
Sawdust (Soft Wood)	7.88%	9.59%	1.81%	17.68%	52.04%	10.66%	-	Ogundele et al. 2017; Philippoussis 2009
Sawdust (Hardwood)	8.93%	9.83%	1.72%	11.05%	41.57%	11.05%	-	Hoa et al. 2015; Ogundele et al. 2017; Philippoussis 2009
Sawdust (General)	91.06%	5.90%	1.32%	19.52%	51.26%	22.00%	295.00	Girmay et al. 2016; Hoa et al. 2015
Sugarcane Bagasse	91.56%	6.68%	2.00%	27.13%	34.94%	29.25%	266.28	Hoa et al. 2015; Philippoussis 2009
Wheat Straw	90.16%	0.82%	0.15%	1.66%	7.22%	-	110.23	Fanadzo et al. 2010; Girmay et al. 2016; Masevhe et al. 2016; Salama et al. 2016; Upadhyay et al. 2002.

Table 4 Proximate mineral composition of *P. ostreatus* based on cultivation with common lignocellulosic substrates.

	Mineral (mg/100g dry weight)								
Substrate	Ca	Cu	Fe	K	Mg	Mn	P	Zn	References
Corn Cob	340.08	2.55	14.64	2,624	225.17	3.68	717.49	11.45	Hoa et al. 2015
Soft Wood Sawdust	3.42	-	-	21.36	1.04	-	10.09	0.95	Ogundele et al. 2017
Hardwood Sawdust	3.51	-	-	21.81	1.25	-	10.36	0.96	Ogundele et al. 2017
General Sawdust	336.02	2.08	14.83	1424.37	217.76	2.06	620.35	8.69	Hoa et al. 2015
Sugarcane Bagasse	345.06	2.22	14.85	2573.79	237.07	3.06	732.27	8.56	Hoa et al. 2015
Wheat Straw	-	-	-	1.12-1.13	-	-	1.82-1.84	-	Masevhe et al. 2016; Salama et al. 2016

followed by sugarcane bagasse and other substrates such as general sawdust, softwood sawdust, hardwood sawdust, and wheat straw. Furthermore, cultivation of *P. ostreatus* on both corn cob and sugarcane bagasse produced mushrooms with high carbohydrate content resulting in more than 265 Kcal/100 g of calories, indicating a higher nutritional content with a comparatively lower calorie value. This could be because a significant amount of the carbohydrate content in mushrooms consists of dietary fiber (Nakalembe et al. 2015; Kumari 2020). Although the individual substrates such as corn cob and sugarcane bagasse seem to produce *P. ostreatus* with high protein and carbohydrate content, it would be interesting to study the effects of mixing substrates on the growth and nutritional content of this mushroom. Owaid et al. (2015) reported an increase in the productivity and biological efficiency of *P. ostreatus* in mixed substrates compared to using wheat straw alone. This indicates that more studies on the utilization of mixed substrates from agro-industrial waste and their effects on the growth output and nutritional content of *P. ostreatus* should be carried out.

Mushroom fruiting bodies have a high concentration of easily absorbed mineral components which could be due to their capability of bio-accumulation of metals (Gebrelibanos et al. 2016). Since the fungal hyphae come in contact with the compound and absorb its important components, the substrate has a direct impact on the mineral composition of the mushroom (Bellettini et al. 2019). *P. ostreatus* has been known to contain generally high levels of nutritionally essential minerals, including potassium and phosphorus, and sufficient quantities of ferum with high bioavailability (Raman et al. 2021). Table 4 shows the approximate mineral composition of *P. ostreatus* based on cultivation with common lignocellulosic substrates. These data show that growing *P. ostreatus* on sugarcane bagasse leads to mushrooms with significantly high mineral content, particularly in comparison with sawdust which is the common substrate utilized to cultivate edible mushrooms. It has to be noted that the mineral content in mushrooms is important as they are considered a "dietary supplement" with low calorific value and high protein content (Kakon et al. 2012). The high mineral content in combination with the large-cap diameter of *P. ostreatus* makes sugarcane bagasse a more suitable substrate for the cultivation of this species of edible mushroom.

4 Conclusion and Recommendations for Future Research

Substrates directly impact mushroom composition, so the choice of appropriate substrate is of utmost importance. Based on the reviewed data, sugarcane bagasse has been concluded as the best substrate for *P. ostreatus* cultivation. The nutritional content of *P. ostreatus* grown on sugarcane bagasse is comparable to cultivation on other substrates. Furthermore, sugarcane bagasse is also well-researched for *Pleurotus* growth. As long as parameters such as pH, moisture content of the substrate, etc. are critically analyzed beforehand, sugarcane bagasse is an appropriate choice of substrate for *P. ostreatus* cultivation.

As with the majority of studies, the design of the current study is subject to limitations. The data that have been quoted in this review are from multiple studies which are different from each other in methodology (such as pre-treatment sterilization) or environmental conditions and differences in microclimates. Although this paper theoretically concludes based on data from several papers that sugarcane bagasse is the most suitable substrate for *P. ostreatus* cultivation, there is a need to confirm this based on actual results from the field compared with other substrates simultaneously while maintaining similar culture conditions, and other factors such as temperature and nitrogen levels.

Conflict of Interest

The authors declare no conflict of interest. This paper has not been submitted for publication in any other journal.

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A Review on DNA Vaccines in Pre-Clinical Trials Against SARS-CoV-2

Dinesh Kumar Lakshmi Narayanan[1], Sinouvassane Djearamane[2], Shivkanya Fuloria[3], Saminathan Kayarohanam[1], Vetriselvan Subramaniyan[4], Mahendran Sekar[5], Neeraj Kumar Fuloria[3*]

[1]Faculty of Bioeconomics and Health sciences, Geomatika University College, Kuala Lumpur, 54200 Malaysia
[2]Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Kampar, 31900 Malaysia
[3]Faculty of Pharmacy & Centre of Excellence for Biomaterials Engineering, AIMST University, Bedong 08100, Kedah, Malaysia
[4]Department of Medicine, Bioscience and Nursing, MAHSA University, Bandar Saujana Putra, Jenjarom Selangor, Malaysia.
[5]Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Health Sciences, Universiti Kuala Lumpur Royal College of Medicine Perak, Ipoh 30450, Perak, Malaysia

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ABSTRACT

COVID 19 Pandemic is caused by the viral pathogen severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Scientific fraternity worldwide swiftly developed various types of vaccines for the prevention and as mitigation measures for curbing the pandemic. Traditional inactivated vaccines, mRNA vaccines (protein subunits such as spike proteins), and viral vector vaccines (non-replicating vectors with protein subunits) have been approved by World Health Organisation (WHO) for emergency use. The emergence of many mutated variants has been a worrying factor in the fight against the pandemic. There has been continuous research in the quest for more therapeutics, especially vaccines to curb and stop the pandemic. According to WHO, there are 194 vaccines in pre-clinical trials belonging to various types out of which sixteen is DNA vaccines. In this review, we have discussed the advantages and disadvantages of the DNA vaccines for Covid - 19. This article tried to explore the available information on DNA vaccines and their current status against Covid – 19 which are in pre-clinical trials.

* Corresponding author
E-mail: neerajkumar@aimst.edu.my (Neeraj Kumar Fuloria)

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1 Introduction

Covid - 19 was first reported from Wuhan, China in December 2019. The severe respiratory syndrome was reported with symptoms of fever, dizziness, and cough. The RNA sequencing was done with the bronchoalveolar lavage fluid sample of the patient revealing it belongs to the family *Coronaviridae* (Wu et al. 2020). Further phylogenetic analysis results revealed that the virus belongs to the order of *Nidovirales*, suborder *cornidovirineae*, family *coronaviridae*, subfamily *orthocoronavirinae*, genus *Betacoronavirus*, and subgenus *Sarbecovirus* and to the species of severe acute respiratory syndrome coronavirus. It was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the Coronaviridae Study Group (CSG), the working group of the International Committee on Taxonomy of Viruses ICTV (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses 2020). As the outbreak spread, worldwide World Health Organisation declared it as a pandemic on 11 March 2020.

2 SARS-COV-2

Severe acute respiratory syndrome coronavirus 2(SARS-COV-2) belongs to the family of *coronaviridae*. There are four groups among this family namely alpha, beta, gamma, and delta. SARS-CoV2 belongs to the beta coronavirus which is known to cause respiratory and gastrointestinal diseases. In the past also, there have been outbreaks from two pathogenic species from this genus namely severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002 and Middle Eastern respiratory virus (MERS-CoV) in 2012. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a spherical single-strand RNA virus that is in a positive sense (Michael et al. 2021). The genome size of the coronaviruses is largest when compared to other RNA viruses. The genomic length of the SARS-CoV-2 is about 30kb and it has fourteen open reading frames (ORFs) in which genes encode for its structural proteins, accessory proteins, and non-structural proteins (Finkel et al. 2021). ORF1a and ORF1ab encode for the two largest polyproteins which are cleaved to form sixteen non-structural proteins, four structural proteins, and eight accessory proteins. These translated polyproteins are cleaved by a virus-encoded protease. These non-structural proteins play an essential role mainly as enzymes in genome replication and initial transcription regulation. The non-structural proteins included proteases, RNA-dependent polymerase, helicase, exoribonuclease, endonuclease, and methyl transferases (Gordon et al. 2020; Alexandra et al. 2020). Further, the four structural proteins are spike protein (S), an envelope protein (E), membrane (M), and nucleocapsid protein (N). The structural proteins play an important role in the virus entry, pathogenicity, immune evasion, and many more. Spike protein (S) present on the surface is a glycoprotein and it is involved in the viral entry into the host. Spike protein is significant for the binding of the virion with host Human Angiotensin-converting Enzyme -2 (hACE2) which are distributed in the lungs, intestine, heart, and kidney. Spike proteins contain a trimer which is composed of two subunits namely S1 and S2. Among these, the S1 subunit contains the receptor binding domain (RBD) which is responsible for recognizing the receptor of the host hACE2 and binding to it. Once the binding of the virus to the host is completed, host cell protease namely TM protease serine 2 (TMPRSS2) activates the S protein and cleaves it into subunits. S2 subunit contains the Heptad repeat (HR) domain and contains fusion proteins (FP) which are also responsible for the viral fusion with the host hACE-2 cells (Miyuki et al. 2019; Alexandra et al. 2020; Huang et al. 2020). Envelope protein (E) is the smallest structural protein with a size of 8-12 kDa and has three domains namely the N-terminus domain, a transmembrane domain, and C terminal region. The E protein is responsible for assembly, budding of the virions, envelope formation, and pathogenesis (Schoeman and Fielding 2019; Sarkar and Saha 2020). Membrane protein (M) is a glycoprotein with a size of 25–30 kDa and it is the most abundant protein amongst the structural proteins and is composed of three transmembrane domains. These proteins are associated with the other structural proteins in the molecular pathogenesis of the SARS-CoV2 and facilitate the molecular assembly of the virus particles by associating with the nucleocapsid protein (N), thereby assembling the virions in hACE2 cells. M proteins were also found to join the molecules together in the endoplasmic reticulum thereby inducing apoptosis of host hACE2 cells (Yadav et al. 2021; Giuseppina et al. 2020). Nucleocapsid protein (N) is the viral protein coat with the size of 46 kDa and helps in the formation of capsids in coronaviruses. It contains five domains namely the N-terminal domain (NTD), RNA-binding domain (RBD), disordered central linker (LINK), a dimerization domain, and C-terminal domain (CTD). N protein is responsible for recognizing its viral genome and forming a capsid by oligomerization. N protein apart from this important viral life cycle event is also involved in various pathogenic effects such as deregulating its life cycle, inhibiting its cytokinesis, inhibiting its translation machinery, inducing apoptosis, inhibiting interferon, etc. in the host cells (Rota et al. 2003; Surjit and Lal 2009; Cubuk et al. 2021;).

The role of the accessory proteins of the SARS-CoV2 in the molecular mechanism and pathogenesis has not been completely understood to an extent but there is a need for continuous research to elucidate more, yet the available studies and research have indicated these accessory proteins do have an important function in molecular mechanism and pathogenesis of the SARS-CoV2. The positive-strand genome encodes for the ORF3a, ORF3b, ORF3c, ORF3d, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10 accessory proteins of SARS-CoV2 (Narayanan et al.

2008). The accessory proteins along with non-structural proteins are found to be important in the replication, and transcription, in evading host immune responses as well as the viral dissemination. They are also found to inhibit the interferons, inducing apoptosis, inhibiting the translation machinery of the host cells, arresting the host cell cycle, etc., thereby playing an important role in the activation of host cell death pathways and pathogenesis (Silvas et al. 2021).

3 SARS-COV2 and Immune System Response

SARS-CoV2 infection induces natural immune response. In this section, we will discuss the natural immune response as well as the vaccine-induced immune response. SARS – CoV2 infection is known to induce innate and adaptive immune responses in the host. The innate and adaptive immune responses involve the synthesis of pro-inflammatory cytokines which leads to the activation of T cells. As a result of this, CD4 and CD8+ T cells become activated and play a significant role in the viral cycle inhibition and clearance of already infected cells with various molecular mechanisms. It has been found that this innate immune response is activated initially by pathogen recognition receptors (PRRs) 3, 7, and 8 which are present in the immune cells. The innate immune response involves the production of various cells like IFN-ɣ interferon, interleukins such as IL-1β, IL-1RA, IL-7, IL-8, IL-10, monocyte chemoattractant peptide (MCP)-1, macrophage inflammatory protein (MIP) such as 1A and MIP-1B, granulocyte colony-stimulating factor (G-CSF) and tumor necrosis factor-alpha (TNF-α) (Abdurrahman et al. 2020; Shah et al. 2020). These signaling cascades of immune response proteins are very important for recognizing and eliminating the virus so that it is not spreading to the neighboring cells in the host. Interleukin IL-6 is found to be the main factor in the initiation of various pathologic mechanisms thereby leading to acute respiratory distress syndrome in the infected individuals. Increased/exaggerated production of IL-6 has been reported in patients with inflammatory disorders and autoimmune disorders which will lead to endothelial cell damage, cytokine storm, and capillary leak. IL-6 is also known to induce the complement system thereby inducing complex reactive proteins (CRP) (Jordan 2021).

The innate immune response which is very fast is followed by the adaptive immune response involving the T and B cells. The adaptive immune response is initiated by the complement pathway activation as well as the presentation of the virus in the epithelium by antigen-presenting cells, MHC-Class II molecules. The affected cells which may be subjected to cell cycle lysis are destroyed by CD8 T cell and Natural Killer (NK) cells with the perforin and enzymes like gran-enzymes they possess. It is followed by the recognition and presentation by dendritic cells to the CD 4 T cell. CD 4 T cell initiates the polyclonal memory cells such as Th1, Th17, and T follicular helper cells. CD4 T cell also helps the plasma cell in the production of virus-specific B cell antibodies such as IgM, IgA, and IgG (Ahmet et al. 2020). The vaccines in clinical use presently are intended to induce the neutralizing B cell antibodies. These clinically approved vaccines are proven to induce more neutralizing antibody titres when compared to the naturally infected antibody titres (Xaquin et al. 2020). The Replicating vector vaccine has been proved to induce the CD4 helper T-cell responses as well as cytolytic CD8 T-cell responses (Corbett et al. 2020). The present clinically used vaccines are also known to induce a balanced humoral and cellular immune response, especially the IgG subclass (van Doremalen et al. 2020). Previous studies also showed that inactivated SARS-CoV-2 vaccine candidate induces high levels of neutralizing antibody (Wang et al. 2020).

4 DNA Vaccines

DNA vaccines involve the introduction of plasmids which contains the gene encoding for the antigen with promoter/terminator to express. DNA vaccines are advantageous since they can induce both B and T cell immune responses involving the T Cytolytic cells, T helper cells, and B cells. The one striking advantage of DNA vaccines is they induce CD8 cytolytic T lymphocytes when compared to the other vaccines. In inactivated virus vaccines and recombinant protein vaccines, the antigen-presenting cells are MHC – class II molecules that are involved in the presentation which may induce T helper cells. In contrast DNA vaccine antigen-presenting cells are MHC – class I molecules are involved in the presentation. These MHC – Class I molecules induce the cytolytic T cells (CTL) (Liu 2003). In a scenario of emerging variants like in the case of Covid- 19, an ideal candidate should be easy to manufacture, have fewer requirements for storage, and cost of manufacturing should be less. DNA vaccines are addressing these issues and are also capable of inducing both humoral, and cellular responses (Ebony and David 2020). DNA vaccine manufacturing does not require the growth of a live virus. Swift up-scale processing can be achieved with the DNA vaccines as they employ the synthetic DNA of the antigen and this will be very impactful in the case of pandemic situations like the one, we have facing right now (Leo et al. 2018).

4.1 Advantages of DNA Vaccines

DNA vaccines have the major advantage of inducing broader immunity when compared to other vaccines. Manufacturing DNA vaccines do not require more expenses as in the case of other vaccines. DNA vaccines do not require specific transportation like RNA vaccines because of their heat stability (Abdulhaqq and Weiner 2008; Stachyra et al. 2014). Some of the common advantages of DNA vaccines are (i) induces both humoral and cellular immune response, (ii) safer when compared to inactivated vaccines which may cause infection, (iii) DNA plasmids are very

simple to create, (iv) have high stability, (v) do not require specific storage conditions like the protein/mRNA vaccines, (vi) genetic manipulation as required can be achieved with DNA plasmids, (vii) genes encoded in DNA vaccines induces stronger immune reaction as the protein expressed from the DNA vaccines are properly conformed, and (viii) manufacturing of DNA vaccines is easy, low cost and easy to transport.

4.2 Disadvantages of DNA Vaccines

Some disadvantage of DNA vaccines are also reported, among these some common are (i) activation of oncogenes, inducing anti-DNA antibodies have been reported in the experimental animals, (ii) adjuvants has to be added to increase the immunogenicity in vivo, (iii) antibody induction may be slower, (iv) lower efficacy is reported in humans, (v) need multiple doses, and (vi) there may be incorporated into the host genome (Abdo Hasson et al. 2015; Kowalczyk and Ertl 1999)

4.3 DNA vaccines for COVID-19 in preclinical trials

According to the World Health Organisation vaccine tracker and landscape as per the WHO, Covid-19 vaccines track there are sixteen DNA vaccines in Phase I/II clinical trials which are presented in Table 1 (Prompetchara et al. 2021; Meyers et al. 2021).

5 DNA vaccines in clinical trials

5.1 DIOS-CoVax2

The synthetic gene encoding the antigen of SARs–CoV2 is constructed with the 3–D model. It is aimed to induce the humoral responses (B cell immunity) as well as the T cell immunity to stop the replication of virus and cytolysis of infected host cells. DIOS-CoVax2 also aimed to reduce the adverse effects by designing the plasmid antigen without the parts of the virus known to induce such reactions in the host. The vaccine is in Phase 1/ 2 trials and found to be effective and it is said to be employing multiple delivery systems for the delivery (News University of Cambridge 2020).

5.2 SN14 vaccine

SN14 vaccine is the product of Joint research and development of Scancell, the University of Nottingham, and Nottingham Trent University. It is aimed to induce a humoral response to neutralize the spike proteins and to induce T cell response against the Spike & Nucleocapsid proteins. It is also aimed to be effective in various mutant strains as the Nucleocapsid portion is found to be conserved in the variants. The SN14 also employs monoclonal AvidiMab™ to increase the immunogenicity and specificity and

Table 1 Covid-19 DNA Vaccines in Clinical Trails

No	DNA Vaccine	Developers
1	DNA - Multiple Delivery system	DIOSynVax Ltd + University of Cambridge
2	DNA vaccine	Ege University
3	DNA plasmid vaccine RBD&N	Scancell/University of Nottingham/ Nottingham Trent University
4	DNA with electroporation	Karolinska Institute / Cobra Biologics (OPENCORONA Project)
5	DNA with electroporation	Chula Vaccine Research Center
6	Plasmid DNA, Needle-Free Delivery	Immunomic Therapeutics, Inc./EpiVax, Inc./PharmaJet
7	DNA plasmid vaccine (S,S1,S2,RBD &N)	National Research Centre, Egypt
8	DNA vaccine	BioNet Asia
9	ms DNA vaccine	MediphageBioceuticals/University of Waterloo
10	DNA vaccine	Entos Pharmaceuticals
11	DNA plasmids containing S-gene	Biosun Pharmed
12	DNA plasmid vaccine	Globe Biotech Limited, Bangladesh
13	Plasmid DNA, nanostructured RBD	National Institute of Chemistry, Slovenia
14	DNA plasmid vaccine encoding RBD	Vaccibody, Oslo Research Park, Norway
15	DNA Immunostimulatory sequences	Inserm
16	The 3 regions of SARS-Cov-2 Spike-protein: NTD, RBD, and HR1-HR2 inserted into the plasmid of PcDNA3.1.	Center of Genomics and Bioinformatics of Academy of Science of the Republic of Uzbekistan

soon it is expected to go for clinical trials (FDA news 2020; Scancell Holdings plc 2020).

5.3 Vaccine developed by Karolinska Institute and Cobra Biologics

The OPENCORONA vaccine consortium synthesized the code for N protein and other structural proteins to incorporate into the plasmid. This DNA vaccine employs a synthetic whole virus gene and electroporation technique to deliver. It is expected to undergo clinical trials in 2021 (CovidVax 2020a; Gustaf et al. 2020; Mohamadian et al. 2021).

5.4 Vaccine developed by Chula Vaccine Research Center

Researched and developed by Chula Vaccine Research Center, National Research Council of Thailand, and BioNet-Asia using electroporation for the delivery. It is constructed with synthetic DNA encoding various regions of pike protein and it is inserted into the pCMVkan expression vector. Phase 1/2 clinical trials are being conducted for this candidate (Covidvax 2020b).

5.5 EPV-CoV19 vaccine

Developed by ImmunomicTx, EpiVax, and PharmaJet, it is named EPV-CoV19. This vaccine employs synthetic peptides of T-cell epitopes from spike, membrane, and envelope aimed to induce the T cell response when vaccinated. This vaccine is expected to be effective against all strains distributed worldwide and has long-lasting T cell memory. The vaccine is expected to undergo trial sooner (CovidVax 2021a; Gustaf et al. 2020).

5.6 Mini string DNA Vaccine

It is researched and developed by Mediphage Bioceuticals with the University of Waterloo. In this DNA vaccine, ms DNA is constructed to encode the Virus-Like Particle (VLP) derived from the SARS-CoV-2 genome. This vaccine is developed to present via the nasal spray and aimed to induce both cell-mediated and humoral responses. Soon it may enter the clinical trials (CovidVax 2021b; Press Release MediphageBioceuticals, March 31st, 2020)

5.7 Covigenix VAX-001

Researched and developed by Entos Pharma and Cytiva it is named Covigenix VAX-001. This vaccine is constructed with the DNA encoding for the spike glycoprotein of the SARS – CoV2 with two genetic adjuvants. It is aimed to induce both innate and adaptive immune responses. The vaccine employs the Fusogenix delivery system for better intracellular delivery. The vaccine has completed the Phase 1 trials and has entered the Phase 2 clinical trials (CovidVax 2021c; Press Release Entos 2021; U.S. National Library of Medicine 2021).

Conclusion

With the scenario of emerging SARS-CoV2 variants and reported immune evasion by the variants, it is important to look upon the new therapeutics to curb the pandemic situation. At present, inactivated virus vaccines, mRNA vaccines, and RNA-based replicating vector vaccines are in clinical use. Even though these vaccines have been developed and approved rapidly we are facing the issue of the emergence of variants that are reported to possess immune evading properties. DNA vaccines provide various advantages such as inducing the cytolytic T cell response apart from the humoral B cell responses which cumulatively be effective in arresting viral life cycle, cytolysis of infected cells, and neutralizing antibodies. Apart from these therapeutic advantages, DNA vaccines also have the advantage of storage conditions, rapid upscaling process, and rapid manufacturing, etc., In this review, we have discussed the general properties of DNA vaccines and the candidates from this category in pre-clinical trials. We may have various DNA vaccines against Covid - 19 shortly.

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Potential of Zinc Oxide Nanoparticles as an Anticancer Agent: A Review

Hemaroopini Subramaniam[1], Sinouvassane Djearamane[1*], Lai Hock Tey[2], Ling Shing Wong[3], Piyush Kumar Gupta[4,6], Ashok Kumar Janakiraman[5]

[1]Department of Allied Health Sciences, Universiti Tunku Abdul Rahman, Perak, Malaysia
[2]Department of Chemical Sciences, Universiti Tunku Abdul Rahman, Perak, Malaysia
[3]Life Science Division, Faculty of Health and Life Sciences, INTI International University, Nilai, Malaysia
[4]Department of Life Sciences, School of Basic Sciences and Research, Sharda University, Greater Noida, India
[5]Faculty of Pharmaceutical Sciences, UCSI University, Malaysia
[6]Department of Biotechnology, Graphic Era Deemed to be University. Dehradum, Uttarakhand, India

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ABSTRACT

According to reports, one of the leading causes of mortality is cancer. Over the years, numerous approaches have been devised to lessen chronic pain and death as well as to elevate the quality of life. However, a scarcity persists in the effectiveness of cancer treatments. Early cancer identification and medication delivery with excellent specificity to reduce toxicities are two critical elements in ensuring effective cancer treatment. As a result of severe systemic toxicities and issues with current cancer diagnostic and treatment procedures, alternative nanotechnology-based techniques are being employed to improve detection and minimize disease severity. Nanotechnology has shown promising breakthroughs in cancer therapy by eliminating tumours with minimal damage to surrounding healthy cells. Since zinc is one of the necessary trace elements found in large amounts in human body tissues, zinc oxide nanoparticles (ZnO NPs) are said to be the most cost-effective and have the least hazardous characteristics of all metal oxide nanoparticles. In addition, ZnO NPs have several biological uses, notably in the field of drug administration. In this review, we tried to explore the advantage of ZnO NPs in the biomedical field, particularly in the treatment of cancer which can help to facilitate future research progress.

* Corresponding author
E-mail: sinouvassane@utar.edu.my (Dr. Sinouvassane Djearamane)

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1 Introduction

Nanoscience and nanotechnology refer to a rapidly growing field of study that includes structures, technologies, and systems having unique features and functionalities attributing to the positioning of their size on the 1–100 nm scale. This area of research attained promising awareness among the public and controversies in the early 2000s and commenced commercial applications. Nanotechnologies have a huge impact on biology, chemistry, materials science, and physics (Bayda et al. 2019). Nanomaterials' unique size-dependent property makes them essential in a variety of fields, including material sciences, medical and diagnostics, agriculture, and cosmetics (Salata 2004). Scientists have been drawn to various nanomaterials such as zinc oxide nanoparticles (ZnO NPs), gold nanoparticles (Au NPs), copper oxide nanoparticles (CuO NPs), titanium dioxide nanoparticles (TiO_2 NPs), and silver nanoparticles (Ag NPs) as a result of their distinct optical and chemical reactions that may be changed by changing the shape in various ways (Yaqoob et al. 2020). Further, the role of ZnO NPs in the pharmaceutical industry is also well known and these have antimicrobial, acaricidal, larvicidal, and anti-diabetic activities (Bala et al. 2016). In this review, we tried to explore the advantage of ZnO NPs in the treatment of cancer which can facilitate the future research program.

2 Nanotechnologies in Therapeutic Application

Nanotechnology has been used in diagnostics and molecular imaging with promising results. Cancer biomarkers such as cancer-linked proteins, circulating tumour DNA and cells, and exosomes can be detected using nanoparticles (Jia et al. 2017). In addition, visualizing tumour tissue with nanoparticles has allowed for cancer diagnosis in its preliminary phases. The metastases in lung cancer may be identified by producing superparamagnetic iron oxide nanoparticles (SPIONs) that can be employed in MRI imaging of the cancer cells as the SPIONs' target (Wan et al. 2016).

Nanotechnologies can assist physicians to diagnose at the cellular and molecular levels. Moreover, nanotechnologies further assist in point of care diagnostics, therapeutics, and the development of medicine (Riehemann et al. 2009). Many nanomaterials-based drug delivery systems including liposomes, nanomicelles, branched dendrimers, nanostructured lipid formulations, and nanoconjugates have been developed. The nanomaterials-based drug delivery systems offer present various advantages such as nontoxicity, biocompatibility, good biodegradability, and anti-inflammatory (Barani et al. 2021). For instance, liposomes can be PEGylated to extend the circulation shelf duration and prevent their elimination by the mononuclear phagocytic system (Suk et al. 2015). Qi et al. (2018) examined the efficiency of paclitaxel containing PEGylated liposomal nanoformulation (PL-PTX) in suppressing the multiplication of ovarian carcinoma cells *in vivo* and *in vitro* models. The PL-PTX-mediated treatment markedly inhibited the aggressiveness and growth of ovarian tumor cells.

The nanoparticles (NPs) used in medical treatment typically have specific sizes, shapes, and surface characteristics because these three factors have a significant impact on the effectiveness of nano-drug delivery and therefore regulate therapeutic efficacy (Bahrami et al. 2017). NPs with diameters ranging from 10 to 100 nm are commonly used in cancer therapy since they can efficiently implement drug delivery while also achieving enhanced permeability and retention (EPR) (Greish 2012). Nano-carriers in the treatment of cancer target tumour cells after absorption via the carrier effect, targeting substance, and release of the drugs into the target tumour cells to initiate cytotoxicity (Senapati et al. 2018). Meanwhile, the targeting system shields normal cells from drug cytotoxicity, which helps to mitigate the side effects of cancer (Bahrami et al. 2017).

The potential benefits of nanobiotechnologies raise great hopes in therapies for cancer and chronic lung diseases, antimicrobial agents, diabetes, and gene therapy. Metal nanoparticles, such as gold, silver, copper, and zinc nanoparticles, have been extensively studied due to their unique chemical and optical characteristics (Maslanka Figueroa et al. 2021). For instance, due to their propensity to scatter visible light, gold nanoparticles have been utilized as contrast agents in cancer detection and therapy (Kolhe and Parikh 2012). On the other hand, silver nanoparticles have attracted the interest of biomedical researchers due to their high inherent antibacterial efficacy and non-toxicity. Among the numerous possible uses of silver nanoparticles, much attention and effort have been focused on their prospective implications in wound dressing, tissue scaffolding, and protective clothing applications (Gudikandula et al. 2017). Copper has inherent antibacterial and anti-inflammatory properties, making copper-based nanomaterials a good candidate for designing microbe-resistant medical devices (Verma and Kumar 2019). Whereas ZnO nanostructures have high catalytic efficiency as well as a great adsorption ability and are more widely utilized in the production of sunscreens (Sabir et al. 2014). The characteristics of nanomaterials, such as shape, size, surface structure and charge, chemical composition, agglomeration, aggregation, and solubility, can impact how nanoparticles interact with biomolecules and cells. Biomolecules such as protein, cell surface receptors, DNA, cell membrane, and haemoglobin have similar diameters to metal nanoparticles. As a result, nanoparticles with a diameter within 50 nm have been demonstrated to interact with human cells both extracellularly and intracellularly (Barua and Mitragotri 2014).

3 Application of Nanotechnology in Cancer

Chemotherapy, radiation therapy, and surgery have long been recognized as the primary cancer treatment options. On the other

hand, these therapy approaches have a slew of negative side effects owing to their non-selective effects on cells, which means they may harm healthy cells as well. As a result, it's critical to create therapies that can kill cancer cells with minimal or no side effects to normal cells. Although metals and metal ions can control a variety of biological processes in live creatures, larger dosages of metal or metal ions supplied externally for therapeutic or diagnostic purposes might be harmful. Furthermore, it has been observed that the usage of bulk materials in medicine has disadvantages such as non-specificity and low bioavailability (Rizvi and Saleh 2018). In this light, scientists predicted that converting bulk chemicals to nanoparticulate forms could change the materials' physicochemical and biological characteristics, therefore minimizing the disadvantages (Jeevanandam et al. 2018).

Cancer nanotechnology is a branch of nanotechnology that combines many fields, including material science and physics, as well as cancer biology. This multidisciplinary collaboration has resulted in the development of devices or materials with critical components ranging from 1 to 100 nanometers, allowing for advancements in cancer treatments and tumour imaging. This developing field of nanohealth might enable the early identification of human tumours, regardless of where the original tumour or metastases are located, as well as ways to efficiently eliminate tumours and their accompanying vascular supply with minimum consequences (Zhang et al. 2019). According to Wu et al. (2013), the pH response to fluorescent nanoprobes can assist in the detection of fibroblast activated protein-a on the cell membrane of fibroblasts associated with tumours. Furthermore, after being absorbed, nanocarriers in cancer therapy target tumour cells via the carrier impact of nanoparticles and the positioning effect of the targeted material. The medications are then delivered to tumour cells to destroy them (Chen et al. 2010). Due to their size and surface features, nanocarriers can elevate permeability and retention which lead to the enhanced half-life of medications and cause their accumulation in tumour tissues (Bertrand et al. 2014; Kalyane et al. 2019).

3.1 Zinc Oxide Nanoparticles in Cancer Therapy

ZnO NPs have a molecular weight of 81.38 g/mol and appear as white odourless powder. It has a wurtzite crystal structure and is a semiconductor with a large band gap (3.37 eV at ambient temperature). The tiny size of ZnO NPs allows zinc to be absorbed more easily by the body. Zinc stands out among metals due to its strong reducing potential, mild reactivity, and five stable isotopes, as well as its wide range of applications and environmental suitability (Keerthana and Kumar 2020). ZnO NPs' easier synthesis and functionalization due to the presence of -OH group, biodegradability, biocompatibility, multiple loading of drugs or therapeutic molecules, and economical biodegradability and low toxicity have enhanced their use in cancer drug delivery more than other nanoparticles (Mocchegiani et al. 2013).

Since zinc oxide does not interact with key pharmacological active ingredients, it is an excellent option for drug delivery (Beyene et al. 2021). ZnO NPs were found to be capable of targeting multiple cancer cell types as well as inhibiting cancer cell proliferation, sensitizing drug-resistant cancer, preventing cancer recurrence and metastasis, and reviving cancer immune surveillance (Wang et al. 2017; Moghaddam et al. 2017; Aalami et al. 2020). The use of NPs in targeted medication delivery has been a promising area of study for cancer therapy. The number of medicines used to treat malignant cells will be reduced. Due to the intricacy of cancer, an ideal anticancer treatment would target cancer cells and the tumour microenvironment.

Combining conventional cancer treatments with NPs is one way to minimize their toxicity (Vinardell and Mitjans 2015). *In vitro* combination of ZnO NPs with paclitaxel, cisplatin or daunorubicin improves the efficacy of these chemotherapeutics (Hackenberg et al. 2012). Different-sized ZnO NPs may substantially increase drug targeting and accretion of daunorubicin in leukemia cancer cells and therefore function as an effective agent to improve drug delivery (Guo et al. 2008). In a similar study, it was reported that loading doxorubicin, an anticancer medication, inside ZnO NPs resulted in greater anticancer activity than either ZnO NPs or doxorubicin alone. It was believed that the drug's cytotoxic potential was enhanced as a result of the synergy created by the drug's anticancer effect combined with that of ZnO NPs (Sharma et al. 2016).

The Food and Drug Administration (FDA) has authorized zinc oxide nanoparticles as a new and effective anticancer treatment (Anvarinezhad et al. 2020). In addition to producing reactive oxygen species (ROS), ZnO NPs can cause selective cytotoxicity against cancer cells by inducing disequilibrium of zinc-dependent protein activity (Table 1) (Wahab et al. 2014). ZnO NPs harm cancer cell lipids, protein, and nucleic acid when there are high amounts of ROS and oxidative stress (Valko et al. 2006) (Figure 1). Elevated ROS can damage cell membranes by protein denaturation and lipid peroxidation, resulting in necrosis, and DNA damage, culminating in apoptosis (Wang et al. 2017). Highly reactive ROS species can react with DNA components, changing DNA composition and causing mutations. The OH radical, a highly reactive oxygen species, induces single-stranded DNA breaking (Phaniendra et al. 2015). These DNA breaks and crosslinks cause DNA damage, which activates a mitochondrial apoptotic pathway, resulting in cell death by apoptosis (Mishra et al. 2017). The primary mechanism of cell death in this cytotoxic reaction of ZnO NPs is thought to be apoptosis, or programmed cell death (Bisht and Rayamajhi 2016).

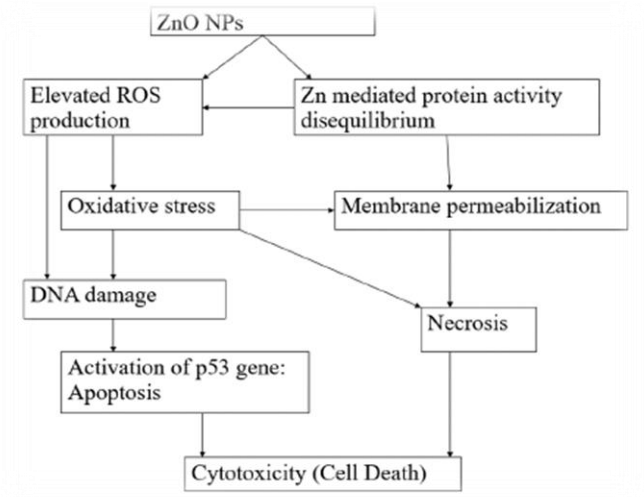

Figure 1 A schematic outline of the mechanism of cytotoxicity of ZnO NPs which leads to cell death (Bisht and Rayamajhi 2016).

Table 1 Anticancer activity of ZnO NPs on different types of cancer cell lines

Cancer cell line	Key observations	Reference(s)
Human ovarian cancer cells (SKOV3)	Dose-dependent loss of cell viability and presence of characteristic apoptotic features such as loss of adherence to enhanced ROS generation and loss of mitochondrial membrane potential	Bai et al. 2017
Human epidermal carcinoma cell line (A431)	Generation of ROS and cell cycle arrest in S and G2/M phase with the higher uptake in G2 M phase compared with other phases	Patel et al. 2016
Human liver hepatocellular carcinoma (Hep-G2)	Dose-dependent cytopathic effects, caspase-3 activation, and apoptosis	Chung et al. 2015
Human breast cancer cell line (MCF-7)	Disequilibrium of zinc-dependent protein activity	Wahab et al., 2014
Human pulmonary adenocarcinoma cell line (LTEP-a-2)	Increase in ROS coincided with depletion of GSH in apoptotic cells, suggesting that oxidative stress may be the primary toxicological mechanism	Wang et al., 2015
Human neuroblastoma cells (SHSY5Y)	ZnO NPs induced significant cytotoxicity in a size-dependent manner and reactive oxygen species generation was the main factor that lead to a decrease in cell viability and apoptosis	Liu et al., 2017
Human cervical adenocarcinoma cells (HeLa)	mRNA expression of apoptotic gene p53 and level of ROS increased in a dose-dependent manner	Pandurangan et al., 2016
Human skin melanoma (A375)	Significant decrease in cell viability and generation of ROS. Apoptosis confirmed by chromosomal condensation assay and caspase-3 activation	Alarifi et al., 2013; Mishra et al., 2017
Human epithelial colorectal adenocarcinoma (Caco-2)	Significant reduction in glutathione and increase in ROS and lactate dehydrogenase	Kang et al., 2013; Song et al., 2014

According to previous studies, ZnO NPs might be used as a pH-dependent medication carrier with improved cellular absorption and tumour spheroid penetration (Muhammad et al. 2011; Zare et al. 2019). ZnO NPs have been shown to efficiently inhibit cell adhesion, migration, and carcinogenesis in cancer (stem-like) cells, as well as sensitize medication therapy. According to these findings, ZnO NPs offer a lot of promise as a multifunctional nanomedicine for anticancer research (Hamrayev et al. 2020).

Nanomedicine is still a technology-driven field with many scientific difficulties ahead of it. It does, however, represent a developing sector with the potential to meet the long-standing demand for new and improved anti-cancer medicines. According to Rasmussen et al. (2010), NPs are not always similar, different from batch to batch, and they may change in surface chemistry or size distribution and thus eliciting diverse biological responses. There is currently inadequate *in vivo* evidence to determine the biological impact of these materials on inflammation and functional modifications at the cellular or whole-body level. ZnO NPs can accumulate in the body and induce organ toxicity or disintegration in unanticipated ways. The high solubility of the particles was attributed to the harmful consequences, which

included cytotoxicity and mitochondrial dysfunction. There is a need to assess if the potential benefits of these nanomedicines exceed the possible risks associated with nanotoxicity (Condello et al. 2016). ZnO NPs have shown remarkable potential in cancer detection and therapy; nevertheless, further in-depth and sophisticated study of ZnO NPs, extensive knowledge of the cellular and molecular processes, and clinical trials are necessary in the future for improved cancer theranostic outcomes and to assess their long-term health concerns (Anjum et al. 2021).

3.2 The Advantages of Utilization of Zinc Oxide Nanoparticles

ZnO NPs can be synthesized via physical, chemical, and biological methods or top-down or bottom-up processes. For the production of NPs, the green synthesis technique is more advantageous than other traditional methods (Gour and Jain 2019). This is owing to its environmentally responsible strategy, which avoids the use of harmful chemicals. Furthermore, this technique does not need high pressure or temperature. The production of ZnO NPs using environmentally friendly ingredients such as plant extract, bacteria, fungus, and enzymes offer advantages in terms of compatibility with pharmaceutical and other biomedical applications (Kalpana and Devi Rajeswari 2018). The bottom-up method of nanoparticle biosynthesis is based on the reduction/oxidation process. Metal compounds are reduced into nanoparticles by microbial enzymes or plant phytochemicals with antioxidant or reducing capabilities (Ovais et al. 2018). For the method to work, the solvent medium utilized for synthesis, the choice of an eco-friendly reducing agent, and a non-toxic substance for nanoparticle stabilization are also critical (Gour and Jain 2019).

Another characteristic of ZnO NPs is their ability to produce ROS, which can cause cell death when the level of ROS exceeds the cell's antioxidant capacity (Ancona et al. 2018). Zinc is an essential cofactor in a variety of cellular mechanisms. This element is crucial in the regulation of enzymes in the body that are involved in the synthesis and degradation of lipids and proteins, thereby maintaining homeostasis (Skrajnowska and Bobrowska-Korczak 2019). Furthermore, the neutral hydroxyl groups of ZnO NPs can be easily functionalized by a variety of biocompatible molecules and thus increasing their biocompatibility (Abdelmigid et al. 2022).

Aside from specificity, targeted NPs provide other therapeutic benefits such as multidrug conjugation, simple release kinetics adjustment, selective localization, and bypassing multidrug resistance mechanisms (Jiang et al. 2018). Many functionalizations and surface modification strategies to reduce unwanted toxicity and improve the biocompatibility of NPs have been studied (Kim et al. 2017). For instance, surface functionalization of ZnO NPs can be done with various types of biological molecules, such as proteins, peptides, and nucleic acids (Namvar et al. 2016; Othman et al. 2016). The anticancer action of ZnO NPs is unaffected by the biocompatible coating, but the targeting effects on cancer cells are enhanced, and the safety against normal cells is improved (Jiang et al. 2018).

Namvar et al. (2016) used green synthesis to create a hyaluronan-ZnO nanocomposite (HA/ZnO) for cancer therapy. In a dose- and time-dependent manner, the HA/ZnO nanocomposites produced morphological alterations and reduced the growth of pancreatic adenocarcinoma PANC-1 cell, ovarian adenocarcinoma CaOV-3 cell, colonic adenocarcinoma COLO205 cell, and acute promyelocytic leukemia HL-60 cell. The normal human lung fibroblast (MRC-5) cell line did not show any toxicity after being exposed to the HA/ZnO nanocomposite for 72 hours. In another research, peptide-conjugated ZnO NPs were shown to improve the targeting effects of ZnO NPs on MDA-MB-231 cells at lower dosages (Othman et al. 2016). Also, when HT29 cells were treated with peptide ZnO NPs conjugates, there was an increase in cytotoxicity compared to ZnO NPs or peptide therapy alone (Bai Aswathanarayan et al. 2018). This indicates that functionalizing NPs improves their ability to kill cancer cells, making them a promising class of nanodrugs for cancer treatment (Bai Aswathanarayan et al. 2018).

Conclusion

Nanotechnology has had a transformative influence on biomedicine, with remarkable progress over the previous several decades. Nanomaterials can display characteristics unique from molecules and bulk solids, and enable novel interactions with biomolecules on the surface and inside cells. Because of their intrinsic capacity to promote ROS production and trigger apoptosis, ZnO NPs, like other metal oxides NPs, offer great medicinal potential. ZnO NPs have these properties, making them effective anticancer and antimicrobial agents. ZnO nanoparticles are ideal candidates as biodegradable, biocompatible, "deliver and dissolve" platforms for cancer therapy because of their numerous appealing physicochemical characteristics and huge promise for different biomedical applications. When loaded and given with other medicinal treatments, ZnO NPs have been shown to have synergistic effects. Given the future potential of ZnO NPs, a greater understanding of their toxicity is crucial. Over the next few decades, it is predicted that research into biomedical uses of ZnO NPs would thrive, with new advances in this burgeoning field.

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Application of Soil Bacteria as Bioinoculants to Promote Growth of Cowpea (*Vigna unguiculata*)

Kedsukon Maneewan, Sirirat Deeseenthum, Worachot Saengha, Thipphiya Karirat, Vijitra Luang-In*

Natural Antioxidant Innovation Research Unit, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Maha Sarakham 44150, Thailand.

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ABSTRACT

This work aimed to evaluate the capacity of soil bacteria as bioinoculants (biofertilizers) to promote cowpea (*Vigna unguiculata*) growth. Three pure bacterial cultures namely *Acinetobacter pittii* PT1.3.4 (AP), *Achromobacter* sp.C2.23 (AS), and *Achromobacter xylosoxidans* N3.4 (AX) were used as bioinoculants to enhance germination and development of cowpea seeds. Pre-decide formulations of single or mixed cultures were prepared, soaked with cowpea seeds, and cultivated on agar in a growth chamber for 7 days at 25°C. Shoot and root length were measured and percentage germination was determined. Similarly, bacterial formulations were prepared in talcum powder and were used as bioinoculants to adhere to cowpea seeds. The inoculated seeds were cultivated in pots for 28 days for the shoot and root length, fresh and dry weight, and percentage germination. Among the tested various formulations, treatment has *A. pittii* (AP) displayed the highest shoot length (14.67 cm) and fresh weight (0.58 g/plant) of cowpea under laboratory conditions after seven days of inoculation. Similarly, cowpea plants treated with *A. pittii* (AP) also have the tallest shoots (14.25 cm) under natural conditions after 7 days of inoculation, while the highest root length (10.5 cm) and fresh weight (1.57 g/plant) were recorded from the treatment of *Achromobacter* sp. (AS). Further, the results of the study also revealed that soil bacteria can survive for one month in talcum powder at 4°C and room temperature storage. These bioinoculants can be used for agricultural application by local farmers to mitigate the cost of chemicals that cause environmental concerns to promote sustainable agriculture in Thailand.

* Corresponding author

E-mail: vijitra.l@msu.ac.th (Vijitra Luang-In)

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1 Introduction

Bioinoculation of plants with nitrogen-fixing bacteria is considered more ecofriendly, reduced the cost of fertilization, helps in sustainable agriculture, and can be used as an alternative to agrochemicals. Bacterial bioinoculants have been widely used to improve plant growth and yields and minimize the threat of plant diseases (Kalia et al. 2020). Among the most commonly available bioinoculants, *Azotobacter*, *Rhizobium*, *Azospirillum*, and *Burkholderia* strains are now marketed as agricultural biofertilizers and bioinoculants (Maitra et al. 2021). Reena Josephine and Thomas (2022) isolated *Acinetobacter pittii* F25 (Accession no. KM677194) from the maize rhizosphere soil and found plant growth stimulating effects including ammonia production, inorganic and organic phosphate solubilization, and nitrogen fixation. The capacity of this strain to promote plant development in sustainable agriculture was demonstrated under laboratory and greenhouse conditions by improved shoot and root length as well as increased biomass in treated maize seedlings compared to uninoculated groups. Intriguingly, Liu et al. (2022) constructed a synthetic bacterial community (SynCom) from the bacteria isolated from the wheat rhizosphere; this synthetic bacterial community included three species of *Bacillus*, two species of *Acinetobacter*, one species of each *Enterobacter*, *Xanthomonas*, and *Burkholderia*. These researchers reported a significant effect of this SynCom on the growth, root development, and biomass output of wheat plants.

Achromobacter is a gram-negative straight rod-shaped motile bacterial genus that belongs to the family Alcaligenaceae order Burkholderiales. Members of this genus are found in soil and both fresh and saltwater (Isler et al. 2020) and have various agricultural benefits. In addition to nitrogen fixation, *Achromobacter xylosoxidans* BOA4 exhibits some other significant plant growth promoting biochemical features, including siderophore and IAA synthesis and phosphate solubilization (Rai et al. 2018). Recently three important bioinoculant bacteria namely *A. pittii* PT1.3.4 as phytase producer (AP), *Achromobacter* sp. C2.23 as chitinase producer (AS) and *A. xylosoxidans* N3.4 as nitrogen fixer (AX) were isolated from the untapped resource of Nasinuan Community Forest, Kantharawichai District, Maha Sarakham Province, Thailand (Luang-In et al. 2021) were used as bioinoculants to study the impact on germination and growth of the local economic plant cowpea both in the laboratory environment and in soil.

In Thailand, cowpea (*Vigna unguiculata*) is an economic crop widely grown in all regions, especially in the north and northeast regions. It can be consumed as fresh pods or dry seeds, and are a rich source of carbohydrates and proteins (Haisirikul et al. 2020). Cowpea cultivation has various advantages including short harvest, drought tolerance, and easy cultivation on farmlands and paddy fields after harvest. In this study, the capacity of three bacterial strains i.e. AP, AS and AX isolated from the soil of Nasinuan Community Forest (both single strains and multi-strain formula) were evaluated to promote the growth of cowpea under laboratory and field conditions.

2 Materials & Methods

2.1 Isolation and cultivation of bacterial isolates

Three *Achromobacter* bacterial strains i.e. *A. pittii* PT1.3.4 (AP), *Achromobacter* sp. C2.23 (AS) and *A. xylosoxidans* N3.4 (AX) were isolated from the soil of Nasinuan Community Forest, Kantharawichai District, Maha Sarakham Province, Thailand (Luang-In et al. 2021). Phytase-specific agar, chitin agar, and nitrogen-free agar were used as selective media to isolate AP, AS and AX, respectively as described by Luang-In et al. (2021). Phytase producers and chitinase producers' bacteria displayed clear zones around the colonies on selective media while nitrogen fixers turned green to blue media. The positive colonies were point inoculated on selective agars. The diameter of the clear zone or the blue area over the diameter of the colony was measured as the halo:colony ratio for identifying the most potential isolates as a phytase producer, chitinase producer, and nitrogen fixer. The bacteria AP, AS and AX with greater potential for use as bioinoculants were cultured in nutrient broth for 20 h and then spun down for 15 min at 10,000g. The cell pellets were resuspended in sterile water until OD_{600nm} reached 1.0 (1×10^8 CFU/mL).

2.2 Effect of bioinoculants on *in-vitro* cowpea seed germination and sprout growth

Cowpea seeds were purchased from Chia Tai Co., Ltd. Seeds with uniform size were selected and surface-sterilized by soaking in 10% Chlorox with 2 drops of Tween 20 for 15 min, this was followed by the washing of these seeds with sterile water. After this, seeds were soaked in a bacterial suspension of 1×10^8 CFU/mL concentration for 1 hour and following treatments (10 seeds per treatment in triplicate) were imposed: (T_1) AP (15 mL), (T_2) AS (15 mL), (T_3) AX (15 mL), (T_4) AP + AS (7.5 + 7.5 mL), (T_5) AS + AX (7.5 + 7.5 mL), (T_6) AP + AX (7.5 + 7.5 mL), (T_7) AP + AS + AX (5 + 5 + 5 mL) and (T_8) 15 mL water (control). Then seeds were placed on a Petri dish filled with ½ strength of Murashige and Skoog (MS) medium and allowed to germinate and grow for 7 days at 25°C in a 16 h light/8 h dark period in a growth chamber. Seed germination percentage, length of cowpea sprouts, fresh weight, and dry weight were recorded after 7 days.

2.3 Effect of bioinoculants on *in-vivo* cowpea growth in a pot experiment

For the pot experiment, 10 g of autoclaved talcum powder was mixed with predefined bacterial suspension concentration i.e. 1

$\times 10^8$ CFU/mL, and various treatments from T_1 - T_8 were formulated as per laboratory experiments while for field study an additional treatment T_9 (Talc powder) was formulated. Ten healthy, equal-sized cowpea seeds were adhered with each bioinoculant formulation uniformly per treatment in triplicate. Cowpea seeds were allowed to grow for 7, 14, 21, and 28 days in a pot containing autoclaved soil (1.5 kg per pot) under field conditions. Within one week, three or four plants per pot were reduced to one plant per pot. The plants were watered every day until harvested. At harvest, the pots were poured into a 2 mm sieve and the soil was carefully cleansed to separate the shoots and roots. The proportion of seeds that germinated, length of cowpea plants and roots, fresh weight, and dry weight were all reported.

2.4 Soil analysis

The pH and levels of phosphorus, nitrogen, and potassium in the top layer of the soil (0-20 cm) in the pot experiment were determined before and after the experiment using a Rapitest 1835 Digital 3-way soil analyzer (Luster Leaf, USA).

2.5 Storage condition of bioinoculants

The bioinoculants were stored in talcum powder at room temperature (35°C) and in a refrigerator at 4°C for 28 days. Microbial count (CFU/mL) was measured by the viable plate count method at intervals of 7 days until 28 days.

2.6 Statistical analysis

Completely randomized design (CRD) was implemented to conduct the study. Mean differences ± standard deviations of triplicates were compared using analysis of variance (ANOVA) and Tukey's multiple comparisons with GraphPad Prism 8.0 (GraphPad Software, Inc.). Statistically significant differences were considered at $p< 0.05$.

3 Results

3.1 Bioinoculants promoted the growth of cowpea

Seed germination percentages in all treatment and control were reached 100% after 7 days of inoculation on ½ strength MS agar medium. Among the tested formulations highest fresh weight (0.58 g/plant) and longest shoots (14.67 cm/plant) were reported in the seeds treated by AP after 7 days of treatment under laboratory conditions (Figure 1A; Table 1). The longest roots (7.17 cm/plant) were reported from the combined application of AP + AX. Further, plant fresh weight was not showing any significant difference among various formulations, while dry weight was significantly different among the various treatments (Table 1). This showed that bioinoculants significantly promoted the growth of cowpea on ½ MS agar under laboratory conditions compared with the control.

The effect of bioinoculants on cowpea growth in a pot experiment under field conditions for 7, 14, 21, and 28 days was also studied (Figure 1; Table 2). Like laboratory conditions, the longest shoot on days 7^{th}, $14^{th,}$ and 21^{st} (14.25, 19.50, 21.75 cm/plant respectively), was reported in the plants treated with bioinoculants AP (T_1) (Figure 1B, 1C, 1D; Table 2), while, on the day 28^{th}, longest shoots i.e. 32.00 cm/plant were recorded in the pot treated with AS (T_2) (Figure 1E and 1F; Table 2). Similarly, on day 7^{th}, the longest roots (10.5 cm/plant) was reported from the pots treated with bioinoculants AS (T_2), while on day 14 and 21, the highest root length (10.13 cm/plant and 11.75 cm/plant, respectively) was reported from the pot treated with AX (T_3) and on day 28, it was reported 12.67 cm/plant from the plants treated with AP (T_1), in this manner a mixed response was reported in case of root length and it varies with the time spent. In the case of a combination of various bioinoculants, no significant differences were reported during the study. Further, in the case of fresh weight, on day 7^{th} and 14^{th}, the highest fresh weight (1.57 g/plant and 1.96 g/plant, respectively) was reported in the plants treated with bioinoculants

Table 1 Effect of bioinoculants on cowpea growth in ½ MS media under laboratory conditions for 7 days

Treatment	Germination (%) on day7	Length (cm/plant)		Fresh weight (g/plant)	Dry weight (g/plant)
		shoot	root		
AP	100	14.67± 0.75^a	3.07±0.98^c	0.58 ± 0.09	0.06 ± 0.01^a
AS	100	11.83± 0.50^b	4.73 ± 0.85^b	0.35 ± 0.09	0.03 ± 0.00^c
AX	100	7.67± 0.35^c	2.67 ± 0.51^d	0.28 ± 0.16	0.02 ± 0.00^c
AP+ AS	100	13.87 ± 0.64^a	6.33 ± 0.85^a	0.58 ± 0.12	0.06 ± 0.01^a
AS + AX	100	12.93 ± 0.65^b	7.17 ± 0.35^a	0.51 ± 0.16	0.05 ± 0.00^a
AP + AX	100	11.67 ± 0.55^b	2.23 ± 0.68^d	0.41 ± 0.18	0.04 ± 0.00^b
AP+ AS+ AX	100	10.83 ± 0.31^b	3.87 ± 0.76^b	0.41 ± 0.19	0.04 ± 0.01^b
Water (control)	100	2.47 ± 0.81^d	2.87 ± 0.71^d	0.24 ± 0.10	0.03 ± 0.00^c

* Disinct superscripts in columns represent statistically significant differences ($p< 0.05$)

Table 2 Effect of bioinoculants on cowpea growth in the pot experiment under field conditions

Treatment	Length (cm/plant)								Fresh weight (g/plant)			
	Day 7		Day 14		Day 21		Day 28		Day 7	Day 14	Day 21	Day 28
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root				
AP (T_1)	14.25 ± 0.25^{a}	5.50 ± 0.36^{c}	19.50 ± 0.50^{a}	3.70 ± 0.61^{b}	21.75 ± 0.75^{a}	8.25 ± 0.75^{b}	29.50 ± 1.80^{a}	12.67 ± 0.58^{a}	1.14 ± 0.07^{a}	1.61 ± 0.00^{a}	2.60 ± 0.36^{a}	3.95 ± 0.31^{a}
AS (T_2)	13.75 ± 0.75^{a}	10.50± 0.00^{a}	14.07 ± 0.12^{c}	5.23 ± 0.40^{b}	20.75 ± 0.75^{a}	10.50 ± 0.50^{ab}	32.00 ± 2.65^{a}	9.30 ± 0.58^{b}	1.57 ± 0.14^{a}	1.96 ± 0.15^{a}	2.07 ± 0.21^{a}	3.72 ± 0.45^{a}
AX (T_3)	13.25 ± 0.75^{a}	8.75 ± 0.25^{b}	14.07 ± 0.12^{c}	10.13 ± 2.23^{a}	20.25 ± 0.75^{a}	11.75 ± 1.25^{a}	23.00 ± 0.87^{b}	9.17 ± 1.04^{b}	1.33 ± 0.04^{a}	1.53 ± 0.15^{a}	1.37 ± 0.40^{c}	3.14 ± 0.45^{a}
AP+ AS (T_4)	12.5 ± 1.00^{a}	6.25 ± 0.25^{c}	14.83 ± 0.29^{c}	7.07 ± 0.12^{b}	16.50 ± 1.00^{d}	7.00 ± 1.00^{bc}	24.33 ± 0.58^{b}	9.67 ± 1.53^{b}	1.25 ± 0.18^{a}	1.58 ± 0.24^{a}	1.83 ± 0.25^{a}	3.55 ± 0.29^{a}
AP+ AX (T_5)	11.75 ± 0.25^{a}	9.00 ± 0.50^{b}	13.00 ± 0.00^{d}	4.33 ± 0.58^{b}	18.00 ± 0.50^{b}	4.50 ± 1.50^{c}	25.00 ± 0.00^{b}	10.67 ± 0.58^{a}	1.12 ± 0.17^{a}	1.67 ± 0.36^{a}	1.33 ± 0.06^{c}	3.30 ± 0.35^{a}
AS+ AX (T_6)	11.25 ± 0.25^{b}	8.00 ± 0.00^{b}	14.47 ± 0.57^{c}	7.53 ± 0.84^{b}	17.50 ± 0.50^{c}	10.25 ± 0.75^{ab}	21.17 ± 1.26^{b}	8.50 ± 1.04^{c}	1.29 ± 0.23^{a}	1.40 ± 0.20^{a}	1.67 ± 0.21^{b}	3.01 ± 0.11^{a}
AP+ AS+ AX (T_7)	10.75 ± 0.75^{b}	7.25 ± 1.25^{b}	13.83 ± 0.76^{d}	9.03 ± 0.06^{a}	20.00 ± 0.00^{a}	9.00 ± 0.00^{b}	22.50 ± 1.80^{b}	9.00 ± 0.87^{c}	0.95 ± 0.09^{b}	1.48 ± 0.16^{a}	1.73 ± 0.15^{b}	3.16 ± 0.88^{a}
Water (T_8)	7.75 ± 0.75^{c}	2.70 ± 0.75^{d}	16.33 ± 1.53^{b}	4.33 ± 1.04^{b}	18.75 ± 0.25^{b}	8.00 ± 0.00^{bc}	20.83 ± 1.89^{b}	8.00 ± 1.00^{c}	0.69 ± 0.24^{b}	1.10 ± 0.21^{b}	2.17 ± 0.06^{a}	1.69 ± 0.13^{b}
Talc (T_9)	9.50 ± 2.00^{c}	4.50 ± 1.00^{c}	12.50 ± 0.50^{d}	9.50 ± 3.04^{a}	15.25 ± 2.75^{d}	6.00 ± 2.00^{bc}	20.83 ± 0.29^{b}	10.50 ± 1.32^{a}	0.73 ± 0.33^{b}	1.21 ± 0.32^{b}	1.60 ± 0.46^{b}	2.07 ± 0.39^{b}

* Disinct superscripts in columns represent statistically significant differences (p< 0.05).

Table 3 Effect of bioinoculants on soil N and K levels in the pot experiment

Treatment	Soil N and K levels (ppm)					Soil P level (ppm)				
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 0	Day 7	Day 14	Day 21	Day 28
AP (T_1)	74 ± 12	134 ± 12	100 ± 18^{ab}	80 ± 0^{cd}	80 ± 0^{cd}	5.6 ± 0.8	9.6 ± 0.8	7.3 ± 1.2^{ab}	6.0 ± 0.0^{cd}	6.0 ± 0.0^{cd}
AS (T_2)	80 ± 15	158 ± 15	100 ± 18^{ab}	110 ± 0^{ac}	110 ± 0^{ac}	6.0 ± 0.0	11.2 ± 1.0	7.3 ± 1.2^{ab}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
AX (T_3)	80 ± 0	155 ± 15	110 ± 0^{a}	110 ± 0^{ac}	110 ± 0^{ac}	6.0 ± 0.0	11.0 ± 1.0	8.0 ± 0.0^{a}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
AP+ AS (T_4)	83 ± 12	155 ± 15	100 ± 18^{ab}	110 ± 0^{ac}	110 ± 0^{ac}	6.2 ± 0.8	11.0 ± 1.0	7.3 ± 1.2^{ab}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
AP+ AX (T_5)	83 ± 12	143 ± 12	100 ± 18^{ab}	110 ± 0^{ac}	110 ± 0^{ac}	6.2 ± 0.8	10.2 ± 0.8	7.3 ± 1.2^{ab}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
AS+ AX (T_6)	80 ± 0	143 ± 12	100 ± 18^{ab}	140 ± 30^{ab}	140 ± 30^{ab}	6.0 ± 0.0	10.2 ± 0.8	7.3 ± 1.2^{ab}	10.0 ± 2.0^{ab}	10.0 ± 2.0^{ab}
AP+ AS+ AX (T_7)	80 ± 0	143 ± 12	100 ± 18^{ab}	125 ± 15^{b}	125 ± 15^{b}	6.0 ± 0.0	10.2 ± 0.8	7.3 ± 1.2^{ab}	9.0 ± 1.0^{b}	9.0 ± 1.0^{b}
Water (T_8)	80 ± 0	143 ± 12	80 ± 0^{b}	110 ± 0^{ac}	110 ± 0^{ac}	6.0 ± 0.0	10.2 ± 0.8	6.0 ± 0.0^{b}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}
Talc (T_9)	80 ± 0	140 ± 12	110 ± 0^{a}	110 ± 0^{ac}	110 ± 0^{ac}	6.0 ± 0.0	10.0 ± 0.8	8.0 ± 0.0^{a}	8.0 ± 0.0^{ac}	8.0 ± 0.0^{ac}

* Disinct superscripts in columns represent statistically significant differences (p< 0.05).

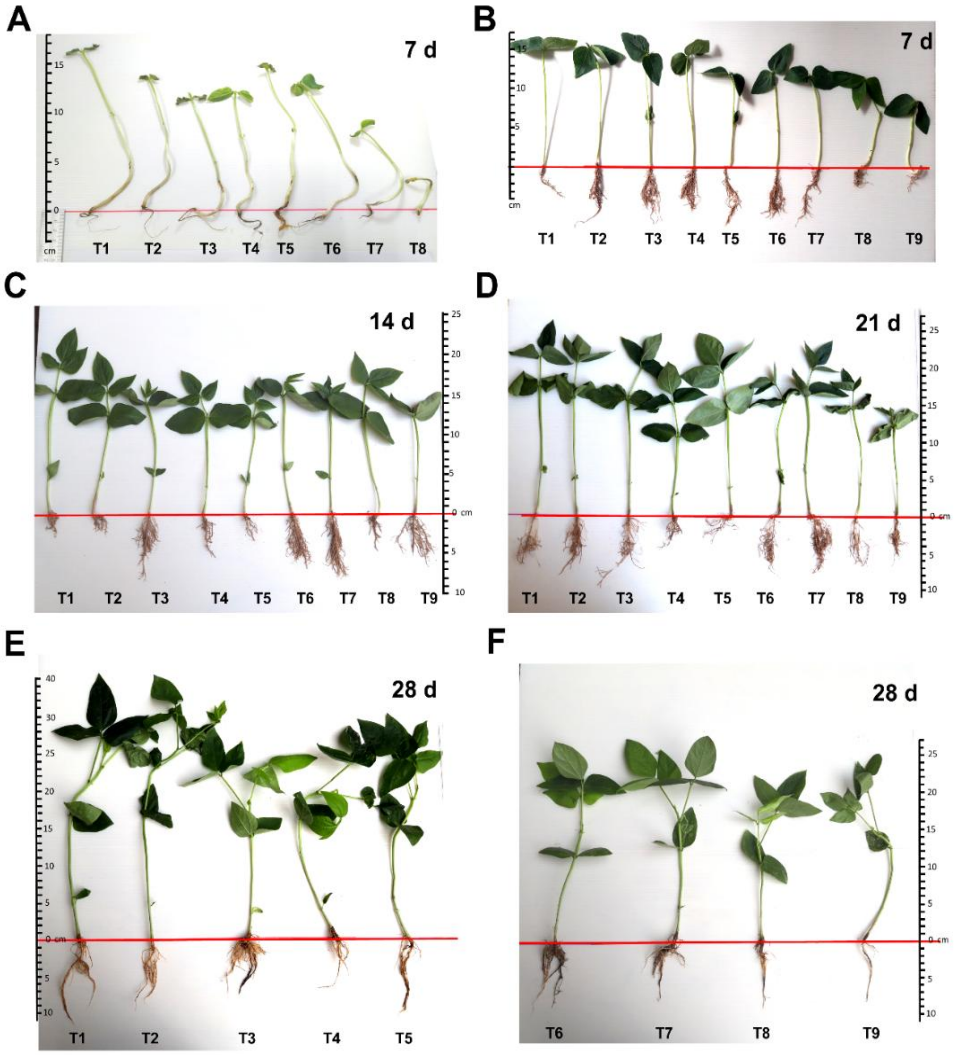

Figure 1 Growth of cowpea (A) on ½ strength MS liquid media under laboratory conditions after 7 days of bioinoculants treatment; Growth of cowpea in pot experiment under field conditions (B) at 7 days; (C) at 14 days; (D) at 21 days; (E) at 28 days (T1-T5); (F) at 28 days (T6-T9)

AS (T_2), while on day 21st and 28th, highest fresh weight (2.60 g/plant and 3.95 g/plant, respectively) was recorded from the plants treated with bioinoculant AP (T_1). Dry weight was not significantly different (data not shown) for all treatments. These findings underscored that bioinoculants significantly promoted the growth of cowpea in the pot experiment under field conditions at 7, 14, 21, and 28 days compared with the controls (water or talcum only).

3.2 Effect of bioinoculants on soil fertility levels and soil pH

The collected soil samples were analyzed as per the manual of a Rapitest 1835 Digital 3-way soil analyzer and reported optimal levels of nitrogen (N), phosphorus (P), and potassium (K) in the range of 50-200 ppm, 4-14 ppm and 50-200 ppm, respectively. Results of soil fertility properties showed that on day 0, soil Nitrogen and potassium levels were 74-83 ppm (Table 3) and these mineral levels significantly increased to 134-158 ppm on day 7; however, on day 21-28, the level of Nitrogen and potassium declined to 80-140 ppm. Likewise, the level of phosphorus followed the same trend (Table 3) and on day 0, the P level was 5.6-6.2 ppm which was significantly increased on day 7 (9.6-11.2 ppm). However, on day 28, the P level dropped to 6-10 ppm (Table 3).

The case of optimal soil pH for general plants ranged from 6.5-7.0. On day 0, soil pH ranged from 6.1-6.8 (normal), but after 7 days there was a significant decrease in soil pH ranging from 5.5-6.0, indicating increased acidity (Table 4). On days 14 and 21, soil pH increased to neutral at 7.0 with no significant difference in all treatments, while on day 28 the pH decreased to a weakly acidic level of 5.93-7.13 (Table 4).

Table 4 Effect of bioinoculants on soil pH in the pot experiment

Treatment	Soil pH				
	Day 0	Day 7	Day 14	Day 21	Day 28
AP (T_1)	6.8 ± 0.0^{a}	6.0 ± 0.3^{a}	7.00 ± 0.00	7.00 ± 0.00	5.93 ± 0.12^{c}
AS (T_2)	5.8 ± 0.3^{e}	5.7 ± 0.0^{a}	7.27 ± 0.46	6.60 ± 0.10	6.00 ± 0.00^{c}
AX (T_3)	6.1 ± 0.1^{de}	5.7 ± 0.1^{a}	7.00 ± 0.00	7.00 ± 0.00	6.00 ± 0.00^{c}
AP+ AS (T_4)	6.3 ± 0.1^{cd}	5.5 ± 0.1^{b}	7.00 ± 0.00	7.00 ± 0.00	5.90 ± 0.17^{c}
AP+ AX (T_5)	6.4 ± 0.1^{bcd}	5.7 ± 0.2^{a}	6.93 ± 0.12	6.60 ± 0.40	6.07 ± 0.12^{c}
AS+ AX (T_6)	6.3 ± 0.1^{cd}	5.5 ± 0.1^{b}	7.00 ± 0.00	7.00 ± 0.00	6.10 ± 0.17^{b}
AP+ AS+ AX (T_7)	6.5 ± 0.1^{abc}	5.7 ± 0.2^{a}	7.10 ± 0.17	6.90 ± 0.10	6.20 ± 0.00^{b}
Water (T_8)	6.2 ± 0.1^{cd}	5.7 ± 0.2^{a}	7.00 ± 0.00	7.00 ± 0.00	6.67 ± 0.58^{ab}
Talc (T_9)	6.3 ± 0.1^{cd}	5.8 ± 0.1^{a}	7.00 ± 0.00	7.00 ± 0.00	7.13 ± 0.23^{a}

* Disinct superscripts in columns represent statistically significant differences ($p< 0.05$).

Table 5 Microbial counts of bioinoculants stored at 4°C and 35 °C (room temperature)

Treatment	Day 0	Microbial counts (CFU/mL) at 4°C				Microbial counts (CFU/mL) at 35°C			
		Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
AP (T_1)	8.68 ± 0.06^{b}	8.71 ± 0.04^{b}	8.78 ± 0.03^{b}	8.83 ± 0.01^{a}	8.85 ± 0.08^{a}	8.85 ± 0.19^{b}	8.98 ± 0.08^{b}	8.99 ± 0.09^{b}	9.19 ± 0.14^{a}
AS (T_2)	8.57 ± 0.04^{b}	8.60 ± 0.05^{b}	8.62 ± 0.02^{b}	8.71 ± 0.02^{a}	8.67 ± 0.01^{a}	8.68 ± 0.01^{b}	8.83 ± 0.06^{a}	8.91 ± 0.16^{a}	9.04 ± 0.08^{a}
AX (T_3)	8.71 ± 0.08	8.70 ± 0.07	8.70 ± 0.09	8.63 ± 0.03	8.59 ± 0.12	8.90 ± 0.09^{b}	9.08 ± 0.01^{a}	9.21 ± 0.06^{a}	9.22 ± 0.13^{a}
AP+ AS (T_4)	8.82 ± 0.10	8.78 ± 0.09	8.80 ± 0.08	8.81 ± 0.07	8.85 ± 0.06	8.86 ± 0.12^{b}	8.99 ± 0.10^{b}	9.19 ± 0.16^{a}	9.35 ± 0.09^{a}
AP+ AX (T_5)	8.58 ± 0.12^{b}	8.60 ± 0.08^{b}	8.62 ± 0.11^{b}	9.00 ± 0.05^{a}	9.05 ± 0.04^{a}	8.78 ± 0.21^{b}	8.84 ± 0.20^{b}	9.38 ± 0.04^{a}	9.43 ± 0.04^{a}
AS+ AX (T_6)	8.60 ± 0.02^{b}	8.63 ± 0.01^{b}	8.62 ± 0.03^{b}	9.01 ± 0.05^{a}	9.07 ± 0.08^{a}	8.78 ± 0.02^{b}	8.92 ± 0.02^{b}	9.34 ± 0.04^{a}	9.46 ± 0.02^{a}
AP+ AS+ AX (T_7)	8.68 ± 0.06^{b}	8.62 ± 0.04^{b}	8.67 ± 0.05^{b}	8.98 ± 0.03^{a}	8.99 ± 0.09^{a}	8.95 ± 0.10^{a}	8.98 ± 0.08^{a}	9.00 ± 0.05^{a}	9.28 ± 0.03^{a}

* Disinct superscripts in rows represent statistically significant differences ($p< 0.05$).

3.3 Microbial counts of bioinoculants under different storage conditions

Microbial counts of bioinoculants during the storage conditions were carried out at 4°C and 35°C (room temperature) for 28 days. For this, bioinoculants along with talc powder are kept in a small glass jar at the selected temperatures. At 4°C, microbial counts were relatively stable at every **7** days at log 8-9 CFU/mL. On day 21st and 28th, except for AX (T_3) and AP + AS (T_4), the microbial counts were increased in most bioinoculant formulations (Table 5). Further, in the case of room temperature (35°C), microbial counts increased after **7** days in all bioinoculant formulations (Tables 5). This indicated that bioinoculants can be stored in a refrigerator or at room temperature in Thailand. This is convenient for farmers who do not need to use expensive instruments to store bioinoculants for at least a month.

4 Discussion

This is the first report demonstrating the utilization of three potential soil bacteria isolated from Na Si Nuan Community Forest, Kantharawichai District, Maha Sarakham Province, Thailand. Bioinoculants from soil bacteria enhanced the growth of cowpea under laboratory and field conditions. Results of the study showed that phytase (AP) and chitinase-producing (AS) bacteria were the most effective in promoting the growth of cowpea in the soil during the selected experimental periods. The *A. pittii* PT1.3.4 bacteria produced phytase which degrades phosphorus into more readily usable forms, thereby facilitating plant growth, photosynthesis, biochemical oxidation, nutritional absorption, and plant cell division.

Our results were consistent with recent findings of Yaghoubi Khanghahi et al. (2021), which found phosphate solubilizing

capacity in *A. pittii*, *Acinetobacter calcoaceticus*, *A. oleivorans*, *Acinetobacter* sp., and *Pseudomonas alcaligenes*. In this study, all soil pH values following bioinoculant treatment on day 28 exhibited a shift toward the mild acidic range, indicating that organic acid exudation is involved in phosphate solubilization. Expectedly, the control groups had the highest pH levels (talc and water). In a recent study, Yaghoubi Khanghahi et al. (2021) also determined *A. pittii* JD-14 as the most active strain in improving the biomass of alfalfa at Hada Al Sham in Saudi Arabia.

The bioinoculant strain *Achromobacter* sp. C2.23 produces chitinase which can be able to degrade fungal and insect cell wall chitin into amino acids and nitrogenous sources for plants. Phosphate-solubilizing bacteria release gluconic, acetic, and oxalic acids, as well as quinic and succinic acids while growing *in vitro*. Organic acids generated on the outside face of the cytoplasmic membrane were shown to be the product of direct oxidation in an acidic (lower pH) environment (De Amaral Leite et al. 2020). An association has been also found between the pH drop and phosphorus solubilization in the liquid culture medium, as shown by the results of the pot experiment in this study where soil pH was reduced due to added bioinoculants.

In this study, N, P, and K contents in soil increased from day 1 to day 7 in every treatment, including the control. The used cultivation soil was of good quality at the start of the experiment. However, ketogluconic, oxalic, gluconic, and succinic acids, as well as other organic acids, were released into the soil over time, causing the pH of the soil to decrease with time.

Plant root exudates and decaying microbial matter are the two most important sources of organic acids in soil (Paul et al. 2021). Bacterial, fungal, and lichen species are significantly contributed to the release of organic acids into the soils. Decomposition of organic waste also adds to high levels of organic acids in the soil and improves the habitat for plants (Paul et al. 2021). Free phosphorus (P) can be released from complex mineral P such as hydroxyapatite or tricalcium phosphate by organic acid-aided chelation of divalent cations (e.g. Ca^{2+}). Thus, plants can take up free P more readily. Several gram-negative bacteria such as *Acinetobacter* sp. SK2 undergoes periplasmic glucose oxidation through pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) enzyme to produce gluconate that can be metabolized or enhanced the P solubilization effects (Bharwad and Rajkumar 2020).

The bioinoculant that contained nitrogen-fixing bacterial strain *A. xylosoxidans* N3.4 created soil acidic. Nitrogen-fixing bacteria are known to convert ambient nitrogen (N_2) into plant-utilizable ammonia (NH_3). This process consumes H^+ and, as a result, changes the pH. Certain NFB strains created neutral pH whereas others produced acidic pH (Oliveira et al. 2017). The pH discrepancies between NFB isolates might be attributed to differing N_2 fixing mechanisms across bacterial taxa (Das et al. 2022).

Strain AP gave the highest growth, shoot, and fresh weight of cowpea on ½ strength MS media in laboratory conditions; however, combined strains AP + AX gave the longest roots. Likewise, for cowpea growth in soil under the field condition, on day 28, strain (AP) recorded the highest growth, shoot, and fresh weight. These results are in agreement with the findings of Daur et al. (2018), who found that introduction of *Achromobacter* sp. 5B1 in *Arabidopsis thaliana* resulted in a fourfold increase in lateral root number and density, which is associated with a significant increase in the overall fresh shoot and root weight. Increased synthesis of photosynthetic pigments occurred as a result of microbial inoculation. Plants with more chlorophyll have a higher photosynthetic rate, which transforms more carbon dioxide and water into glucose, increases metabolic activity and subsequently improves plant development (Kumawat et al. 2022). *Achromobacter* sp. 5B1 has been reported to promote root development of *A. thaliana* via auxin signaling and redistribution (Jiménez-Vázquez et al. 2020). Further, the results of the study suggested that the combination of AS + AX bacterial strains and AP + AS + AX strains produced the highest mineral levels in the soil.

Jha and Kumar (2009) suggested that *A. xylosoxidans* produces indole acetic acid, nitrogenase activity, and phosphate solubilization which may explain the plant development potential of this genus. Similarly, Wang et al. (2020) established the indole-3-acetic acid excretion capabilities of *A. xylosoxidans* GD03 which help in the rice growth. Under drought stress, bioinoculants of the bacteria *E. cloacae* and *A. xylosoxidans* boosted growth, gas exchange characteristics, and nutrient concentrations in shoots and grains, as well as yield in maize (Danish et al. 2020). Additionally, inoculation of PGPR increased the amount of N, P, K, and Mg, these PGPR also improved a variety of processes including increased nutrient availability, greater nutrient absorption, and healthier root development. By releasing regular organic acids, PGPR boosts nutritional availability by solubilizing nutrients (Cataldi et al. 2020).

Conclusions

Results of the study can be concluded that the three bacterial strains namely *A. pittii* PT1.3.4 (AP), *Achromobacter* sp. C2.23 (AS) and *A. xylosoxidans* N3.4 (AX) have the highest potential for producing agriculture and can be used as bioinoculants to enhance cowpea growth. Talcum-based bioinoculants showed positive results on cowpea growth when they were applied to the seeds before planting in the soil. Inoculated plants thrived in cultivated soils significantly better than the controls. Local bacteria can be

used to promote the growth of cowpea, thereby mitigating the cost of chemicals to cultivate local crops in a safe environment for both farmers and consumers. Thai agriculture can develop toward sustainability using biological methods.

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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Phytoremediation of chromium, iron and nickel by Indian Rice Plant (*Oryza sativa* L.): An opportunity for management of multi-metal contaminated tannery wastewater

Arti Singh Katiyar[1*], Amit Singh[2,5], Amar Abhishek[3], Monika Bhaskar[3], Divakar Sharma[4*], Vishal Garg[1]

[1]Department of Chemistry, Maharaj Vinayak Global University, Jaipur, Rajasthan, India
[2]CCRF, All India Institute of Medical Sciences, New Delhi, India
[3]School of Life Sciences, Department of Botany, Guru Ghasidas Vishwavidyalaya, Bilaspur-495009, Chhattisgarh, India
[4]Department of Microbiology, Maulana Azad Medical College, New Delhi, India
[5]Department of Gastroenterology & HNU, All India Institute of Medical Sciences, New Delhi, India

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ABSTRACT

India is the largest producer of leather and leather products. Tannery industries use a large number of synthetic chemicals for the processing of leather and generate a huge amount of wastewater containing a large amount of potentially toxic heavy metals (PTHMs) making them problematic for next-door soil and water system. Currently, phytoremediation is an inexpensive green technology used to move, eradicate, and stabilized heavy metal contamination from contaminated sludge, soil, and wastewater. In this study, the accumulation and distribution of PTHMs found in tannery wastewater and their physio-biochemical effects on *Oryza sativa* L. have been studied by ICP-MS, GC-MS, and biochemical analysis. The plant was grown in the soil spiked with a mixture of metals (Cr, Fe and Ni) and their five-level of treatment T1 (25mg/kg); T2 (50mg/kg); T3 (100mg/kg); T4 (200mg/kg) and T5 (400mg/kg). During the experiments, various morphological attributes, oxidative stress, enzymatic activities, chlorophyll, and protein content at the different stage was measured. Further, metal accumulation pattern in different parts of plants was also measured. Results of the study revealed that plant root, shoot length, chlorophyll content, and enzymatic activities were significantly reduced after the treatment with 200 mg/kg PTHMs; whereas oxidative stress was increase compared to control levels. Further, treatment of PTHMs suggested that the rice plant (*Oryza sativa* L.) is well adapted to tolerate and accumulate a high level of heavy metals (up to 200mg/kg) in the root and shoot of the treated plants. If it is treated above this, then seeds were also affected and not safe for human consumption.

* Corresponding author

E-mail: divakarsharma88@gmail.com (Divakar Sharma);
akihbt@gmail.com (Arti Singh Katiyar)

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1 Introduction

In Asian countries, India is the foremost exporter of leather and its products. The tannery industries generate hazardous chemical waste due to the use of large amounts of synthetic chemicals to convert animal skins into the leather which are harmful to the environment. The released wastewater is characterized by high odors due to the presence of free ammonia, volatile organic compounds, and hydrogen sulphide (Senatore et al. 2021). Tannery wastewater (TWW) also has a high level of potentially toxic heavy metals (PTHMs) like Cr, Cd, etc., this mixture of heavy metals makes the tannery wastewater, problematic for the environment (Urbina-Suarez et al. 2021; Ahmed et al. 2022). Water/soil contaminated with these toxic heavy metals has become a worldwide concern due to their non-specific toxicity and long-lasting exposure to the environment. The accumulation of heavy metals in the environment is also contaminating the food chains. The toxicity of these PTHMs is non-specific and affects organisms via several competing pathways (Lakshmi et al. 2021).

A wide range of chemical, physical and biological treatment policies are being implemented for the reduction of PTHMs contamination from soil or water. Some of the conventional treatment methods are chemical fixation, soil replacement, and thermal desorption (Hansen et al. 2021). In addition, conventional treatment approaches are difficult to operate, as they are quite expensive, laborious, and produce a massive amount of secondary waste that requires further treatment. Therefore, there is need for a suitable treatment technique with minimal waste generation is required for the remediation of PTHMs present in TWW.

Recently, some plant species were used for phytoremediation techniques because they have the capabilities to accumulate PTHMs in their roots and shoot system (Diarra et al. 2021). This technique is an eco-friendly, emergent, and broadly accepted plant-based technology for the removal of PTHMs in polluted water or soil. A range of crops like *Brassica napus, Solanum nigrum,* and *Zea mays* have been discovered which accumulate and extract the residual amount of PTHMs from the soil or water. Gupta and Sinha (2007) evaluated the phytoextraction capability of different plants growing in tannery sludge dumping areas. According to Evangelou et al. (2015), few trees such as Willow (*Salix* spp), poplar (*Populus* spp), and few plants like maize (*Zea mays*), Indian mustard (*Brassica juncea*), have been known as a suitable candidate for the management of these heavy metals (Chowdhary et al. 2018). These plants have some advantages over other crops such as rapid growth, tolerance to harsh environmental conditions such as high salinity, aesthetically pleasant, control of soil erosion, high biomass production, and removal of heavy metal discharge (Lakshmi et al. 2021). The efficiency of rice plants in phytoremediation of PTHMs from water or soil has been reported by various researchers (Liu et al. 2007; Evangelou et al. 2015; Pajević et al. 2016; Yan et al. 2020). Further, Rice is being used in numerous research due to its vast range of adaptability to different environmental conditions and potential to tolerate abiotic stress (Liu et al. 2007; Bharagava et al. 2008; Satpathy et al. 2014; Olawale et al. 2021; Lakshmi et al. 2021). Earlier, the potential of the rice plant to remediate heavy metals like Cd, Pb, and Fe contaminated soil and water have been studied by various researchers (Liu et al. 2007; Satpathy et al. 2014; Olawale et al. 2021). India has high production of rice and many farmers irrigate their crop plants with industrial wastewater having a high load of toxic compounds including PTHMs due to the non-availability of alternative sources of irrigation. In the current study, the rice plant was selected for phytoremediation because the information related to the potential of rice plant in phytoremediation of two or three heavy metals is limited. The accumulation of heavy metals in crop plants can be health hazardous. Therefore, it is obvious that metal stability of experimental plant must be under continuous surveillance because the accumulation of heavy metals in crop plants may be hazardous for health and ecosystem. Thus, in this study, the effect of PTHMs contaminant water irrigation on rice plant root and shoot growth, biochemical changes, oxidative stress, assessment of accumulation of heavy metals, and heavy metal concentrations in seeds were evaluated to determine the safe limit of rice plant irrigation.

2 Materials and Methods

2.1 Description of sampling site and sampling

The TWW samples were collected from M/s Unnao tanneries pollution control company, A-7, Site-II, UPSIDC, Industrial Area. Unnao, 209101, U.P., India, in a pre-sterilized container of 10 litre capacity. Further, the estimation of heavy metal and physio-chemical analysis of the collected water samples has been carried out as per the standard method of APHA (2005; 2021). All the observations were recorded in triplicate and their mean values are reported with their variation. The pH, Electrical Conductivity (EC), and other ions of collected tannery effluent were measured by Orion 960 titrator (Thermofisher, USA) (Singh et al. 2015), while COD, BOD, and color were measured by open reflex method, 5-day method, and cobalt platinum method respectively (APHA 2005; 2021). Further, phosphate and sulphate concentrations were analyzed by the stannous chloride method and gravimetric method respectively (APHA 2005; 2021). The concentration of different toxic metals was measured by using Electron Model IRS intrepid II Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermofisher, USA)(Singh et al. 2015). The organic matters were detected by GC-MS (Agilent, USA).

2.2 Pot Experimental Design

The experiment was carried out during the rainy seasons (i.e. rice growing season from May to -October). Rice variety MTU1010

was selected for this experiment because of its largest production area in India. Only those heavy metals (Cr, Fe, and Ni) were selected for the experiment which has been detected in high load in tannery effluent samples; these were prepared at five different levels i.e. T1 (25mg/kg); T2(50 mg/kg); T3(100 mg/kg); T4(200 mg/kg) and T5(400 mg/kg). The stock solution was prepared with their respective salts such as potassium dichromate ($K_2Cr_2O_7$), ferric chloride ($FeCl_3$,) and nickel chloride ($NiCl_2$), and dissolved in 500 mL water. The unspiked/unexposed and uncontaminated air-dried agriculture field soil collected from the college campus was used for this experiment. In each pot, 10kg of collected soil was filled. The physicochemical analysis of this agricultural soil was carried out as per the standard protocol given in APHA (2021). The concentrations of heavy metals in soil were measured using an ICP-MS (Inductive Coupled Plasma Mass Spectrophotometer) Thermo, Electron Model IRS intrepid II, USA) and organic matters by GC-MS (Singh et al. 2015). Each earthen pot was treated with predefined concentrations of selected heavy metals in 5 replicates.

In this experiment, tap water was used as a control. The seeds of rice were dipped in a water bath for approximately 48 h at room temperature. For germination, seeds were kept under moist conditions (to maintain moisture these seeds are covered with moist two-layer cloth gauze) at room temperature for an additional 30 h and the sprouted seeds were grown in the paddy seedbed under field conditions and raised for seedlings. After 30 days, the seedlings were transplanted into the heavy metal contaminated soil containing pots @ 4 plants per pot. During the whole growth period, water in the pot was maintained 3-4 cm from the soil surface. The experiment was laid out as a Complete Randomized Block Design (CRBD) with five replicates. The harvesting data were recorded after the 4 months of transplantation.

2.3 Measurement of plant growth parameters

Plant samples were taken three times during the time course of this experiment, first: just before the plantation of rice seedlings to the pot (1^{st}month); second, after the 2.5^{th} month and third before harvesting the plant (4^{th} month). Collected plants were uprooted and washed off with running water followed by tap water to remove any contiguous particles. Further, these plants were rinsed with 20 mM calcium chloride solution along with distilled water for further analysis such as root, shoot growth, and lipid peroxidation (Singh et al. 2015).

Physio-biochemical analysis of root and shoot growth parameters were measured as per standard protocol (Singh et al. 2015). The fresh weights (FW) of root, shoot, and panicle were determined through an electronic weighing balance. All the plant samples were oven dried for 48 hrs at 70°C and cooled down to room temperature and dry weights (DW) were determined. Water content (WC %) was calculated as per the formula:

$$\mathbf{WC} = \frac{(\mathbf{FW} - \mathbf{DW})}{\mathbf{FW}} \times \mathbf{100}$$

Here DW = Dry Weight, FW = Fresh Weight; WC = Water Content

The pigments of selected plants i.e. Chlorophyll a, chlorophyll b, and carotenoids were determined by the spectrophotometer method as per the standard protocol given by Lichtenthaler and Wellburn (1983). The protein concentration was estimated by the Lowry method (Lowry et al. 1951).

2.4 Lipid peroxidation (LiP)

Lipid peroxidation (LiP) in plant samples was estimated indirectly in terms of Malondialdehyde (MDA). The total MDA content was estimated by the method of Heath and Packer (1968). In brief, 0.5 gm leaf and root tissues (air dried) crushed and homogenized with 2 mL of 0.25% (w/v) TBA (2- Thiobarbituric acid) in 10% (w/v) TCA (Trichloroacetic acid). Grinding of plant sample was done in a circular motion until small bubbles appeared. Finally, all homogenate was made up to the volume 4 mL with 0.25% (w/v) TBA in 10% (w/v) TCA. Samples were heated at 95°C for 30 minutes in the water bath. After cooling with ice, samples were transferred into fresh centrifuge tubes with proper shaking and centrifuged at 12000 rpm for 5 minutes. The final concentration of MDA was estimated by deducting the absorbance of supernatant (600nm) from an absorbance coefficient (532 nm) of 155 nmol cm^{-1}.

2.5 Preparation of plant enzyme extract for antioxidant activity

For the enzyme extract, 0.5gm of fresh leaf samples were selected and crushed in 4 mL of phosphate buffer (100 µM; pH 7.5) containing 1 mM EDTA and a tiny pinch (~50mg) of polyvinylpyrrolidone (PVP). This homogenate was subjected to centrifugation at 12000 rpm for 10 min at 4 °C and the crude supernatant was used for the quantification of antioxidant enzymes (Sarker and Oba 2018).

2.6 Antioxidant activity

Superoxide dismutase (SOD) activity was estimated as per the earlier published method of Nishikimi et al. (1972) with some modifications. In brief, the test was carried out in two steps. In step one, 1.1 mL pyrophosphate buffer (PPB), 0.2 mL Nitro Blue Tetrazolium (NBT), 0.2 ml PMS, and 20 µL enzyme extract were taken. While in the second step all the above reagents except the enzyme source have been used. The reaction was started simultaneously in both steps by the addition of 0.2 mL NADH.

After 90 sec, for checking the reaction,0.5 mL glacial acetic acid was added to each tube. The absorbance (560nm) of both set of tubes was recorded against a reagent blank. The difference between reference and experimental absorbance gives the inhibition of NBT reduction by enzyme source. The unit of SOD enzyme activity was defined as "the amount of enzyme required inhibiting the optical density (560 nm) of NBT reduction by 50% in one minute under the assay conditions". The results were expressed as a 1U (1 Unit) enzyme.

Catalase (CAT) activity was calculated by the method of Maehly and Chance (1954). Phosphate buffer (2mL) and 1 mL of diluted H_2O_2(0.2 M) were taken in a cuvette, and add 0.02 mL enzyme source in it and mixed carefully. The reduction in absorbance (at 240 nm) was recorded after every 30 seconds for up to 3 minutes against the negative control.

2.7 Analysis of heavy metals in different parts of plant

The plant samples were harvested at the end of the experiment. Plants were chopped into roots, shoots, and seeds and left to oven dry at 50 °C for one week. A crushed form of dry plant matter was ashed in a muffle furnace at 500 °C for 6 hours. Now, 1 gm of ash from all the samples were digested separately in a 100 mL beaker with a digestion mixture (nitric acid and perchloric acid 5:1 ratio) until white fume comes (Singh et al. 2015; Ranieri et al. 2021). Washed down the wall of a beaker with a minimal amount of distilled water and then filtered. Transferred filtrate made up to 20 mL in the volumetric flask as per standard protocol (APHA 2021). The concentration of various heavy metals was measured using an ICP-MS (Singh et al. 2015).

2.8 Metal bioaccumulation studies

Bioaccumulation factor (BAF) is defined as "the level of metal in an organism's tissue divided by its concentration in soil /water which is expressed in the equivalent unit". BAF was calculated by using the formula given by Yoon et al. (2006). Moreover, translocation factor (TF) was calculated for different metals because they played a significant role in different metabolic activities of plants. The TF was calculated as per the standard method given by Gupta and Sinha (2007).

2.9 Statistical Analysis

Kolmogorov Smirov test was used to test the normality of data. Numerical data were presented in the form of mean ±SD. A post hoc Tukey and least significance difference test (LSD) test followed by one-way ANOVA was applied to analyze the significant difference among sampling sites for different heavy metal and physico-chemical parameters; this post hock test was also applied in biochemical parameters during phytoextraction of heavy metals. Statistical analysis was carried out using the SPSS, version 22 (Chicago, USA). The level of significance means, the *p*-value was set as <0.05. Graphics were prepared with the help of Microsoft Excel and Statistical Software.

3 Results and Discussion

3.1 Chemical texture of TWW

The distributions of heavy metals in collected TWW samples were analyzed, and the results are summarized in Table 1. A varied range of metal concentration (mean ±SD) were found. A post hoc analysis showed that chromium (Cr) is a predominant metal in TWW (58±1.66 mg/L). This was followed by iron and its concentration in the untreated tannery effluent was 7.90±1.6 mg/L. The order of dominancy of heavy metals in tannery effluent is Cr>Fe>Ni>Zn>Mn>Cu>Pb. Further, tests on seasonal variation were found homogenous ($p>0.05$). The tannery industry is one of the pollutions generating industries that mainly causes metal pollution especially chromium in the environment (Appiah-Brempong et al. 2022). In India, there are more than 2000 tanneries, and over 80% of tanneries are engaged in the chrome tanning process (Saxena and Bharagava 2016). In the tanning process, the salt of chromium is used to convert hide into leather and eventually release a large amount of organic matter and heavy metals. Further, it was reported that chromium-containing wastewater causes a serious threat to soil and water pollution and poses a serious hazard to human health (Hansen et al. 2021; Ahmed et al. 2022). These industries discharge chromium-containing wastewater into the canal and river. Moreover, chromium toxicity depends on chemical specificity, thus the health related effect of chromium is concerned by the chemical form of exposure. It is well known that chromium (VI) is a potentially toxic substance to humans, animals, and microbes because it enters the cell via the surface transport system and is reduced to the level of chromium (III) which is associated with the breakdown of DNA (Shi et al. 2021; VonHandorf et al. 2021).

The mean pH value of untreated TWW was 8.0±0.6. The seasonal variation in pH and temperature was found to be homogenous (Table 1). The EC (µS/cm) of untreated TWW was 1323.29±25.7 in summer and 1343.42±30.54 in monsoon season ($p>0.05$) which exceeded than the Bureau of Indian Standard (BIS) permissible limit (>1000 µS/cm). Polyvalent ions such as calcium and magnesium are associated with the total hardness of the water. In this study, total hardness ranges between 1205±30.18 to 1312±29.41. Moreover, the presence of fluoride ions was not detected throughout the study. A significant level of nitrate, nitrite, and other pollution parameters like sulphate, phosphate, etc. was found. This may be due to nitrogen coming from animal skins that are processed in the tannery industry (Saxena and Bharagava 2016).

Table 1 Heavy metal and physio-chemical analysis of tannery wastewater

Heavy metals	Summer (in mg/L)	Monsoon (in mg/L)
Cr	59.04±2.04	57.31±1.31
Cu	0.202±0.01	0.26±0.02
Fe	7.904±1.6	7.84±1.84
Mn	0.28±0.02	0.23±0.01
Ni	2.16±0.31	2.31±0.24
Pb	0.04±0.04	0.06±0.02
Zn	1.04±0.12	1.15±0.21
Physic-chemical parameters	**Summer**	**Monsoon**
pH	8.0[a]±0.6	7.82 [a] ±0.5
Temperature (°C)	27.3 [a] ±1.02	26.8 [a] ±1.1
Electrical Conductivity (µS/cm)	1323.29[a]±25.71	1343.42[a]±30.54
Alkalinity	2.20 [a] ±0.35	2.87 [a] ±0.81
Color (CU)	1124 [a] ±11.2	1215 [a] ±14.02
TS (mg/L)	9001 [a] ±55.1	1942 [a] ±38.64
TDS (mg/L)	727.8 [a] ±33.3	6916 [a] ±41.05
TSS (mg/L)	196.0 [a] ±22.1	119.9 [b] ±20.49
Total hardness	1205 [a] ±30.18	1312 [a] ±29.41
Fluoride (mg/L)	ND	ND
Nitrate (mg/L)	28.37 [a] ±6.25	24 [a] ±5.55
Phosphate (mg/L)	129 [a] ±13.49	126.2 [a] ±14.05
Sulphate (mg/L)	881 [a] ±44.04	893.4 [a] ±50.15
Chloride (mg/L)	123 [a] ±11.9	134.0 [a] ±10.19
BOD (mg/L)	4680 [a] ±69.25	4123 [a] ±61.25
COD (mg/L)	12510 [a] ±200	12348 [a] ±205
Nitrite (mg/L)	31.02 [a] ±1.51	32.12 [a] ±1.39

All values are in ppm except pH, Color and EC; ND- Not detectable; Different alphabets in row show significant variation (Student T test at $p<0.05$).

The chemical characteristics of TWW after conventional treatment has revealed that it has a high load of pollution parameters in terms of heavy metals as well as COD, BOD, phosphate, sulphate, and organic pollutant including heavy metals.

3.2 General characteristic of agricultural soil

The pH and organic matter are important controlling factors, which control the availability of metals to plant species. As soil pH and organic matter increase, heavy metal mobility gets decreases due to the precipitation of hydroxides, carbonates, and the formation of insoluble organic complexes (Javed et al. 2019). On the other hand, metals are more mobile at pH below 7 and moderate organic matter. In this study, the pH and organic matter of experimental soil was 6.7±0.15 and 1.52±0.48 %, respectively **(**Table 2**)**. These results are consistent with Kicińska et al. (2022) who suggested that heavy metal mobilization in the soil is a function of pH and organic matter. On other hand, the amount of PTHMs such as Cr, Cd, and Pb in agricultural soil was found to be almost negligible or below the detection limit. The general characteristics of experimental agriculture soil used in this study were presented in Table 2.

3.3 Effect of PTHMs levels on morphological parameters

Only three PTHMs Cr, Fe, and Ni were found in significant quantities in TWW. Therefore, further phytoremediation studies were carried out on these 3 heavy metals. The rice plants were grown for 4 months in soil spiked with a mixture of three metals (Ni, Cr, and Fe), and their five-level of treatment of T1(25 mg/kg); T2(50mg/kg), T3(100mg/kg), T4(200mg/kg) and T5(400mg/kg). In the phytoremediation study, two types of control were used, TC1 (tap water) and TC2 (mimic control). Tap water control was

Table 2 General parameters of experimental soil

Parameter tested	Experimental Soil
pH	6.7±0.15
Color	Dark grey black yellow
Consistency	Less sticky
Sand (%)	43.3±2.2
Slit (%)	35.33±0.5
Clay (%)	17.72±0.2
Organic matter (%)	1.52±0.48
EC (dS m^{-1})	0.53±0.01
Nitrogen (kg/hectare)	189.14±8
Phosphorus (kg/hectare)	10.37±0.64
Potassium ((kg/hectare)	170.43±5
Sulphur (mg/kg)	10±0.4
Metals (mg/kg)	
Cd	BDL
Cr	0.091±0.06
Cu	1.40±0.3
Zn	0.36±0.5
Fe	4.64±0.1
Ni	0.28±0.15
Mn	3.12±0.2
Pb	BDL

BDL: below detection limit

Table 3 Root and Shoot length of rice plant treated with different concentrations of PTHMs after maturation

Treatments	Root Length (cm)	Shoot Length (cm)
TC1 (Tap water)	18.9±1.05 [a]	64.5±6.57 [a]
TC2 (Mimic control)	22.0±1.5 [b]	68.0±2.89 [b]
T1	22.8±3.16 [b]	71.0±8.6 [b]
T2	24.1±1.1 [c]	72.5±2.79 [b]
T3	23.02±1.16 [b,c]	71.5±3.9 [b]
T4	25.6±1.94 [d]	76.0±3.65 [c]
T5	21.3±1.08 [b]	69.0±3.45 [a,b]

Different letters up to down in separate column shows significant variation (p=<0.05).

used to see the effectiveness of the process of phytoremediation, while mimic control (same as a combination of metals found in tannery wastewater) was used to evaluate the effects of the heavy metal status of the wastewater on rice plant.

The root and shoot lengths were significantly high in TC2 (mimic control) as compared to normal control (TC1). No significant differences were reported in the mean value of plant root and shoot lengths, and among all treatments, the highest root and shoot lengths were recorded in the T4 treatment (25.6 and 76.0 cm respectively) while the lowest root and shoot lengths were recorded from the treatment T5 (21.3 and 69.0 cm respectively). These results suggested that higher concentrations of heavy metals significantly inhibit the root and shoot length of rice plants (Table 3). Further, the effect of metal concentration on the fresh as well as dry weight of the rice plant was also evaluated and presented in

Table 4 Distribution of fresh and dry weight of rice plant

Treatments	Fresh Weight in Gram				Dry Weight in Gram			
	Root	Shoot	Penicles	TW	Root	Shoot	Penicles	TW
TC1	1.45	8.90	1.077	11.44[a]±4.46	0.84	3.366	0.538	4.74[a] ±4.52
TC2	4.45	16.8	1.797	23.1[b] ± 14.8	3.099	7.843	1.526	12.47[b] ±5.88
T1	4.10	19.6	1.053	24.8[b] ±10.98	2.684	8.054	1.435	12.17[b] ±6.42
T2	4.92	27.3	2.015	34.32[c] ±9.84	2.87	9.317	0.833	13.02[b] ±4.93
T3	5.12	21.1	0.479	26.77[b] ±6.89	3.393	8.121	0.26	11.7[b] ± 7.83
T4	6.12	32.9	2.606	41.7[d] ± 6.82	3.074	10.898	1.602	15.57[b] ±7.65
T5	5.95	22.1	1.004	29.1[b,c] ±6.15	4.119	8.663	0.574	13.3[b] ±4.05

TW: total weight of plant;Different letters shows significant variation and same letter shows homogeneity of the data set in separate column

Table 4. Compare to plants grown in unspiked soil (controls), treatment T1 (25mg/kg) to T5 (400mg/kg) represent an increment in fresh weight ($p<0.05$). The mean value of dry weight is increasing with increasing metal concentration but this difference was found non-significant ($p> 0.05$).

3.4 Effect of metal concentration on biochemical parameters

Under PTHMs stress, plants stimulate redundant reactive oxygen species (ROS) which commences oxidation harm such as oxidation of proteins and lipid peroxidation, inhibition of enzymes and genetic materials. No significant changes were observed in 1st month of treatment. As compared to the plants grown in unspiked soil/uncontaminated soil (control) plants grown in PTHMs contaminated soil (T1 to T5) have higher oxidative stress (Figure 1). The highest MDA level (nm/gm) was recorded from the T5 treatment pot as compared to its lower level and control after 2.5 and 4th months. The raised MDA content suggested that intracellular generation of free radicals leads to membrane damage and different cytotoxicological effect by polyunsaturated lipid generating MDA as one of the by-products of lipid peroxidation. In plants, high lipid peroxidation blocks the flow of electrons in photosystem II by metallic ions which generates chlorophyll and thereby generates free radicals (Khorobrykh et al. 2020). In addition, fatty acid supports the structure of harmful lipid peroxides that develop MDA in plants (Sidhu et al. 2016). The accrual of MDA at a high rate in plant tissue usually reflects drastic lipid peroxidation. Therefore, this progression of MDA also supports oxidative stress by modulating several biochemical and morphological attributes (Singh et al. 2015; Qamer et al. 2021). The results of the current study are in agreement with the findings of Singh et al. (2022) who have reported that excessive accrual of metals induces MDA levels in different parts of plants. Another study, done by Bharagava et al. (2008) also made similar conclusions and showed an elevated level of MDA in leaves of the mustard plant treated with different metals concentrations.

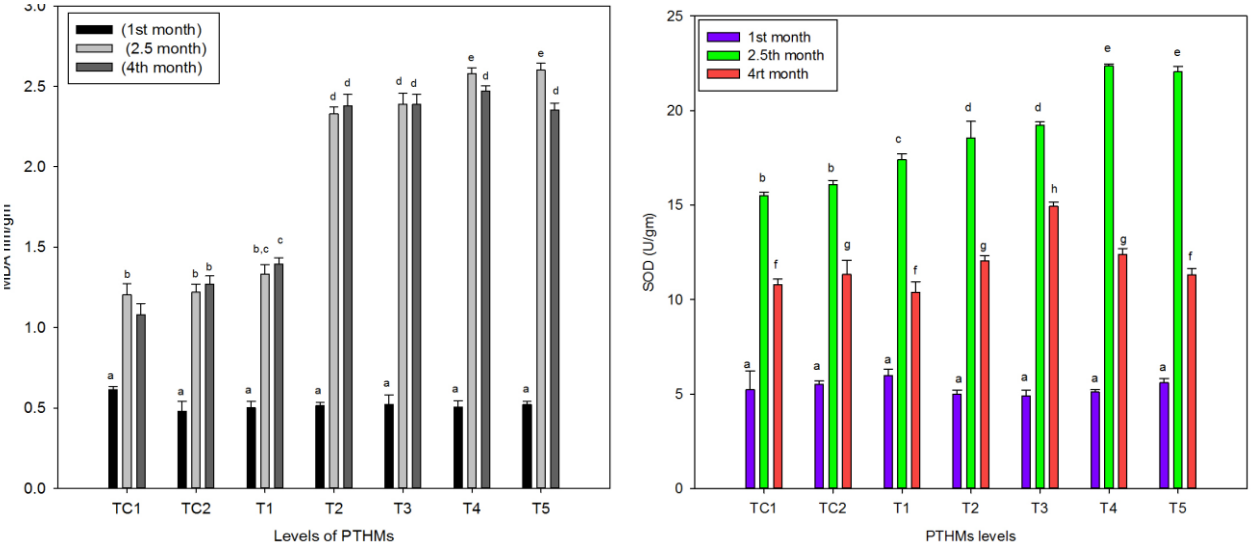

Figure 1 MDA and SOD level in the leaf of rice plant irrigated with different concentration of mixed PTHMs (Different alphabets show significant variation at $p<0.05$).

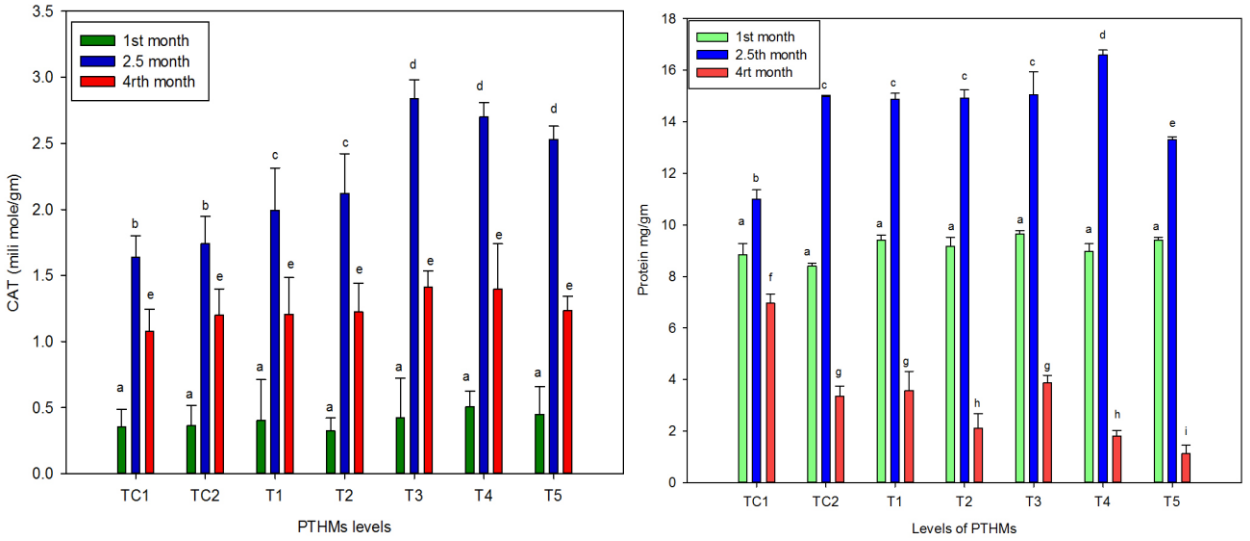

Figure 2 Distribution of Catalase (CAT) and protein level in the leaf of rice irrigated with different concentration of mixed PTHMs (Different alphabets show significant variation at p<0.05).

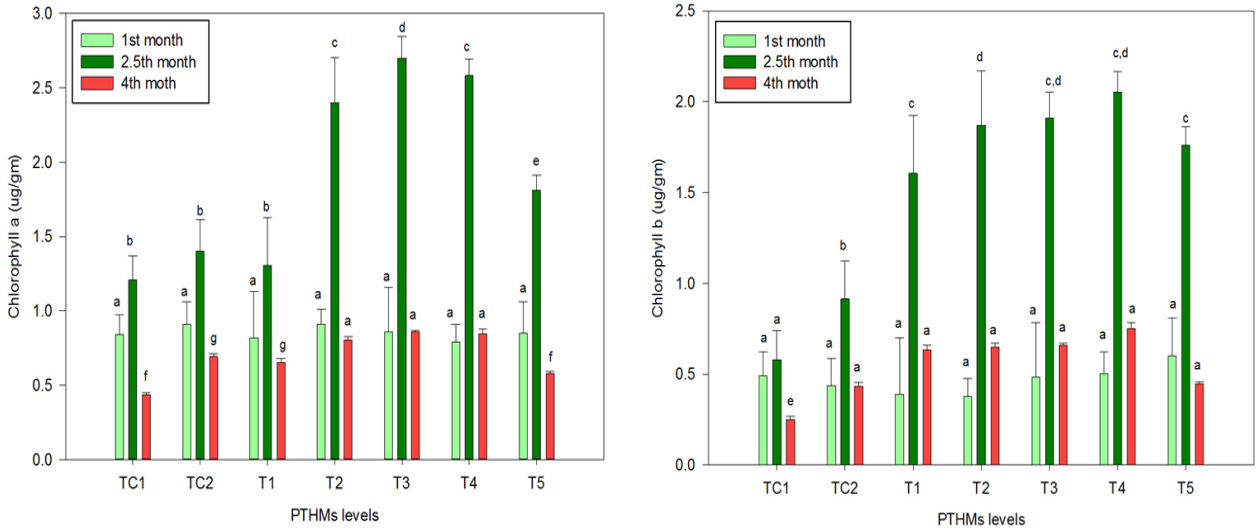

Figure 3 Distribution of Chlorophyll a, b in the Leaf of rice plant irrigated with different concentration of mixed PTHMs (Different alphabets show significant variation at p<0.05).

The defense machinery of plants such as SOD (U/gm) and CAT (millimole/gm) levels were increased significantly with an increase in the concentration of heavy metals T1 (25mg/kg) to T4 (200mg/kg) than control (p<0.05). The maximum increase in SOD (U/gm) was recorded as 23.68±1.73 at T4 (200 mg/kg) treatment after 2.5month of growth period compare to lower level and control. Further, the SOD level was reduced after the 4th month (p<0.05). The Maximum CAT activity was found in plants treated with T3 (100mg/kg) of PTHMs (2.72±0.43 millimole/gm). Whereas, when the concentration increases above T4 (200 mg/kg) the level of antioxidants (SOD and CAT) was reduced. At the end of the 4th month, the enzymatic activities were reduced significantly as compared to 2.5 months (p<0.05). Several independent studies suggest that under sustained environmental stress, the battle between oxidative stress and their scavenging machinery is imbalanced and causes increased oxidative stress which may trigger different types of harmful effects on plants (Farooq et al. 2016; Khorobrykh et al. 2020). The content of protein in the plant leaves was detected higher during the entire growth period as compared to the controls. Like the other two parameters, in the case of protein also, maximum protein content was detected in T4 (200mg/kg), and hereafter this, when the concentration of PTHMs increases the level of protein was decreased gradually (Figure 2).

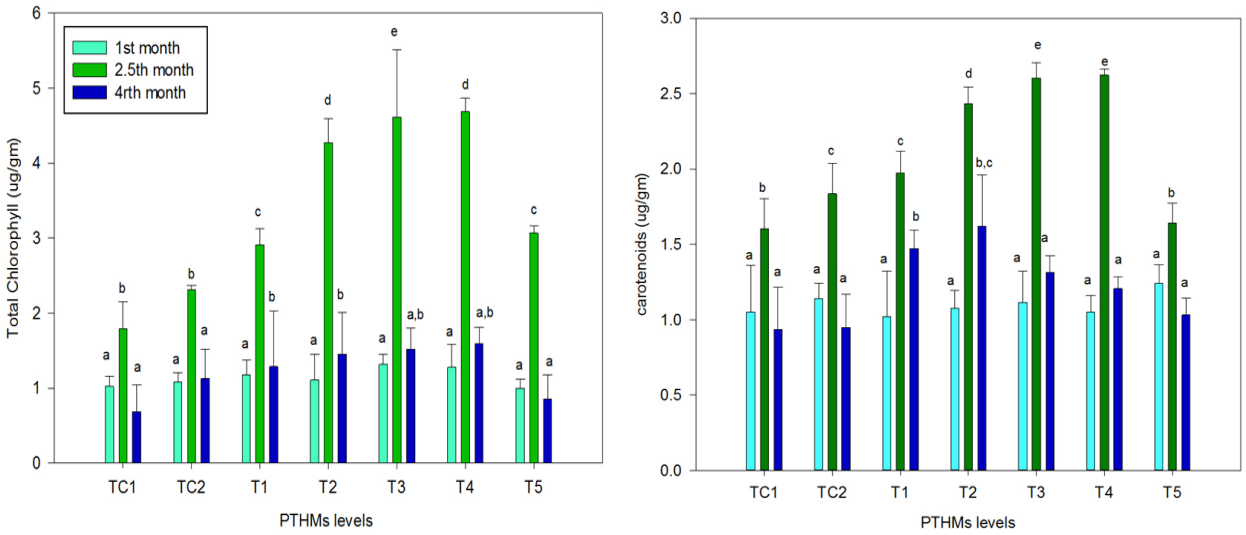

Figure 4 Distribution of total Chlorophyll and carotenoids in rice plant irrigated with different concentration of mixed PTHMs (Different alphabets show significant variation at p<0.05)

The maximum elevations of all chlorophyll content were recorded after 2.5 months of the growth period. The concentration of these pigments reduced drastically after 4 months as compared to 2.5 months. The maximum increase in total chlorophyll, chlorophyll a, b, and carotenoid level were found at 2.5 months in treatment T4 (200 mg/kg) (Figures 3 and 4). This rise in chlorophyll levels in the leaves of the rice plant may be due to the presence of high Cr, Fe, Ni, etc. These findings are in agreement with the earlier results reported (Farooq et al. 2016; Amari et al. 2017; Singh et al. 2022). Moreover, a reduction was observed in chlorophyll and carotenoid level after the T4 (200mg/kg) PHTMs treatment levels (p<0.05). This change may be due to the interference of metals in chlorophyll biosynthesis through direct inhibition of enzymatic steps. For example, high Cr is well reported to hinder the biosynthesis of chlorophyll and decrease total chlorophyll as well as chlorophyll a/b ratio (Singh et al. 2022).

3.5 Accumulation and distribution of heavy metals in plant parts

The mean values of metal accumulation in different parts of rice plants irrigated with different concentrations of Cr, Fe, and Ni with the statistical measures are presented in Table 5. Different type of crop plant works differently for the accrual and dispersal of metals in their plant parts (roots, shoots, fruits, or seeds). Results revealed that this rice plant has shown maximum accrual of Fe (330.2±0.7

Table 5 A Comparative accumulation pattern of metals (mg/kg) in different parts of Rice plant irrigated with different concentration of metals.

Metals	TC1 (Normal control)	TC2 (TWW)	T1 (25)	T2 (50)	T3 (100)	T4 (200)	T5 (400)
Chromium							
Root	BDL	7.77[a]±0.08	2.23[a] ±0.1	13.34 [a] ±0.04	34.68 [a] ±0.57	41.96 [a] ±0.05	46.69 [a] ±0.1
Shoot	BDL	1.30 [a] ±0.02	0.84 [a] ±0.1	9.88 [a] ±0.05	28.46 [a] ±0.05	37.87 [a] ±0.05	40.78 [a] ±0.8
Seed	BDL	BDL	BDL	0.01 [a] ±0.01	0.02 [a] ±0.02	1.07 [a] ±0.05	1.12 [a] ±0.07
Nickel							
Root	1.8[a] ±0.1	2.16 [a] ±0.04	10.01 [a] ±0.04	16.67 [a] ±0.6	21.21 [a] ±0.42	26.34 [a] ±0.81	30.22 [a] ±0.7
Shoot	1.1[a] ±0.1	1.65 [a] ±0.07	7.09 [a] ±0.01	14.67 [a] ±0.8	18.89 [a] ±0.76	24.44 [a] ±0.58	26.0 [a] ±0.76
Seed	BDL	0.62 [a] ±0.08	1.29 [a] ±0.071	1.43 [a] ±0.05	1.72 [a] ±0.011	2.06 [a] ±0.014	2.70 [a] ±0.02
Iron							
Root	3.37[a] ±0.05	6.07 [a] ±0.52	30.07 [a] ±1.6	79.95 [a] ±4.5	160.24 [a] ±12	222.25 [a] ±13	330.2 [a] ±19
Shoot	1.50[a] ±0.1	3.85 [a] ±0.01	20.17 [a] ±0.05	68.6 [a] ±1.82	141.13 [a] ±8.1	202.9 [a] ±10.5	277.7 [a] ±12
Seed	0.8[a] ±0.1	2.75 [a] ±0.05	5.69 [a] ±0.85	12.28 [a]±1.4	23.33 [a] ±1.75	46.38 [a] ±1.71	57.91 [a] ±2.8

Different letters in a row show significant variation and same letter shows homogeneity of the data set

mg/kg) followed by Cr (46.6±0.1 mg/kg) and Ni (30.22±0.7 mg/kg) in roots. The accrual of all tested heavy metals was found maximum in roots followed by shoot and/or seeds, which increased with increasing concentration of metals T1 (25mg/kg) to T5 (400mg/kg). The reason for the high accrual of heavy metals in roots may be due to the high metabolic rate of other plant parts (Phusantisampan et al. 2016; Singh et al. 2021).

This study revealed that concentrations of all heavy metals in seeds of rice plants were found below as recommended by different environmental agencies up to T4 treatment (200 mg/kg) PTHMs. But, as the concentration of heavy metals increased above T4 (200 mg/kg) and onwards, metals concentration in seeds of rice plant were increased and exceeded the permissible limit i.e. 0.02 mg/kg, 1.63 mg/kg, 20.0 mg/kg for Cr, Ni, and Fe respectively for human consumption (Bhargava et al. 2008). Therefore, it may be advisable that the rice plants treated with a high concentration of heavy metals (above 200 mg/kg) are not suitable for human and cattle consumption (Ahmad et al. 2022).

3.6 Translocation factors

According to Baker and Walker (1990), plants are categorized into three groups as per their strategies for growing on metal-polluted soils; indicators, accumulators or hyperaccumulators, and metal excluders. The efficacy of plants for the absorption and distribution of metals is judged based on the bioaccumulation factor (BAC) and translocation factor (TF). Results showed that experimental plants growing at different PTHMs levels have the potential to accumulate metals.

In this study, metal concentrations in the potting soil were taken up to 400 mg/kg (T5) and the maximum accumulation by roots and shoot of rice plant was 330.17±19.56 for iron. Hence, the calculated bioaccumulation factor was less than <1 (Yan et al. 2020). Hence, we can state that rice plants can effectively limit the heavy metal translocation within them and maintain relatively low levels of metals in their shoot over the huge amount present in the soil. Further, metal distribution in different parts of the rice plant (root to shoot and shoot to seed) was also evaluated based on the translocation factor (TF). TF is a relative translocation of metals from root to shoot or shoot to seed in plants. The TF for root to shoot was gradually increased as the concentration of Cr, Fe and Ni increased (Figure 5). The maximum TF (Cr: 0.9, Fe: 0.88 and Ni: 0.89) was recorded in the 200 mg/kg (T4) PTHMs treatment whereas minimum TF was found in the control (TC1=Cr: 0.0; Fe: 0.43 and Ni: 0.6). Further, this pattern was also followed by the translocation factor from shoot to seed. These results were

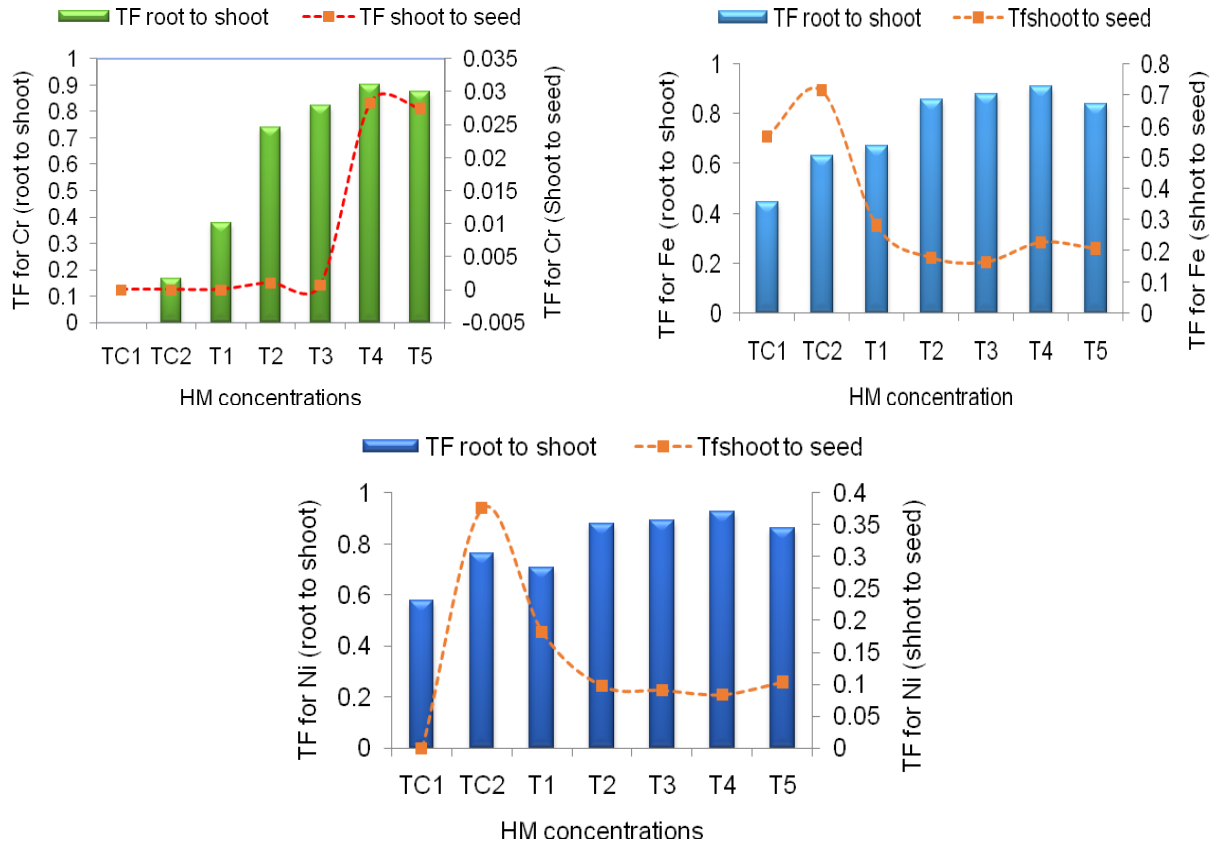

Figure 5 Distribution of metals on the basis of translocation factor (TF) from root to shoot and shoot to seed at different treatment

consistent with Alrawiq et al. (2014) and Kahangwa et al. (2021). The TF for root to shoot was increased as the concentration of metals increased (Figure 5). Plants generally not accumulated those heavy metals that are not needed in metabolic use. The translocation of toxic metals (Cr, Ni) may be restricted by roots to other organs due to blockage by casparian strips in the endodermis. These results were corroborated with the findings of Jamla et al. (2021).

Conclusion

Significantly higher accrual of Cr, Fe, and Ni were observed in different parts of the rice plant treated with different levels of heavy metals. This accrual may directly or indirectly impede various metabolic and biochemical activities along with oxidative damage by altering the enzymatic structure and their regulation. Accumulation and TF of heavy metals suggested that it is effective in removing PTHMs up to 3 times higher than the level of heavy metals present in TWW at present. Therefore, it can be used for the management of multi-metal polluted wastewater such as tannery wastewater or other wastewater but at certain limits (not more than 200 mg/kg). If it is treated above this, then seeds were also affected and not safe for human consumption. Further, the study revealed that plants irrigated with above T4 (200mg/kg) concentration have accumulated toxic metals in seeds beyond the permissible limit as suggested by different environmental agencies for human consumption.

Author contribution

ASK performed, analyze and drafted the manuscript, ASK, MB, DS, AA and AS participated in the experimental design and performed GC-MS and ICP-MS analysis, DS, AS, AA and VG conceptualized and finalize the manuscript. All authors approved the final manuscript.

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Conflict of interest

None

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Optimal condition for Propagation and Growing of *Dendrobium thyrsiflorum*

Nguyen Thi Oanh[1], Nguyen Thi Diem[1], Nguyen Huu Tho[1,2], Nguyen Thi Kim Cuc[1,*]

[1]Institute of Biotechnology, Hue University, Road 10, Phu Thuong, Phu Vang, Thua Thien Hue, Vietnam
[2]College Electro-Mechanics, Construction, and Agro-Forestry of Central Vietnam, QL1A, Cat Hanh, Phu Cat, Binh Dinh, Vietnam

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Dendrobium thyrsiflorum

In vitro

Medicinal orchid

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ABSTRACT

Dendrobium thyrsiflorum is a medicinal orchid that is being gradually destroyed due to over-exploitation. This research focuses on *in vitro* production of *D. thyrsiflorum* plantlet under optimal acclimatization conditions. The results of the study showed that the best medium for seed germination was MS basal medium (full-strength MS nutrient plus 100 ml L^{-1} coconut water; 7 g L^{-1} agar, 30 g L^{-1} sucrose, and 0.5 g L^{-1} active charcoal) supplemented with 60 g L^{-1} mashed sweet potatoes, which induces 97.8% of seed germination. The shoot was well developed in MS basal medium supplemented with 60 g L^{-1} mashed sweet potatoes and 0.4 mg L^{-1} 6-Benzylaminopurine (BA) and, corpulent and green in shoot morphology. The shoot multiplication rate was greatest on MS basal medium supplemented with 0.4 mg L^{-1} BA and 0.4 mg L^{-1} Kinetin with 4.53 times, and the shoot height was reported at 3.45 cm after 8 weeks of subculture. Further, The shoot was 100% rooting, with an average of 4.51 roots/shoot and 5.34 cm per root in length when the shoot was implanted on MS basal medium plus 60 g L^{-1} mashed sweet potatoes and 0.75 g L^{-1} active charcoal. Especially, plantlets after transplanting to the orchid net house reached a 94.8% survival rate on tree fern after 24 weeks. Hence, the results of the study suggested a successful production of *D. thyrsiflorum* plantlet on the selected media compositions.

* Corresponding author
E-mail: ntkcuc.huib@hueuni.edu.vn (T.K.C. Nguyen)

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1 Introduction

Vietnam is a tropical country with diverse natural resources, especially medical plants (Tran 1998). Among the medicinal herbs, orchids are one of the most important groups that have been researched so far. Different medicinal orchids such as *Dendrobium* species, *Gastrodia elata, Bletilla striata, Anoectochilus formosanus, A. koshunensis, Cremastra appendiculata, etc.,* were successfully identified (Bulpitt et al. 2007) and are used as a herbal medicine in Taiwan, Korea, Japan, China, Vietnam. etc., for treatment of various diseases (Pant 2013). Notably, some orchid species like *D. aphyllum, A. setaceus,* and *D. nobile* were successfully cultured under *in vitro* conditions that not only provided disease-free seedlings but also help in preserving these medicinal herbs. Recently, *D. thyrsiflorum* has been reported as a valuable medicinal plant (Bhattacharyya et al. 2015) since several phytochemicals including coumarins, polysaccharides, scoparone, and ayapin have been successfully isolated from this orchid (Yan et al. 2009; Liu et al. 2013; Tikendra et al. 2018) which help protect smooth muscles, blood vessels, and have anti-cancerous properties (Yan et al. 2009).

D. thyrsiflorum is considered abundantly distributed in China, India, Thailand, Myanmar, Laos, and Vietnam, previously (Li et al. 2013; Phan and Nguyen 2017; Dang et al. 2018). However, several research groups, recently, suggest that *D. thyrsiflorum* has been dramatically destroyed under its natural habitat, mainly due to the low rate of natural seed germination (<5%), human deforestation, and excessive illegal exploitation (Martin and Madassery 2006; Hossain et al. 2013; Da Silva and Ng 2017). To preserve and develop this species, some research groups tried to generate quality seedlings by *in vitro* propagation that focused on germination and *in vitro* production of plantlets in test tubes (Da Silva and Ng 2017; Chu and Dao 2018; Adhikari and Pant 2019; Maharjan et al. 2019; Lin et al. 2020) which is still far from a practical application.

D. thyrsiflorum accumulates different bioactive compounds especially, coumarins which have different pharmacological properties (Wu et al. 2009). Further, in *D. thyrsiflorum*, coumarins accumulated in different tissue from stems to roots as well as being present at different ages (Yan et al. 2009). Therefore, to preserve and prepare high-quality plantlets at different stages for comprehensive research to analyze different phytoconstituents in *D. thyrsiflorum* in Vietnam, the current study was carried out. The aim of this study was to rapid multiplication of *D. thyrsiflorum* under in vitro seed germination on different media compositions and to acclimatize the developed plantlets of *D. thyrsiflorum* after transplanting in a net house.

2 Materials and Methods

2.1 Plant materials and aseptic culture

Seed pod of six months old *D. thyrsiflorum* collected from Nam Tra My forest, Quang Nam, Vietnam was used as a sample in this study. All experiments were carried out at the Laboratory of Cells-Institute of Biotechnology, Hue University from November 2020 to October 2021; under the controlled condition with a temperature of $25^{o}C \pm 2^{o}C$, 8 hours of light photoperiod, and between 1000 and 2000 lux light intensity. The culture media used in this study were adjusted to pH 5.8 before autoclaving and then poured into special Polypropylene (PP) plastic bags with square bottoms with approximately 70 mL media/bag (Le et al. 2022). These bags were kept aseptic on the clean bench with paper clips until used.

2.2 Methods

2.2.1 Seed germination and protocorm formation

Six-month-old fruits of *D. thyrsiflorum* were gently washed twice with cotton wool and disinfected with soap under tap water. Further, in a sterile incubator, the fruits were thoroughly washed twice in sterile distilled water for about 90 seconds. Then, the fruits were dipped in 96% alcohol and shaken for 20 seconds before being burned over an alcohol lamp flame for three seconds (Le et al. 2022; Nguyen et al. 2022).

After sterilization, the fruits were placed in a petri dish before being cut into two parts longitudinally to collect seeds. The seeds were then spread evenly over the surface of the prepared basal MS medium (MS nutrition + 100ml L^{-1} coconut water + 30g L^{-1} sucrose + 0.5 g L^{-1} activated carbon + 6.5 g L^{-1} agar) or Knops basal medium (Knops nutrition + 100ml L^{-1} coconut water + 30g L^{-1} sucrose + 0.5 g L^{-1} activated carbon + 6.5 g L^{-1} agar) supplemented with 60 g L^{-1} sweet potatoes or potatoes (Nguyen et al. 2014; Nguyen et al. 2016).

There were four different combinations of media used in this experiment, each medium had three replications and each replication had 10 culture medium bags containing sterilized seeds. The germination and contamination rate was analyzed after 6 weeks of culture; the protocorm morphology was assessed after 8 weeks of inoculation, and the optimal medium in this experiment was used for subsequent experiments. The percentage of seed germination was calculated by (a/b)*100, where a is the number of culture medium bags containing germinated sterilized seeds, and b is the total bags of particular treatment. The formulated four different combination media are

Medium 1: Basal MS medium + 60 g L^{-1} potatoes

Medium 2: Basal MS medium + 60 g L^{-1} sweet potato

Medium 3: Basal Knops medium + 60 g L^{-1} potatoes

Medium 4: Basal Knops medium + 60 g L^{-1} sweet potato

2.2.2 Shoot formation from protocorm

The protocorm was inoculated on the optimal medium from the "seed germination and protocorm formation" experiment and added with BA at a concentration of 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg L^{-1} to evaluate the ability of shoot formation from protocorm. The percentage of shoot formation, shoot height, number of leaves/shoot, and morphological characteristics of the shoot were obtained after 6 weeks of culture. The percentage of shoot formation was calculated by (a/b)*100, where a is the number of shoots induced, and b is the total number of protocorms. The shoot height was measured using a tape measure and the average number of leaves per shoot was calculated. There were three replications for each treatment and each replication had 10 samples.

2.2.3 Shoot multiplication

In this experiment, the shoots were cultured on the optimal medium supplemented with the best concentration of BA from the "shoot formation from protocorm" experiment in combination with concentrations from 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg L^{-1} of KIN to evaluate shoot multiplication after 8 weeks of culture. The monitoring indicators include: shoot multiplication coefficient, shoot height, and morphological characteristics of shoots.

2.2.4 Rooting

Most of the plants required auxins to induce rooting. In the *in vitro* propagation process, authors widely used α-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA) for shoot establish roots (Nguyen et al. 2012; Nong et al. 2016; Nguyen and Nguyen 2020). In addition, activated charcoal (AC) was added to the culture medium to improve cell growth as well as adsorb plant cell growth inhibitors. The role of AC has been demonstrated in the rooting of different plants (Nguyen et al. 2012). Therefore, in this experiment, we used the growth regulators, including Indole-3-acetic acid (IAA), NAA (Naphthalene acid acetic), and AC to determine the role of these substances in the rooting of *D. thyrsiflorum*.

After the shoot proliferation stage, shoots reaching a height of 2.5 to 3.0 cm were transferred to basal MS medium supplemented with IAA or NAA at a concentration of 0; 0.1; 0.3; 0.5 and 0.7 mg L^{-1} or AC with a concentration of 0.5; 0.75; 1 and 1.25 g L^{-1} for rooting. The rate of root induction in shoots, the number of roots, and the root length were identified after 6 weeks of culture.

2.2.5 Acclimatization plantlets

Plantlets with well-developed roots had 4.5-5 cm in height and 4-5 leaves which were removed under the controlled condition and acclimatized in the orchid net house for around two weeks to train gradually with the ambient environment before transplanting (Da Silva et al. 2017).

The orchid net house was about 60 m^2 in size and set up with an irrigation and shading system to avoid direct sunlight to plantlets and keep the temperature in the net house between 25^{o}C and 32^{o}C, light intensity was set between 1000 and 2000 lux, and about 12 to 16 hours light photoperiod.

In the transplanting process, plantlets were taken out from the culture bags and then washed thoroughly several times under tap water to remove the culture medium from the plantlet's roots before being grown on three different substrates to identify the survival rate and the development of plantlets after transplanting (Le at al. 2022). Plantlets were grown in pots (4x4cm) with a substrate consisting of coconut fiber/husk (1:1 ratio, w/w) (Nguyen et al. 2021), or mounted on milk apple wood (20x15cm) (Pradhan et al. 2014) or on tree fern (20x15cm) (Kang et al. 2020) with sphagnum moss.

Each treatment has three replications, each replication has 50 plantlets. The survival rate and growth indicator were analyzed after 24 weeks. Stem height was calculated from the base to the highest leaf using a tape measure.

2.3 Statistical analysis

Statistical analysis was performed using Microsoft Excel 2016, and by one or two ways analysis of variance (ANOVA) followed by Turkey's test, using the SPSS statistic 20.0 software (SPSS Inc., Chicago, IL, USA). Data represented significant differences as $p< 0.05$.

3 Results and Discussion

3.1 Germination of *D. thyrsiflorum* on Plant Growth Regulator-free medium

Results presented in Table 1 revealed that the starting medium had a significant effect on the germination of seeds after six weeks of sowing. The basal culture medium supplemented with mashed sweet potato or potato has a positive effect on *D. thyrsiflorum* seed germination (>80%) and the highest percentage of seed germination was 97.8% when seeds were cultured on basal MS medium (Medium 2) supplemented with 60 g L^{-1} mashed sweet potato. This figure was dramatically higher than those cultured on Medium 1 (86.0%), Medium 3 (85.7%), and Medium 4 (80.5%). Further, like seed germination, protocorm morphology was also

reported superior in Medium 2 and this was corpulent, round, and green in color (after eight weeks of sowing) which was better than those in other culture treatments (Figure 1b, c). Thus, Medium 2 is the most effective medium for *D. thyrsiflorum* orchid seed germination.

These results suggested that the *D. thyrsiflorum* seeds can germinate on the culture medium without plant growth regulator (PGR) supplemented, which will significantly contribute to reducing the cost of commercial plantlet production. In contrast to this, Tikendra et al. (2018) determined that the seeds of five-month-old *D. thyrsiflorum* germinated the best on the culture medium having different concentrations and combinations of BAP (BA), KIN, IBA (Indole-3-butyric acid), IAA, and NAA. In addition, previous studies have also identified that the PGR needs to be supplemented in the cultured medium to improve the seed germination rate, for example, *D. anosmum* had the best germination rate, reaching > 85% when cultured on MS basal medium supplemented with 1.0 mg L^{-1} BA and 0.2 mg L^{-1} NAA (Nguyen et al. 2021). Further, Le et al. (2020) reported that *D. adasatra* germinated on MS medium supplemented with 0.1 mg L^{-1} BAP at a rate of 98.5% after 6 weeks of culture, Asghar et al. (2011) reported that *D. noblie* germinated on the MS medium supplemented with 2.0 mg L^{-1} BAP. Hence, using MS basal medium supplemented by 60 g L^{-1} sweet potato could be an alternative medium for *Dendrobium* species seeds inoculation.

The microbial contamination rate was the most important figure to evaluate the success of the study. In this current study, this number was less than 5% which is an acceptable percentage in tissue culture (Cassells 1991).

Table 1 The influences of culture media on the seed germination and protocorm morphology

Time	6 weeks	8 weeks	Microbial contamination Rate (%)
Culture media	Germination rate (%)	Protocorm morphology (%)	
Medium 1	86.0[b]	Medium, light green	2.2
Medium 2	97.8[a]	Corpulent, green	2.2
Medium 3	85.7[b]	Corpulent, light green	2.2
Medium 4	80.5[c]	Medium, yellowest-green	4.4

Different letters on the same column indicate a statistically significant difference in the sample means with the p-value = 0.05 (Ducan's test)

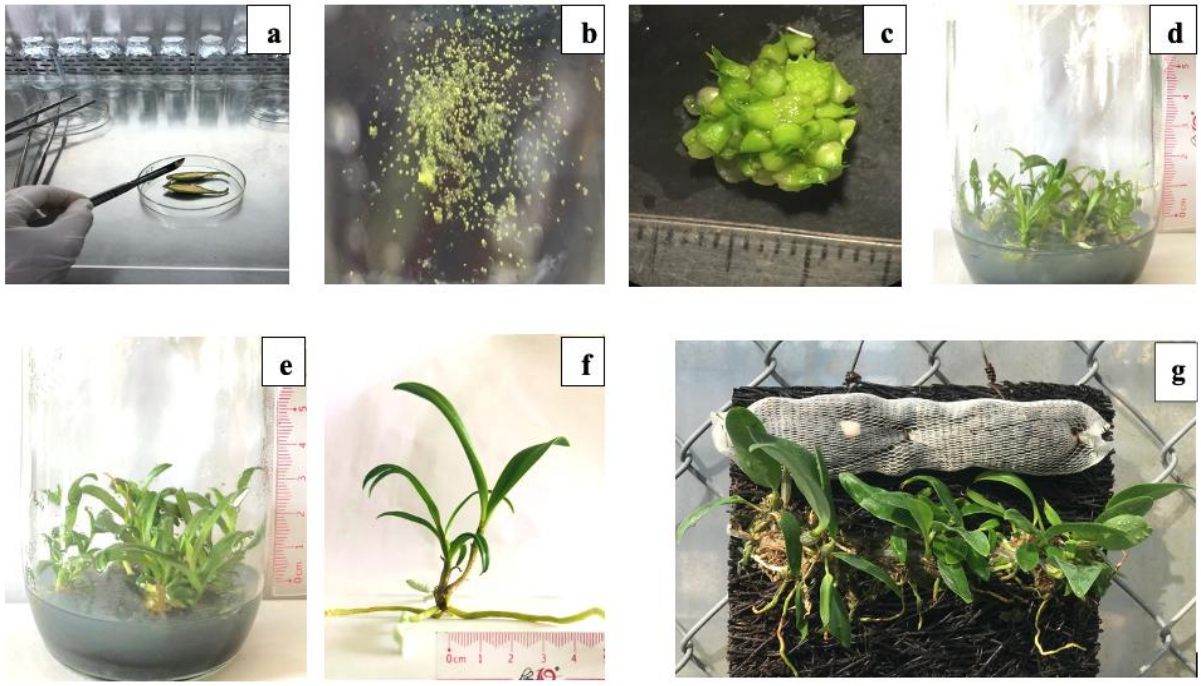

Figure 1 In vitro propagation of D. thyrsiflorum; (a) Collect seeds; (b), (c) Protocorm formation on medium 2; (d) Shoot development on medium containing 0.4 mg L^{-1} BA; (e) Shoot multiplication on medium adding 0.4 mg L^{-1} KIN and 0.4 mg L^{-1} BA; (f) Plantlets; (g) Plantlets growth in fern roots after 24 weeks.

Table 2 Effects of BA on the shoot formation from protocorm

BA (mg/L)	Shoot formation rate (%)	Shoot height (cm)	Number of leaves/shoot	Shoot morphology
0.0	64^{e}	1.17^{c}	2.40^{d}	Thin, light green
0.2	82^{d}	1.33^{b}	2.67^{cd}	Thin, green
0.4	98^{a}	1.53^{a}	3.67^{a}	Big, green
0.6	91^{b}	1.48^{ab}	3.27^{ab}	Big, green
0.8	84^{c}	1.29^{b}	3.07^{bc}	Medium, green
1.0	76^{d}	1.16^{c}	2.53^{d}	Thin, light green

Different letters on the same column indicate a statistically significant difference in the sample means with the p-value = 0.05 (Ducan's test).

Table 3 The effects of BA combined with KIN on the shoot multiplication capacity

BA (mg/L)	KIN (mg/L)	Numbers of shoots/shoot	Shoot height (cm)	Shoot morphology
0.4	0.0	2.40^{e}	2.24^{e}	Thin, green
0.4	0.2	3.67^{bc}	2.85^{c}	Medium, green
0.4	0.4	4.53^{a}	3.45^{a}	Big, green
0.4	0.6	3.93^{b}	3.02^{b}	Big, green
0.4	0.8	3.27^{bcd}	2.95^{bc}	Medium, light green
0.4	1.0	2.93^{d}	2.64^{d}	Thin, light green

Different letters on the same column indicate a statistically significant difference in the sample means with the p-value = 0.05 (Ducan's test)

3.2 Effects of BA on shoot formation from protocorm

As per the results of the first experiment, the basal MS medium containing 60 g L^{-1} sweet potato was used as the culture medium for this stage supplemented with various concentrations of BA to evaluate the formation and development of shoots from the protocorm. After six weeks of culture, protocorms were mostly induced to form shoots, and Table 2 clearly shows that the control medium (without BA addition) had the lowest average rate of shoot induction (64%) and the height of shoot length was only 1.17 cm, with 2.4 leaves/shoot, and light green shoots morphology while the increasing BA concentration from 0.2 to 0.6 mg L^{-1} in the medium led to a rise in the shoot formation rate.

Specifically, the concentration of 0.4 mg L^{-1} BA gave the best results on shoot growth and showed an average of 98% shoot formation, 1.53 cm of the average shoot height, and 3.67 leaves/shoot (Figure 1c). The shoot morphology in this medium was big and green. When gradually increasing the concentration of BA added to the culture medium from 0.8 to 1.0 mg L^{-1}, the percentage of shoot induction, the shoot height, and the number of leaves/shoot were reduced along with the smaller and light green color shoot body (1.0 mg L^{-1} BA). Maharjan et al. (2020) worked on the shoot proliferation in D. *chryseum* Rolfe and reported that ½ strength MS medium supplemented with 2.0 mg L^{-1} KIN has a significant effect on the shoot generation. Similarly, Nguyen (2021) identified that MS medium supplemented with 1.0 mg L^{-1} BA was good for shoot derived from *D. anosmum* protocorm. Meanwhile, Nguyen et al. (2016) combined three different PGRs in the medium culture (Basal Knops nutrient medium supplemented with 0.5 mg L^{-1} KIN + 0.3 mg L^{-1} BA + 0.2 mg L^{-1} NAA) to initiate shoot from the protocorm of *D. amabile* (Lour.) and suggested that each orchid species had a different requirement for their regeneration process.

3.3 Shoot multiplication

The combination of 0.0-1.0 mg L^{-1} KIN and 0.4 mg L^{-1} BA concentrations had a significantly positive effect on the shoot multiplication of *D. thyrsiflorum* (Table 3).

In general, the rate of shoot proliferation in all experiments was more than 2.4 shoots/shoot. The shoot multiplication gradually increased with the rise of KIN concentration from 0.0 mg L^{-1} to 0.4 mg L^{-1} in the culture medium. Remarkably, combining BA with KIN at a concentration of 0.4 mg L^{-1} gave the best results with a shoot multiplication number of 4.53 shoots/shoot with an average shoot height of 3.45 cm, and the proliferate shoots are big and the leaves are green in color (Table 3, Figure 1e). However, the shoot proliferation, as well as the shoot height, decreased substantially when the KIN concentration increased up to 1.0 mg L^{-1} (2.93 shoots/shoot; 2.64 cm in shoot height) and the leaves were light green, and the shoots were small and thin.

The use of BA and KIN for *D. thyrsiflorum* shoot proliferation in this study (4.53 shoots/shoot) gave better results as compared to the Tikendra et al. (2018) study conducted on the same species collected from India when they used KIN and IAA for shoot multiplication (3.83 shoots/shoot). The number was also higher

than those of *D. chryseum* shoot multiplication (3.73) shoot/shoots. On the other hand, for rapid multiplication of *D. amabile* (Lour.) shoot, Nguyen et al. (2016) used a combination of all three PGRs including KIN, BA & NAA, and it led to rapid multiplication and it is up to 12.57 shoots/shoot.

3.4 Effect of IAA, NAA and AC on rooting

The shoots reaching a height of 2.5 to 3.0 cm in the proliferation stage were transplanted to the rooting medium to generate plantlets. The base of MS medium containing 60 g L^{-1} sweet potatoes was supplemented with IAA or NAA at a concentration of 0.1-0.7 mg L^{-1} or adjusted with AC with a concentration of 0.5-1.25 g L^{-1} to evaluate the rooting ability of *D. thyrsiflorum* collected in Vietnam.

The growth regulator IAA and NAA both had a concentration-dependent effect on the rooting ability of *D. thyrsiflorum* orchid (Table 4). With regard to IAA, the concentration of 0.5 mg L^{-1} IAA gave the highest rooting response of 93%, which was significantly higher than those in control (82.93%), 0.1 mg L^{-1} (85.20%), 0.3 mg L^{-1} (87.87%); and 0.7 mg L^{-1} (91.30%), correspondingly. However, this percent was considerably lower than that of *D. thyrsiflorum* shoot inoculation on culture medium supplemented with 0.5 mg L^{-1} or 0.7 mg L^{-1} NAA with 100% of shoot promoting root. The shoot height and the number of roots per shoot in culture medium supplemented with 0.5 mg L^{-1} NAA was the best with 5.31 cm in root height and 4.4 roots/shoot. These number was greater than those in culture medium fortified with 0.7 mg L^{-1} NAA (5.28 cm in root height, and 3.6 roots/shoot) and 0.5 mg L^{-1} IAA (4.3 cm in root height and 3.53 roots/shoot), respectively (Table 4).

Table 5 shows that when gradually adding the amount of AC in the medium, the percentage of rooting and the root length increased, but the number of roots tended to decrease. At a concentration of 0.74 g L^{-1} AC, the rooting rate reached the highest with 100%, the root height was 5.34 cm, and the roots per shoot were 4.51. The rooting rate was also 100% in the medium having 1.0 g L^{-1} AC and 1.25 g L^{-1} AC but the roots height and the number of roots per shoot were significantly lower (Table 5). Hence, the addition of 0.75 g L^{-1} AC to the culture medium was most effective, and it helped the plantlets to grow and develop well, positively affecting the rooting ability of the plant (Figure 1f).

Comparing the effect on the rooting ability of IAA, NAA and AC, the results of this study revealed that when media supplementing NAA with a concentration of 0.5 mg L^{-1} or AC with a concentration of 0.75 g L^{-1}, the rooting rates were similar (100%). However, compared with the cost and optimal environmental conditions, it is better to replace NAA with an AC growth regulator. The use of AC in the medium was also studied by Nguyen et al. (2022) on *D. anosmum* Lindl rooting The authors determined that 1.0 g L^{-1} AC was the best for *D. anosmum* Lindl shoot initiating root with 3.17 roots/shoot which was lower than that of this current study. Therefore, the optimal environment to create plantlets for *D. thyrsiflorum* was basal MS medium supplemented with 60 g L^{-1} mashed sweet potato and 0.75 g L^{-1} AC.

Table 4 Effects of IAA and NAA on the rooting capacity of shoots

IAA (mg/L)	NAA (mg/L)	Rooting rate (%)	Root height (cm)	Number of roots/shoot
0		82.93^{e}	3.45^{c}	2.67^{b}
0.1		85.20^{d}	3.82^{b}	2.93^{b}
0.3		87.87^{c}	3.92^{b}	3.07^{ab}
0.5		93.00^{a}	4.30^{a}	3.53^{a}
0.7		91.3^{b}	4.05^{ab}	3.13^{ab}
	0.1	89.53^{c}	3.56^{d}	2.87^{c}
	0.3	95.13^{b}	3.90^{c}	3.20^{bc}
	0.5	100.00^{a}	5.31^{a}	4.40^{a}
	0.7	100.00^{a}	5.28^{ab}	3.60^{b}

Different letters on the same column indicate a statistically significant difference in the sample means with the p-value = 0.05 (Ducan's test)

Table 5 Effects of AC on the rooting capacity of shoots

AC (g/L)	Rooting rate (%)	Root height (cm)	Number of roots/shoot
0.50	83.33^{b}	3.54^{d}	2.73^{d}
0.75	100.00^{a}	5.34^{a}	4.51^{a}
1.00	100.00^{a}	5.02^{b}	3.86^{b}
1.25	100.00^{a}	4.05^{c}	3.33^{c}

Different letters on the same column indicate a statistically significant difference in the sample means with the p-value = 0.05 (Ducan's test).

Table 6 Effects of substrate cultures on the survival rate and growth indicator of plantlets

Substrate culture	Survival rate (%)	Stem height (cm)		Numbers of leaves/plantlets	
		Initial	After 24 weeks	Initial	After 24 weeks
SC1	82.2	4.5	7.3[b]	4	6.2[b]
SC2	87.8	4.5	7.4[b]	4	6.0[b]
SC3	94.8	4.5	7.9[a]	4	6.5[a]

Different letters on the same column indicate a statistically significant difference in the sample means with the p-value = 0.05 (Ducan's test)

3.5 The growth and development of plantlets after transplanting

After 24 weeks of growing on different substrate cultures, the plantlets were successfully acclimatized in the net house (Figure 1g**).** The survival rate was 94.8% when plantlets were grown on tree fern root mounts and mulched with sphagnum moss (SC3) which was 7% and 12.6% higher than those planted on pot substrate consisting of coconut fiber/husk (1:1 ratio, w/w; SC1), and on tree milk apple and mulched with sphagnum moss (SC2), respectively (Table 6). The stem height and the number of leaves per plant increased significantly when plantlets grown on SC3 were compared to the initial number and those in two other substrates. There was 7.9 cm in plant height and 6.5 leaves/plant on SC3 compared to 7.4 cm in plant height and 6.0 leaves/plant (SC2), and 7.3 cm in plant height and 6.2 leaves/plant (SC1) in turn.

The survival rate after transplanting in this study was substantially higher than that of Tikendra et al. (2018) study in this species with 91% after hardening in greenhouse conditions using a potting mixture of brick, charcoal species, and coconut husk (1:1:1). This identified that the substrate in this current study could be more efficient for *D. thyrsiflorum* plantlet growth than other substrates.

Conclusion

We have firstly investigated the optimal conditions for *in vitro* propagation of *D. thyrsiflorum* collected in Vietnam. The appropriate seeding medium was MS basal medium supplemented with 60 g L^{-1} sweet potato. The suitable medium for shoot development was MS basal supplemented with 60 g L^{-1} mashed sweet potato and 0.4 mg L^{-1} BA, the best rapid shoot multiplication was a combination of 0.4 mg L^{-1} BA and 0.4 mg L^{-1} KIN in the culture medium. 100% of the rooting shoot was identified in the medium with 0.75 g L^{-1} AC. Plants in the nursery on fern roots have a 94.8% survival rate.

Author contribution

TDN and TKCN designed the experiment. TDN, HTN, and TON carried out the experiments and performed data analyses. TDN and TON prepared all of the figures, and all authors contributed to data interpretation. TON wrote the first draft of the manuscript, and TDN and TKCN 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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Prevalence of Respiratory Symptoms and Associated Risk Factors among Street Food Vendors in Klang Valley, Malaysia

Agnes Pang Jia Yi[1], Sumedha[1,2*], Vinodhkumar Ramalingam[1*]

[1]Faculty of Health & Life Sciences, INTI International University, 71800 Nilai, Negeri Sembilan, Malaysia.
[2]Centre for Physiotherapy and Rehabilitation Sciences, Jamia Millia Islamia, Okhla, New Delhi, 110025, India.

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Working Hours

ABSTRACT

The street vendors in Malaysia are at an increased risk of developing respiratory symptoms owing to the continuous exposure to road dust, vehicle emissions, extreme weather conditions, and air pollutants from industrial sites. Hence, the current study aimed to establish the prevalence of respiratory symptoms and the risk factors associated with it among street food vendors in Klang Valley, Malaysia through a cross-sectional study among 237 street food vendors. The socio-demographic data, work characteristics, and information on respiratory symptoms were collected using a self-administered questionnaire. The data analysis was done by using the Chi-square test of association and frequency distribution. The study results revealed that the most frequently reported respiratory symptoms among the street food vendors were sore throat (30.8%), followed by cough (29.1%). No significant association was found between age, gender, duration of job and cough, sputum production, breathing difficulty, chest pain, irritated nose, and sore throat. A statistically significant association was found between working hours and sputum production (p=0.014). Further, the working hours were significantly associated with breathing difficulty (p=0.011). A significant association was also found between the type of cooking fuel used and the presence of cough (p=0.001). Results of this study demonstrated a positive association between work-related risk factors such as working hours with breathlessness and sputum production, and also between cough and the type of cooking fuel used. Based on the aforementioned findings, various control measures such as regular monitoring of lung functions and health education programs can be undertaken. Moreover, vendors need to consider using clean fuels instead of charcoal.

* Corresponding author

E-mail: vinodh.ramalingam@newinti.edu.my (Dr. Vinodhkumar Ramalingam); sumedha.svn@gmail.com (Sumedha)

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1 Introduction

Air pollution has emerged as one of the biggest threats that humankind is combating in the twenty-first century (Usmani et al. 2020). As per World Health Organization, 11.6% of all global deaths (translated to 6.5 million deaths) occurred due to 92% of the population breathing in dirty air (WHO 2016). The association between various health-related variables such as mortality rate, reduced lung function, hospital admissions, and traffic-related air pollution, have been well established through various epidemiological studies (Prabhu et al. 2019; Sepadi and Nkosi 2022). Similar to the global scenario, air pollution has also become a prominent environmental challenge in Malaysia as well (Othman and Latif 2021). Industrialization, haze episodes, and the increasing trend of using private transportation have all significantly contributed to the same (Usmani et al. 2020). Klang Valley being an important economic hub in Malaysia has a noticeably deteriorated air quality owing to the fast-paced urbanization, infrastructure development, and industrialization among other factors (Rahman et al. 2015). This region sees varied factors contributing to the increase in air pollutants from commercial, and industrial to motor vehicles and transboundary haze. In 2009, respiratory diseases were among the 10 principal causes of mortality in Malaysia (Rahman et al. 2015). The air pollution in Klang valley significantly contributed to this spike in data vis-a-vis respiratory disease.

Street food vending is a popular business activity in Malaysian cities and to garner more foot traffic, the vendors are usually situated along major roads in thriving urban environs. Although this may lead to an increase in business, it also causes a proportional increase in exposure to traffic emissions which are a known risk to respiratory health (Jones et al. 2008). The roadside vendors in Malaysia are a high-risk population for developing respiratory symptoms due to continuous exposure to the traffic pollutants as well as the particulate matter arising from the use of fossil fuels during cooking. The common respiratory symptoms reported include cough, sputum production, irritated nose, sore throat, and upper & lower respiratory tract symptoms (Kongtip et al. 2008).

A similar study conducted in Bangkok has also concluded that roadside street vendors were at higher risk for developing respiratory and other adverse health-related symptoms (Kongtip et al. 2008). In another survey conducted among street vendors in Bangkok, vehicular emissions were attributed as the major cause of respiratory symptoms (Noomnual and Shendell 2017). Further studies have also been conducted which support the above findings. For instance, in Serdang, Malaysia, respiratory symptoms, lung function, and their association with traffic-related exposures were studied among the roadside vendors. The study concluded that roadside vendors had impaired lung function, and were at increased risk for developing respiratory symptoms owing to excessive exposure to the traffic-related pollutants (Amaran et al. 2016).

The literature focusing on the association between respiratory health of street food vendors and air pollution exposures in Malaysia is sparse (Amaran et al. 2016). Therefore, it is prudent to conduct further studies in Malaysia regarding the prevalence and associated risk factors of respiratory symptoms among street food vendors. This will help to close the existing gap of knowledge in the literature and help to provide practical suggestions to the policymakers to safeguard the health of this vulnerable population. This will also help to generate public awareness and enable the street food vendors to understand the threats posed by air pollution and steps to take to minimize the exposure from the same. Hence, the current study primarily aimed to establish the prevalence of respiratory symptoms and associated risk factors among street food vendors in Klang Valley, Malaysia.

2 Material and Methods

The research design utilized in the current study was cross-sectional via the purposive sampling method. The inclusion criteria were healthy street food vendors with age ranges between 20 - 50 years old. Cigarette and tobacco users (past and/or present users); people with a history of any cardiopulmonary conditions, abdominal or thoracic surgeries; passive smokers and/or pet owners were excluded from the scope of this study. Sample size estimation was done using the Raosoft sample size calculator. With a 90% confidence interval; 5% margin of error and 50% response distribution, the sample size calculated was 267.

The data was collected via a self-administered validated questionnaire in the English language, which included questions regarding general demographic data (age, gender), working details (working years, daily working duration in hours), type of cooking fuel used and respiratory symptoms (shortness of breath, wheezing, cough, chest pain, sputum production, nose irritating, sneezing, sore throat). The questionnaire was validated before data collection by three experts in this field. The data was collected between January 2021 and March 2021. The questionnaires were given out physically where street food vendors displayed their stalls or via online platforms. All participants were explained regarding the purpose and procedure of the study, and written informed consent was taken before data collection. This study was approved by the Research and Ethics Committee of INTI International University [INTI-IU/FHLS-RC/BPHTI/7NY12020/004]. IBM SPSS Statistics Version 21 was used for data analysis. The descriptive data such as general and work characteristics as well as the respiratory symptoms were presented by frequency and percentages. The association between the various risk factors and respiratory health symptoms has been determined using the Chi-Square test of association, with the significance level taken as $p< 0.05$.

3 Results

3.1 Respondent characteristics

A total of 300 questionnaires were distributed among street food vendors in Klang Valley. Out of this, 237 met the inclusion criteria and were analyzed in this study. As shown in Table 1, most of the street food vendors were male (59.1%) and were primarily working in the street for 6 to 10 years (40.1%). Most of them were aged around 30 to 39 years old (43.9%), and mostly worked for 5 to 8 hours a day (62.4%). The majority of them used liquefied petroleum gas (LPG) (88.2%) to cook the food.

Table 1 General and Work Characteristics of Street Food Vendors in Klang Valley, Malaysia

Respondent Characteristics		Frequency (%) (n=237)
Age	20 to 29 years old	52 (21.9)
	30 to 39 years old	104 (43.9)
	40 to 49 years old	81 (34.2)
	50 years old	0 (0)
Gender	Male	140 (59.1)
	Female	97 (40.9)
Duration of job (years)	0 to 5 years	87 (36.7)
	6 to 10 years	95 (40.1)
	11 to 15 years	38 (16.0)
	> 15 years	17 (7.2)
Working hours	< 5 hours	50 (21.1)
	5 to 8 hours	148 (62.4)
	> 8 hours	39 (16.5)
Type of cooking fuel	Charcoal	28 (11.8)
	LPG	209 (88.2)

3.2 Respiratory symptoms

Table 2 represents the prevalence of respiratory symptoms among street food vendors in Klang Valley, Malaysia. The most frequently reported respiratory symptoms among the street food vendors were sore throat (30.8%), followed by cough (29.1%), irritated nose (27.4%), breathlessness (7.2%), sputum production (5.1%), and least was chest pain (1.7%). Further, the case of wheezing was not reported among the selected street food vendor.

3.3 Associated risk factors

3.3.1 Age

No significant association was found between age and cough (p=0.282), sputum production (p=0.387), breathing difficulty (p=0.744), chest pain (p=0.724), irritated nose (p=0.580) and sore throat (p=0.737), as shown in Table 3.

3.3.2 Gender

No significant association was found between gender and cough (p=0.609), sputum production (p=0.616), breathing difficulty

Table 2 Prevalence of Respiratory Symptoms among Street Food Vendors in Klang Valley, Malaysia

Respiratory Symptoms		Frequency (%)
Cough	Presence of cough	
	Yes	69 (29.1)
	No	168 (70.9)
	Duration of cough	
	< 1 month	1 (4)
	1 to 3 months	6 (2.5)
	4 to 6 months	15 (6.3)
	7 months to 1 year	19(8.0)
	> 1 year	27 (11.4)
	Occurrence	
	Before working	6 (2.5)
	After working	63 (26.6)
Sputum production	Yes	12 (5.1)
	No	57 (24.1)
Breathing difficulty	Presence of SOB*	
	Yes	17 (7.2)
	No	220 (92.8)
	Aggravating factor	
	At rest	0 (0)
	With activity	17 (7.2)
	Severity	
	I did not do the activity today	0 (0)
	Not at all	0 (0)
	Slightly	12 (5.1)
	Moderately	5 (2.1)
	Severely	0 (0)
	So severe I did not do the activity today	0 (0)
Wheezing	Presence of chest sound	
	Yes	0 (0)
	No	237 (100)
Chest pain	Presence of chest pain	
	Yes	4 (1.7)
	No	233 (98.3)
	Occurrence	
	Before working	0 (0)
	After working	4 (1.7)
	Duration	
	< 1 month	2 (0.8)
	1 to 3 months	0 (0)
	4 to 6 months	1 (0.4)
	7 months to 1 year	0 (0)
	> 1 year	1 (0.4)
Irritated nose	Presence of irritated nose	
	Yes	65 (27.4)
	No	172 (72.6)
	Excessive sneezing	
	Yes	26 (11.0)
	No	39 (16.5)
Sore throat	Presence of sore throat	
	Yes	73 (30.8)
	No	164 (69.2)

*SOB: Shortness of breath

Table 3 Association between respiratory symptoms and risk factors among street food vendors in Klang Valley, Malaysia.

Parameters	Cough	Sputum Production	Breathing Difficulty	Chest Pain	Irritated Nose	Sore Throat
Age	0.282	0.387	0.744	0.724	0.580	0.737
Gender	0.609	0.616	0.130	0.162	0.635	0.372
Duration of Job	0.223	0.648	0.262	0.308	0.188	0.650
Working hours	0.330	0.014*	0.011*	0.155	0.736	0.829
Type of cooking fuel	0.001*	0.147	0.120	0.460	0.134	0.300

*Chi-square test, $p<0.05$

(p=0.130), chest pain (p=0.162), irritated nose (p=0.635) and sore throat (p=0.372) (Table 3).

3.3.3 Duration of job (years)

No significant association was found between duration of job and cough (p=0.223), sputum production (p=0.648), breathing difficulty (p=0.262), chest pain (p=0.308), irritated nose (p=0.188) and sore throat (p=0.650) (Table 3).

3.3.4 Working hours

No significant association was found between working hours and cough (p=0.330), chest pain (p=0.155), irritated nose (p=0.736), and sore throat (p=0.829). However, working hours and sputum production showed a statistically significant association (p=0.014). Working hours were also significantly associated with breathing difficulty (p=0.011) (Table 3).

3.3.5 Type of cooking fuel

No significant association was found between types of cooking fuel and sputum production (p=0.147), breathing difficulty (p=0.120), chest pain (p=0.460), irritated nose (p=0.134) and sore throat (p= 0.300). However, the type of cooking fuel used and the presence of cough demonstrated a statistically significant association (p=0.001) (Table 3).

4 Discussion

This study primarily aimed to establish the prevalence of respiratory symptoms and the associated risk factors among street food vendors in Klang Valley, Malaysia. Various previous studies explored the respiratory health of roadside hawkers in countries such as Nigeria (Nwankwo et al. 2018), Ghana (Amegah et al. 2021), Thailand (Kongtip et al. 2008), India (De et al. 2019; Prabhu et al. 2019), Brunei Darussalam (Wahid et al. 2014) Egypt (Serya et al. 2019) and South Africa (Sepadi and Nkosi 2022). However, there are only limited number of studies undertaken in Malaysia to understand the prevalence and risk factors of respiratory symptoms among the high-risk population of street food vendors. To the best of our knowledge, only one comparative study examined the respiratory health symptoms among roadside vendors in Serdang, Malaysia focusing mainly on traffic-related exposures (Amaran et al. 2016). Therefore, the findings of the present study might provide a deeper insight into the respiratory health of street food vendors in Malaysia and complement the current literature.

4.1 Prevalence of Respiratory Symptoms

The findings of the current study indicated that sore throat (30.8%) was the most common respiratory symptom, followed by cough (29.1%), irritated nose (27.4%), breathlessness (7.2%), sputum production (5.1%), and chest pain (1.7%), among the street food vendors in Klang Valley. These findings are in agreement with the results of a study done in Thailand which showed that sputum production, sore throat, and cough were among the most frequent respiratory symptoms reported by the street vendors. This comparative study also reported no wheezing by residential street vendors which are similar to the present study's findings (Kongtip et al. 2008). Comparable results regarding sore throat were shown in a study undertaken in India (36.3%) (Saxena et al. 2019) and Egypt (31.6%) (Serya et al. 2019). Further studies carried out in Brunei Darussalam (Wahid et al. 2014) and Nigeria (Awopeju et al. 2017) reported the prevalence of cough among roadside hawkers as 28.3% and 29.6%, respectively, which is similar to the present findings. In contrast to the current study, a comparative study undertaken in Serdang, Malaysia showed wheezing (68.3%) as the most common respiratory symptom followed by chest tightness, cough, and sputum production among the exposed roadside hawkers (Amaran et al. 2016). This may be because the current study was undertaken during the lockdown period owing to the COVID-19 pandemic. This resulted in a considerable reduction of vehicular emissions and other air pollutants such as those generated due to construction activities (Othman and Latif 2021). Apart from this, street food vendors were also using face masks, which might have reduced exposure to particulate matters, and hence no wheezing was reported.

4.2 Associated risk factors

Various associated risk factors including age, gender, and duration of job showed no statistically significant association with respiratory symptoms such as cough, sputum production, breathing

difficulty, chest pain, irritated nose, and sore throat. These findings are in agreement with the results of other studies which also showed no significant association between age, gender, and respiratory symptoms (Wahid et al. 2014; Amaran et al. 2016). However, contrasting results were reported in other studies regarding the total job duration (years), showing a significant association with the respiratory symptoms (Wahid et al. 2014; Amaran et al. 2016). A probable reason could be that these studies have included individuals with preexisting medical conditions, smokers, and pet owners. These might be the confounding factors that affected the results for the studied association, whereas the same has been excluded under the present study.

The results of the present study showed a statistically significant association between working hours and sputum production (p=0.014). The working hours were also significantly associated with breathing difficulty (p=0.011). A prevalence study conducted in Egypt among 152 female food vendors showed that 92.8% of them worked for 8 hours or more per day. The most frequent respiratory symptoms amongst them were shortness of breath (67.8%), tightness of the chest (66.4%), sputum production (54.6%), nasal congestion (47.7%), and cough (42.8%) (Serya et al. 2019). However, the study did not verify the associated risk factors.

In addition, the observed significant association between the type of cooking fuel used and cough (p=0.001) in the current study was also consistent with mounting evidence provided via other studies on people with domestic biomass exposure. As per a study conducted in Nepal, the group of participants exposed to biomass smoke showed a significantly higher prevalence of airflow obstruction (8.1%) as compared to the control group (3.6%), with similar findings for males (7.4% vs 3.3%; p=0.022) and females (10.8% vs 3.8%; p<0.001) (Kurmi et al. 2013).

The present study has limitations due to the use of a self-administered questionnaire which might lead to recall and response bias. Future studies can be done using a longitudinal study design to study the effect of various risk factors on the respiratory health of street food vendors. Based on the present findings, various control measures such as regular examination of the work environment via air sampling, the use of face masks, and health education programs may be undertaken. Apart from this, vendors are also required to consider using clean fuels instead of charcoal.

Conclusion

The present study reported an association between work-related risk factors and respiratory health symptoms. Breathlessness and sputum production were associated with working hours whereas cough was associated with types of cooking fuel used. The results of this study show that street food vendors in Klang valley are at the risk for various respiratory symptoms that can lead to morbidity or mortality.

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Conflict of Interest

No conflict of interest was declared.

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A pilot study of Resilience Programme through Group Dynamics on Academic Problems among the Matthayom Suksa 1 Students of Chiang Mai University Demonstration School

Chanakarn Kumkun, Supat Chupradit*, Pornpen Sirisatayawong

Department of Occupational Therapy, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, 50200, Thailand

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ABSTRACT

The objective of this study was to develop a Resilience Programme through Group Dynamics on Academic Problems among Matthayom Suksa 1 Students at Chiang Mai University Demonstration School. For this, four junior high school students were selected as respondents. The effect of the resilience program was evaluated through a general questionnaire, the Canadian Occupational Performance Measures ***(***COPM***)***, and Resilience Inventory. Further, the resilience program was developed by using cognitive behavioral therapy combined with acceptance and commitment therapy, group dynamics, and resilience according to the concept of Grotberg. The total period of the program was 11 weeks, with 1 session per week lasting for 60 minutes. Results of the study revealed that all the selected respondents had higher academic performance and most of them (75%) had higher academic satisfaction and resilience score. After participating in this program, the samples had a higher average resilience score (114.5) as compared to those before participating in the program (107.5). The results of this study can be concluded that the newly developed resilience program can improve the resilience component in almost all the students. Hence, it can be practiced in junior high school students to manage their academic problems. This program can also be a prototype for developing future resilience programs.

* Corresponding author

E-mail: supat.c@cmu.ac.th (Dr. Supat Chupradit)

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1 Introduction

Students of junior high school are in their early teens, ranging from the age of 10 to 15 years (World Health Organization 2010). This is the right age of learning life skills that will help them to manage their emotions and the challenges of life. The changes at this age include the changes in the classroom from primary to secondary school. The changes in the body will also affect their perception of self-image. The changes in the mind which would like to have more freedom and the changes in the society which will give importance to the friends of same age more than the family, etc. The learning of early teens will happen from participation in everyday life activities (American Occupational Therapy Association 2014). The study activities are very important for early teens. The teens may face academic problems such as lacking discipline, using rude words, sleeping during class, lacking responsibility, and many others (Jullasub and Ativittayaporn 2008). They may also face challenges or adversity which put them in stress and anxiety (De Bruyn 2005; Sacker and Schoon 2007). It has been found that such pressure has a negative relationship with learning achievement (De Bruyn 2005). Hence, mental health promotion should be promoted for teens to help them grow completely according to their development and success in their studies (Fallon 2010). It has been found that resilience is a needed skill for teens. Resilience will help them to manage the challenges and academic problems effectively (Fallon 2010).

Resilience is the positive ability to efficiently manage or face hardship situations that might create stress and challenges in life (Tuntipivattanasakul 2008; Comas-Diaz et al. 2013; Ninthachan 2015; Bowden et al. 2018). The resilience can be contributed at the levels of personal, family, school, and community. Resilience consists of 3 components including external supporting factors (I have), personal realization (I am), and the realization of one's capacity (I can) (Grotberg 1995). If people have resilience, it will help them to create good feelings for their well-being and lead to suitable wellness (Abolghasemi and Varaniyab 2010). This is especially important in learning activities as resilience and participation in the activity will help to forecast the studying capacity of teens (Ayala and Manzano 2018).

From previous studies, it was reported that the research related to the resilience program has used various curing guidelines such as Cognitive Behavioural Therapy (CBT), which will focus on acceptance and commitment as known as Acceptance and Commitment Therapy (ACT), which is a therapy in group dynamics form **(**Burton et al. 2010; Towsyfyan and Sabet 2017; Joyce et al. 2018). Group dynamics have seven steps which are introduction, activity, sharing, processing, generalizing, application and summary. This curing guideline will provide efficiency for contributing to resilience (Kittisoonthorn 2016). Kittisoonthorn (2016) developed a cognitive behavioral therapy program in the form of groups, wherein they found that the experimental group had higher resilience and capacity in controlling emotions than the control group. In the experimental group, it was found that the scores for resilience and capacity in controlling emotion were higher after participating in the program. Moreover, Burton et al. (2010) developed a READY program based on acceptance and commitment therapy combined with cognitive behavioral therapy. Previous studies examine the efficiency of the contribution of resilience to wellness. The results found that the sample had more positive emotions, self-acceptance, and personal value. Besides, it was found that the efficiency of group dynamics on the contribution of resilience will help to create trust and collaboration in therapy. According to Cara (2013), a person will learn and develop via interaction with others through group dynamics.

In the present study, a resilience program was developed by using group dynamics on related problems with Matthayom Suksa 1 Students attending Chiang Mai University Demonstration School. The resilience program was developed under the idea of the resilience theory according to the concept of Grotberg, together with cognitive and behavioral therapy. The therapy focuses on acceptance and commitment by using group dynamics. The main objective of this study was to develop a study on the resilience program for Matthayom Suksa 1 students of Chiang Mai University Demonstration School.

2 Materials and Method

The quasi-experimental design was used in this research for evaluating the results of the newly developed resilience program. For this, a general information questionnaire, Canadian Occupational Performance Measures (COPM), which evaluates the academic performance and satisfaction (Law et al. 2020**)**, and the Resilience Inventory which evaluate the resilience score was used to extract the data. The resilience Inventory was developed based on the Grotberg Resilience concept **(**Grotberg 1995**)**. The developed questionnaire had 28 questions with Likert scales. The highest score was 140 while the lowest score was 28 (Ninthachan 2015). As respondents, four junior high school students of the academic year 2021, studying at Chiang Mai University Demonstration School were selected. The students were screened with purposive sampling by using the resilience score. The student who had the lowest resilience score in 8 orders of the population were selected for this study. Based on group dynamic theory, the sample size should have 3 to 8 persons in a group (Early 2017) that's why the present study had only 4 volunteers. The program was developed by using cognitive and behavioral therapy focussing on acceptance and commitment, group dynamics (Cole 2018), and resilience according to the concept by Grotberg (1995). The content accuracy of the developed theory was validated by qualified persons from the department of educational foundations and development, faculty of education; department of occupational

Table 1General information of the selected Respondents

General information		Number of persons	%
Gender	Male	0	0
	Female	4	100
Number of Brothers and Sisters	0	0	0
	1	3	75
	2	1	25
	3	0	0
Birth Sequence	1st in Sequence	4	100
	2nd in Sequence	0	0
	3rd in Sequence	0	0
	4th in Sequence	0	0
Socioeconomic Status of the family	Have money left over	3	75
	Have enough money / Not have enough money left	0	0
	Have debts	1	25
Average income of the family (Monthly)	Less than 10,000 THB	0	0
	THB 10,001- THB 25,000	0	0
	THB 25,001- THB 50,000	2	50
	THB 50,001- THB 100,000	1	25
	THB 100,001 up	1	25
Expenses (Monthly)	Not more than THB 5,000	2	50
	THB 5,001- THB 10,000	1	25
	THB 10,001- THB 15,000	0	0
	THB 15,001 or higher	1	25
Current residence type	Home	4	100
	Dormitory	0	0
Currently residing with	Father and Mother	4	100
	Only with Father	0	0
	Only with Mother	0	0
	Other family members	0	0
	Friend	0	0
	Alone	0	0
Residential Style	Housing Estate/Detached House/Townhouse	3	75
	Tenement House / Commercial Building	1	25
	Condominium / Flat Crowed Rally	0	0

therapy, faculty of associated medical science, Chiang Mai University, Thailand; educational psychology and counseling, faculty of education, KhonKaen University, Thailand; Suanprung psychiatric hospital and Galyarajanagarindra Institute, Thailand. The total period for the whole program was 11 weeks with 1 sessions lasting for 60 minutes per week.

2.1 Statistical analysis

Descriptive statistical analysis was used to analyze the general information, the resilience score, and academic performance and satisfaction.

3 Results

3.1 General Information

Results presented in Table 1 revealed that all the selected respondents are female (100%) and are the eldest in the family. Further, almost all of them have a brother or sister (75%). In the case of economic status, 75 percent of the selected respondent's family has enough money left and the acquired income of the family is 25,001-50,000 THB per month. The sample spends money less than 5,000 THB per month (50%). All of the samples live at home with their parents in a housing estate, detached house, or townhouse (75%).

3.2 Family Information

Results presented in Table 2 showed the family information of the selected respondents. Results of the study suggested that the selected maximum respondents are staying together (75%) and their father and mother work as staff in government service (50%) and got a bachelor's degree (50%). Half of the respondents have a warm relationship in their family (50%).

3.3 Academic performance and satisfaction

Results presented in Table 3 suggested that after participating in the program, most of the respondents (75%) have higher academic performance and satisfaction levels as compared the before participating in the program. One respondent has low satisfaction and this might be poor understanding of the term and conditions of the design study.

3.4 The resilience

Results presented in Table 4 revealed that most of the sample (75%) had a higher resilience score after participating in the program (114.5) as compared the before participating in the program (107.50).

Table 2 Family information of the samples

Family information		Number of persons	%
Marital status of father and mother	Stay together	3	75
	Separated	1	25
	Divorced	0	0
	Father and/or mother passed away	0	0
	Father passed away	0	0
	Mother passed away	0	0
Father Employment	Government service	2	50
	State enterprise employee	0	0
	Company employee	0	0
	Trader	2	50
	Agriculture	0	0
	Self business	0	0
	General employee	0	0
Mother Employment	Government service	2	50
	State enterprise employee	0	0
	Company employee	1	25
	Trader	1	25
	Agriculture	0	0
	Self business	0	0
	General employee	0	0
Father Education	Primary School	0	0
	Secondary School	0	0
	Higher School	0	0
	Bachelor's Degree	2	50
	Master's Degree	1	25
	Doctoral Degree	1	25
Mother Education	Primary School	0	0
	Secondary School	0	0
	Higher School	0	0
	Bachelor's Degree	2	50
	Master's Degree	0	0
	Doctoral Degree	2	50
Relationships within the family	Warm family	2	50
	Sometimes quarrel	1	25
	Frequently quarrel	1	25

Table 3 Academic performance and satisfaction level of the selected respondents

Respondents (n=4)	Academic performance			Academic satisfaction		
	Before (PS1)	After (PS2)	Change (PS2-PS1)	Before (SS1)	After (SS2)	Change (SS2-SS1)
A	5.40	8.80	3.40	4.00	9.20	5.20
B	2.25	5.60	3.35	4.16	7.37	3.21
C	6.00	6.00	0.00	2.00	3.00	1.00
D	3.00	6.80	3.80	8.40	4.60	-3.80

Table 4 Resilience scores of the studied respondents

Resilience score	Respondents (n = 4)				(Mean)	(SD)	SEM
	A	B	C	D			
Before participating the programme	136	92	104	98	107.50	19.621	9.811
After participating the programme	134	112	107	105	114.5	13.329	6.665

4 Discussion and Conclusion

Results of the study suggested that the samples had higher academic performance and satisfaction after participating in the program. Furthermore, the average resilience score after participating in the program was also higher than before participating in the program. The higher resilience score might occur from students' realization of their external supporting factors, personal realization, and capacity which is consistent with the resilience component according to the Grotberg resilience concept **(**Grotberg, 1995**)** This resilience program was developed using group dynamics, so it might help them to realize the external supporting factors. They can understand and cope with their academic problems from their perspective and can learn strategies from other members' perspectives. It is consistent with the group dynamics concept which explained that group dynamics can promote people learning and develop interactions between the members of the group (Cara 2013). In the part of personal realization and capacity, students realized themselves through cognitive behavioral therapy, and acceptance and commitment therapy such as drawing, writing, and self-monitoring. The academic performance and satisfaction scores of all the subjects after participating in this program were improved as the resilience program was expected to make the subjects realize their capacity and also analyzed academic problems and improve their skills. The respondents can develop their personal resilience to manage the academic problem they face. Overall, the results of the study revealed that a resilience program can improve the resilience component in almost all the selected respondents. Hence, this newly developed resilience program can be practiced in students to manage the academic problem faced by junior high school students. This research is a pilot study that's why the sample size is very small, so, in future, further studies with large sample sizes are

needed. This program can be a prototype for developing future resilience program.

Ethics Approval

The data collection in this study was approved by the IRB of the Faculty of Associated Medical Sciences, Chiang Mai University, Thailand (Approval Number: AMSEC-64EX-018). The funding was supported by the Graduate school, Chiang Mai University, Chiang Mai, Thailand. There are no potential conflicts of interest to declare.

Conflicts of Interest

The authors declare no conflict of interest.

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Vaccine hesitancy toward the COVID-19 vaccine among the Malaysian population

A. H. Azizan[1], G. Subramaniam[1*], L. A. Sivasamugham[1], S. Gopinathan[2], M. Raman[3], L.S. Wong[1]

[1]INTI International University, Persiaran Perdana BBN, Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia
[2]Multimedia University, Persiaran Multimedia, 63100 Cyberjaya, Selangor
[3]Asia Pacific University of Technology & Innovation, Jalan Teknologi 5, Taman Teknologi Malaysia, 57000 Kuala Lumpur, Wilayah Persekutuan Kuala Lumpur, Malaysia

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ABSTRACT

COVID-19 is a potentially fatal infectious disease that requires effective vaccines to keep the outbreak under control. Despite the ongoing efforts for an effective vaccine, public hesitancy towards vaccines is now one of the main concerns to the global health in containing this global pandemic. Thus, this preliminary study was carried out to assess the degree of COVID-19 vaccine hesitancy among the general public in Malaysia and to identify the underlying reasons for their hesitancy by using 5C psychological antecedents of vaccination. This study was conducted by carrying out a cross-sectional online survey for approximately two months between January to February 2021, involving 385 participants. The survey contained questions based on the 5C model proffered by WHO. The data from the survey were analyzed using Smart PLS 3 for statistical analysis, with the partial least squares structural equation modeling (PLS-SEM). According to the findings, only 62.5 percent out of the 385 participants had planned to get the COVID-19 vaccine, while the remaining 37.5 percent did not. The results also showed that confidence, calculation, collective responsibility, and constraints had a significant influence on vaccine hesitancy but not complacency. There is a degree of vaccine hesitancy towards the COVID-19 vaccines among the Malaysian population, although the data that we have obtained cannot be used to generalize for the entire Malaysian population due to the small sample size. Thus, for the vaccination campaign to be more effective, it should focus more on addressing the issue relating to confidence, calculation, collective responsibility, and constraints and less on complacency.

* Corresponding author
E-mail: geetha.subramaniam@newinti.edu.my (Geetha Subramaniam)

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1 Introduction

An outbreak of a mysterious pneumonia-like disease in Wuhan, China, devastated the world in late December 2019. On March 11, 2020, the disease was renamed as coronavirus disease 2019 (COVID-19) and declared a global pandemic by the World Health Organization (WHO 2020). COVID-19 is a deadly infectious disease caused by acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It causes a wide variety of symptoms, from mild (fever, cough, and shortness of breath) to more severe illnesses that could be fatal (CDC 2020). As of September 25, 2021, WHO had recorded over 230 million confirmed cases and over 4.7 million deaths in all countries worldwide (WHO 2021). The numbers have been steadily rising since the outbreak began even though several measures have been implemented to contain the infection. From the onset of the pandemic, there were no treatment options available against COVID-19, thus non-pharmaceutical interventions were imposed including social distancing, mass economic shutdowns, and lockdowns that aimed at avoiding human-human contact thereby reducing possible exposure to this virus (Wong et al. 2020). However, these measures have had negative impacts on the physical and psychosocial well-being of the general public, social interactions, and economic activity (Wan Mohd Yunus et al. 2021). In November 2020, PfizerBioNTech released the first vaccine against COVID-19 and since then, vaccination campaigns have progressed over the past few months globally to curb this seemingly unquenchable disease.

Nevertheless, if there is vaccine hesitancy in the general population, the presence of the COVID-19 vaccine would become less effective in preventing COVID-19 outbreaks. Vaccine hesitancy is a refusal or being hesitant to accept vaccination, which may result in the resurgence of vaccine-preventable diseases such as measles (MacDonald and Sage 2015). Since vaccine hesitancy can prevent the effective containment of vaccine-preventable diseases, such as COVID-19, WHO has named vaccine hesitancy as one of the top ten challenges to global health in 2019 (WHO 2019). Thus, this preliminary study was conducted to identify the underlying reasons for COVID-19 vaccine hesitancy among the general public in Malaysia so that strategies aimed at raising vaccine acceptance and immunization rates for COVID-19 vaccine and other vaccines in the future can be tailored.

Table 1 Measurement items based on the survey questions adapted from Betsch et al. (2018) and Larson et al. (2016)

Constructs	Items	Questions
Confidence (CD)	CD1	I am familiar with the term “vaccines”.
	CD2	The COVID-19 vaccines will be able to protect my body from the COVID-19 virus.
	CD3	The COVID-19 vaccine can modify my DNA.
	CD4	After vaccination, the COVID-19 vaccines pose a risk of infecting me.
	CD5	Good hygiene and proper nutrition would be a better option to reduce the spread of the COVID-19 virus, rather than taking the vaccine.
	CD6	I have previously experienced serious side effects with any of my previous vaccinations.
	CD7	If I have experienced serious side effects from previous vaccinations, this will discourage me from getting the COVID-19 vaccine.
	CD8	I have encountered a situation where my doctor has discouraged me from being vaccinated.
	CD9	I am worried about the safety of a rapidly-developed vaccine like COVID-19 vaccine.
	CD10	I am concerned about where the COVID-19 vaccine is made.
	CD11	Vaccines made in Europe or America are better than those made in other countries.
	CD12	I have refused a vaccine before because I thought it had porcine or other animal-derived ingredients (non-halal) in it.
	CD13	I am concerned about the safety and efficacy of the COVID-19 vaccines.
	CD14	Having public figures taking the COVID-19 vaccine helps in increasing my confidence to take this vaccine.
	CD15	I am willing to take the COVID-19 vaccine even when many people have not taken it yet.
	CD16	I prefer this mode of vaccine administration for the COVID-19 vaccine (you may choose more than one option)
	CD17	The delivery mode of the COVID-19 vaccine can affect my decision in getting this vaccine.
	CD18	I think the pharmaceutical companies are producing the COVID-19 vaccine solely for profit and not because they are concerned about public health.
	CD19	I have heard about the adverse effects that occurred in the volunteers of the clinical trials after taking the COVID-19 vaccine.

Constructs	Items	Questions
Complacency (CP)	CP1	I am at risk of being infected with the COVID-19 virus.
	CP2	COVID-19 is dangerous to my health and safety.
	CP3	The COVID-19 vaccine can help in overcoming the COVID-19 pandemic.
	CP4	Having the COVID-19 vaccine makes me less worried about the COVID-19 pandemic.
	CP5	There is a better way to prevent me from getting COVID-19 other than taking the vaccine.
Constraints (CT)	CT1	The price of the COVID-19 vaccine will affect my decision to take this vaccine.
	CT2	I agree with some of the global leaders and influencers on not administering the COVID-19 as circulated via social media.
	CT3	The traveling distance to the healthcare facility and the waiting period at the facility will be a hindrance to my being vaccinated.
	CT4	I plan on getting the COVID-19 vaccine when it is widely available.
	CT5	The reason I am not willing to get the COVID-19 vaccine is (Choose one or more than the following answers):
	CT6	I have previously refused a vaccine because I thought it had porcine or other animal-derived ingredients (non-halal) in it
	CT7	My religious beliefs or culture prevent me from taking the COVID-19 vaccine.
	CT8	The COVID-19 vaccines from Pfizer / BioNtech's, Astra Zenecca, and Moderna will require the recipient to take two doses to work effectively. This means that I must come to the clinic/hospital to take another shot after a few weeks. This will be a problem for me.
Calculation (CL)	CL1	I obtain information regarding the COVID-19 vaccine from (you may choose more than one)
	CL2	The information regarding the safety and efficacy of the COVID-19 vaccine on social media is reliable.
	CL3	I am aware of the reported side effects of the COVID-19 vaccines.
	CL4	I am interested in the various information regarding vaccine complications in the media.
	CL5	I am worried about the quality and validity of information being circulated in the media regarding the COVID-19 vaccine.
	CL6	The negative social media posts influence my opinion on the safety of the COVID-19 vaccine.
	CL7	I will handle the information concerning the side effects of the COVID-19 vaccines by (You may choose more than one option)
Collective responsibility (CR)	CR1	Everyone needs to be vaccinated against COVID-19.
	CR2	I know of friends/family members/others who have advised me to not take the COVID-19 vaccine.
	CR3	The opinions of my friends and family influence me against being vaccinated for COVID-19.
	CR4	I am familiar with the term "herd immunity".
	CR5	I need to proceed with vaccination even though other people around me have not been vaccinated.

2 Materials and Methods

The survey was based on the 5C scale for vaccination which assesses the psychological antecedents (depicted as constructs in Table 1) as the theoretical framework (Betsch et al. 2018).

2.1 Sampling method

A web-based, cross-sectional survey using an online Google Form, was carried out from 20th January to 17th February 2021, approximately ten months after the declaration of COVID-19 as a global pandemic by WHO. A few popular social network platforms in Malaysia such as WhatsApp, Facebook, and Instagram were used to circulate and spread the survey link to the general public. The involvement criteria were Malaysian citizens who were 18 years old and above and were literate in English as the survey questionnaire was tailored in English. The survey questions were designed based on several published studies, hence content validation was not required (Larson et al. 2016; Betsch et al. 2018). A total of 385 surveys were returned but only 379 were complete hence the valid response rate was 98.4%. The survey was conducted online involving human subjects; hence ethical approval was obtained from the INTI IU Ethics Committee (INTI/UEC/2018/001). A brief explanatory statement about the aim and purpose of this survey was given to respondents in the introduction part of the survey which included a guarantee of anonymity with regards to the respondents' data. Respondents were informed that their participation was solely voluntary and they were allowed to terminate their participation at any time.

2.2 Survey instrument

This study employed the 5-point Likert scale with a grade scale ranging from 1 (Strongly agree) to 5 (Strongly disagree) (Cook and Beckman 2006). The survey was comprised of two sections, the first of which contained questions that assessed the demographic background of respondents and their health status including their experiences with COVID-19. Section 2 contained questions that assessed vaccine hesitancy among respondents which was measured using the "5C model" of psychological antecedents to vaccination–derived items as listed in Table 1 (Larson et al. 2016; Betsch et al. 2018).

2.3 Statistical analysis

For the statistical analysis, Smart PLS 3 was utilized, where the method of partial least squares structural equation modeling (PLS-SEM) was used to calculate the quantitative results (Benitez et al. 2020). This analysis enabled the determination of the relationship between vaccine hesitancy (VH), depicted by dependent variables (DV), and the factors that influence vaccine hesitancy, depicted by independent variables.

Based on the 5C model and the design of the survey, a measurement model was developed (Figure 2) to test the significance of confidence, complacency, constraints, calculation, and collective responsibility towards vaccine hesitancy based on the hypotheses listed below:

H1: Calculation has a significant effect on vaccine hesitancy

H2: Collective responsibility has a significant effect on vaccine hesitancy

H3: Complacency has a significant effect on vaccine hesitancy

H4: Confidence has a significant effect on vaccine hesitancy

H5: Constraints have a significant effect on vaccine hesitancy.

To ensure validity and reliability of constructs, the Composite Reliability (CR) and Cronbachs' Alpha (CA) were tested for each component in the analysis (Ringle and Sarsted 2016). Two types of validity tests, convergent validity and discriminant validity which used the Fornell-Larcker criterion were used in this analysis (Fornell and Larcker 1981). The resulting structural model was assessed by the significance of path co-efficient using bootstrapping technique and the R-squared (R2) to analyze the determination of the standard path coefficient of each relationship between the variables.

3 Results

The total number of Malaysians who participated in this survey were 385 individuals. However, only 379 (98.44%) respondents' surveys were complete and could be used in the PLS analysis. All

Table 2 Summary of respondents' demographic characteristics

Characteristics	Number (n=)	Percentage (%)
Gender		
Male	122	31.66
Female	259	68.33
Age group		
>60	57	15.03
50-59	66	17.41
40-49	61	16.09
30-39	66	17.41
25-29	57	15.04
18-24	72	18.99
Ethnicity		
Indian	180	47.50
Malay	140	36.90
Chinese	52	13.70
Others	7	1.80
Religion		
Islam	140	36.90
Hindu	146	38.50
Christian	46	12.13
Buddhist	34	9.00
Others	13	3.43
Locality (State of residence in Malaysia)		
Selangor	113	29.80
Kuala Lumpur	43	11.30
Johor	26	6.90
Negeri Sembilan	16	4.20
Melaka	16	4.20
Penang	14	3.69
Perak	14	3.69
Kedah	11	2.90
Pahang	7	1.85
Terengganu	6	1.58
Sabah	4	1.05
Sarawak	3	0.79
Occupation		
Employed in the medical line	57	15.03
Employed in the non-medical line	189	49.90
Homemaker	65	17.10
Self-employed	68	17.90
Education level		
Tertiary (Science)	161	42.48
Tertiary (Non-Science)	151	39.84
Secondary (Science/Non-Science)	67	17.64

demographic characteristics such as religion, age, ethnicity, and gender, were varied (Table 2). From the obtained results, there were more female respondents (n=259; 68.33%) compared to males (n=120; 31.66%). All 6 age groups were almost represented equally with most of the respondents aged between 18 to 24 years of age (n=72, 18.99%). The majority of the respondents were Indian (n=180, %=47.50%), followed by Malay (n=140, %=36.90%), Chinese (n=52, %=13.70%) and others (n=7, %=1.80%). In the context of the respondents' religions, the number of Hindu respondents were the highest (n=146; %=38.50%) which could correlate with the majority of the respondents being Indian. Most of the states in Malaysia were represented with the highest number of respondents being from Selangor (n=113, %=29.80%). With regards to employment, it was found that a majority of the respondents were employed in the non-medical sector (n=189, %=49.90%), followed by those who were self-employed (n=68, %=17.90%), homemaker (n=65, %=17.01%), and employed in the medical line (n=57, %=15.03%). In terms of education level, most of the respondents had at least a tertiary education level whereby a large number of them were from a science-related background (n=161; %=42.48%).

Analysis of the data also indicated that there were only 62.5% of Malaysians expressed their intentions to get the COVID-19 vaccination once it became widely available while the 13.4% did not have any intentions of doing so (Figure 1). The data was subsequently analyzed by the PLS-SEM software to determine the measurement model's reliability and validity, indicator reliability, convergent validity, internal consistency reliability, and discriminant validity (Figure 2).

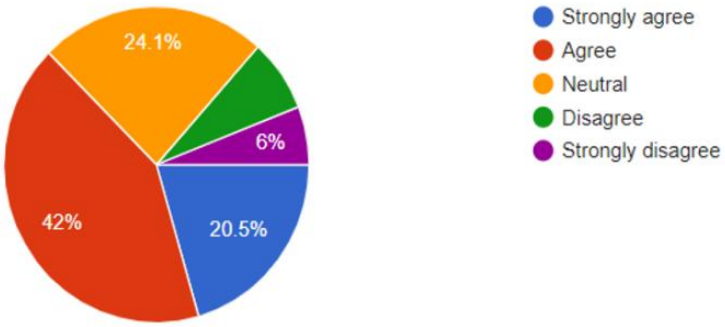

Figure 1 Respondents' willingness to take the COVID-19 vaccine

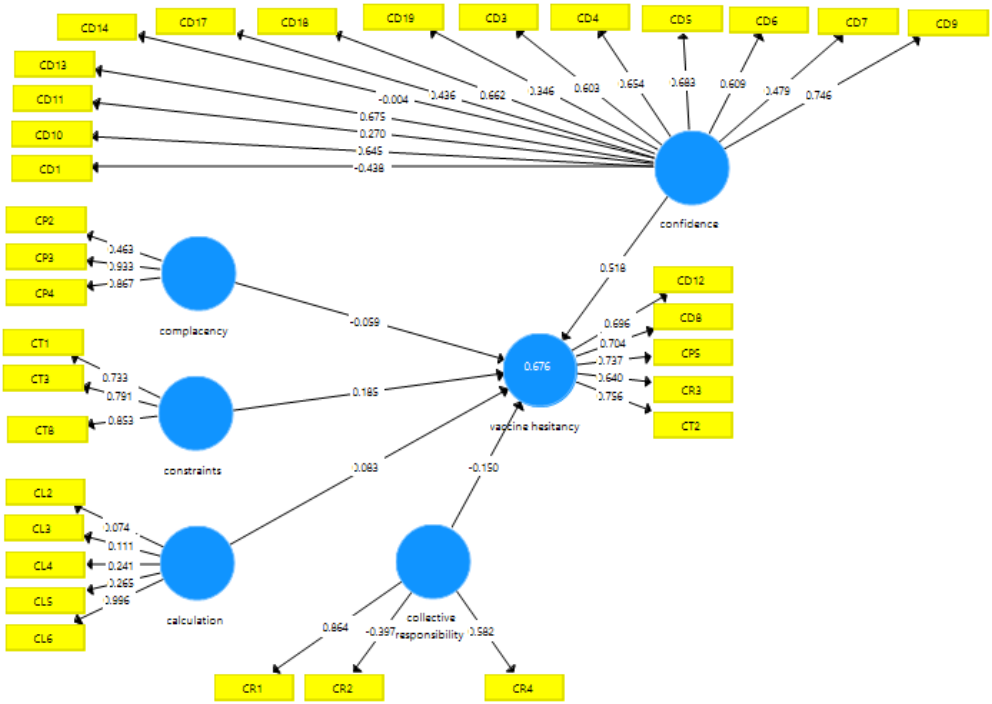

Figure 2 Measurement model showing factor loading values.

As shown in Table 3, Cronbach's alpha values of confidence and constraints variables exceeded the 0.70 thresholds and were considered reliable, while the values for calculation, collective responsibility, and complacency did not exceed the threshold. This indicated that some constructs in this research did not have internal consistency. However, according to the Fornell and Larcker criterion, the average variance extracted (AVE) was higher than the squared correlation between each pair of constructs in Table 4 suggests that discriminant validity was present (values in bold font).

The structural model was assessed after the measurement model had been determined to be accurate and reliable. Figure 3 depicts the structural model generated after bootstrapping.

Table 3 Criterion used to test for internal reliability, construct reliability and convergent validity

Parameters	Cronbach's Alpha	Composite Reliability	Average Variance Extracted (AVE)
Calculation	0.415	0.425	0.228
Collective responsibility	0.156	0.386	0.415
Complacency	0.664	0.815	0.612
Confidence	0.738	0.807	0.307
Constraints	0.710	0.836	0.630
Vaccine hesitancy	0.751	0.834	0.501

Table 4 Fornell and Larcker criterion to test for discriminant validity

Parameters	Calculation	Collective responsibility	Complacency	Confidence	Constraints	vaccine hesitancy
Calculation	**0.477**					
Collective responsibility	-0.314	**0.644**				
Complacency	-0.221	0.583	**0.783**			
Confidence	0.473	-0.643	-0.574	**0.554**		
Constraints	0.407	-0.385	-0.219	0.548	**0.794**	
Vaccine hesitancy	0.464	-0.615	-0.503	0.789	0.573	**0.708**

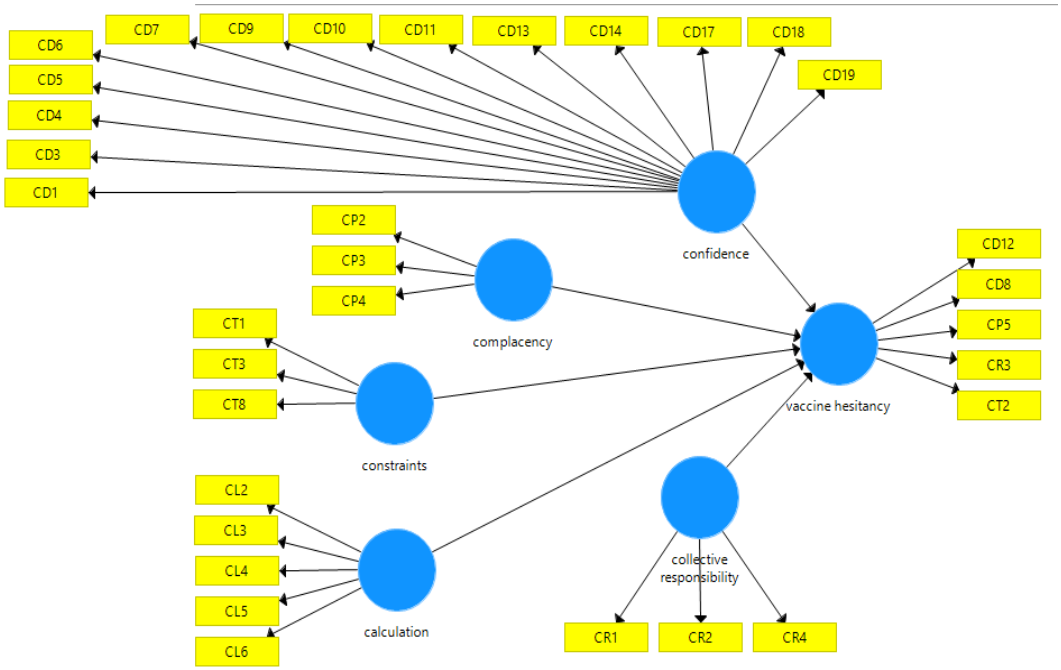

Figure 3 Structural model

Table 5 Hypothesis, Relationships, Original sample (O), Sample mean (M), Standard deviation (STDEV), T-statistics, P-values, and Decisions.

Hypothesis	Relationship	Original sample (O)	Sample mean (M)	Standard deviation (STDEV)	T-statistics	P-values	Decision
H_1	Calculation-> VH	0.083	0.089	0.042	1.982	0.048	Supported
H_2	Collective responsibility -> VH	-0.150	-0.150	0.040	3.732	0.000	Supported
H_3	Complacency -> VH	-0.059	-0.059	0.043	1.369	0.171	Not Supported
H_4	Confidence -> VH	0.518	0.519	0.046	11.281	0.000	Supported
H_5	Constraints -> VH	0.185	0.183	0.043	4.259	0.000	Supported

*Significant at $p<0.05$

The coefficient of determination (R^2) and direction coefficient that was subsequently generated was used to evaluate the structural model. To determine the collinearity problem, the structural model was evaluated for a coefficient of determination (R^2) as well as the statistical significance and relevance of path coefficients. The R^2 value is the coefficient of determination that is used to calculate the percentage of the overall variance. R^2 value in this research was 0.676 which was more than the threshold value of 0.5, therefore it was considered to be a strong and reliable value.

The significance of the route coefficient was determined using bootstrapping. Table 5 shows the T-statistics value and P-values for all the constructs after bootstrapping. T-statistics value must be 1.645 and above at a 0.05 alpha level for a path coefficient to be significant. From Table 5, it was concluded that calculation (H1)(t=1.982; p=0.048), collective responsibility (H2) (t=3.732; p=0.000), confidence (H4)(t=11.281; p=0.000), and constraints (H5)(t=4.259; p=0.00) had a significant positive influence on vaccine hesitancy as the hypotheses H1, H2, H4 and H5 were supported. However, complacency (H3) (t=1.369, p=0.171) was concluded to not have a significant positive influence on vaccine hesitancy as the hypothesis H3, was not supported with its T-values being less than 1.645.

4 Discussion

Vaccine hesitancy is a global issue and has been made more apparent during this COVID-19 pandemic. From January 28 to 31, 2021, a survey about global attitudes on the COVID-19 vaccine was conducted by Ipsos using the online Global Advisor platform for The World Economic Forum (Ipsos 2021; Coronavirus 2021). With a base sample of more than 500 participants, the survey was conducted among adults aged 16 to 74 from 15 different countries spanning Europe, Asia, Africa, Australia, and the United States. The survey showed that the vaccination intent in the United Kingdom was the highest at 89% where 9 out of 10 British adults agreed that they would get the COVID-19 vaccine once it was available. Brazil (88 percent), China (85 percent), Mexico (85 percent), Italy (80 percent), Spain (80 percent), Canada (79 percent), and South Korea (78 percent) were among the other countries with high vaccination intent. The result of these surveys showed that there was increasing demand for COVID-19 vaccine in each of these countries compared to December 2020 when a similar survey was conducted and at that time the vaccines had not yet been approved for use in many of the countries. Besides the vaccination intent, the survey also determined that one of the factors that caused vaccine hesitancy among individuals was that they were worried about the side effects as well as the speed at which the clinical trials of the COVID-19 vaccine were conducted. Other factors that contributed to the vaccine hesitancy included that they doubted the effectiveness of the vaccine which was also reflected by the findings in this study.

In addition to the PLS-SEM analysis, the raw data was also reviewed and it was found that, as of February 17th, 2021, there were only 62.5% of Malaysians who expressed their intentions to get the COVID-19 vaccination once it became widely available while the 13.4% did not have any intentions of doing so. This is concerning as the data indicated that this number does not reach the herd-immunity threshold which is around 70% to 85% of a population (McDermott 2021). Thus, there is a need to carry out intervention to cater to the 24.1% of Malaysians with vaccine hesitancy so we can increase the vaccine uptake and achieve herd immunity against COVID-19.

From the 5C model, five main reasons can lead to vaccine hesitancy which are calculation, collective responsibility, confidence, constraints, and complacency. From the data obtained in this study, it was shown that one of the significant reasons that cause vaccine hesitancy among the Malaysian population is a calculation that was linked to an individual's efforts in searching for validated information regarding COVID-19 vaccines and vaccination. Theoretically, a high level of calculation can have a positive impact on vaccine uptake as they have enough knowledge on the risk of disease as well as knowledge about the importance of the vaccine. However, it is also depending on the source of information. It is a problem if the respondents obtain information regarding vaccines against COVID-19 from social media like Facebook which could be a source of inaccurate information in the form of fake news regarding COVID-19, as was found in the study by Puri et al. (2020). Collective responsibility (H2) which is the desire to protect others by herd immunity by vaccinating oneself,

also had a significant positive influence on vaccine hesitancy. The findings of this study revealed that there was a lack of collective responsibility among the respondents, as they did not seem to understand the importance of each individual to be vaccinated so that we can achieve herd immunity in the efforts to stop the spread of COVID-19 infection in the community (Table 5). Thus, to prevent vaccine hesitancy, the government should also focus on providing knowledge on herd immunity and its importance in combating COVID-19 (Kwok et al. 2021).

Confidence can be described as confidence in the vaccine's safety and effectiveness, as well as the delivery system such as health services and the policymakers, where lack of confidence may reduce the vaccination rate. In this study, it is found that there is a significant positive relationship between confidence (H3) and vaccine hesitancy indicating that there were respondents who lacked confidence in the safety of the COVID-19 vaccine (Table 5). This could have been because it was rapidly developed and only took approximately one year to be produced, unlike any other vaccine which can take up to 10 years (Dror et al. 2020).

Furthermore, constraints (H5), which included any obstacle that can restrict an individual's ability to access a vaccine service, had a significant positive relationship with vaccine hesitancy. From this study, it was found that two main structural barriers can cause vaccine hesitancy among the public. The first was money and the second one was time taken and traveling distance to the vaccination centers (indicated by CT1 and CT3 respectively in Table 1 and Figure 2). These constraints could prevent individuals from getting vaccinated even though they want it. However, the cost is not an issue as the vaccines are provided free by the government. Furthermore, several employers are initiating workplace vaccination programs as has been successfully done in other countries (Zhang and Fisk 2021).

Complacency is when someone perceives the risk of such diseases, in this case, the risk of contracting COVID-19 is low and thus vaccination is not considered mandatory and relevant to be taken. However, in the case of this study, results obtained have shown that there was no significant positive relationship between complacency and vaccine hesitancy (Table 5). This study indicates the Malaysian respondents were already well aware of the danger and seriousness of COVID-19 and believe that preventive measures should be taken, including vaccination. It is just that there are other factors, like what has been mentioned above, that have led to vaccine hesitancy.

There were limitations to this study, the first and most important being the sample size, which was only 379 respondents who could be included in the data analysis. Although this is an indication of vaccine hesitancy and it was sufficient to carry out PLS analysis, it cannot be a true measure of vaccine hesitancy in Malaysia. Furthermore, there should be more even participation from other states to have a better view of the level of understanding on COVID-19 vaccination among Malaysians. There should also be a more targeted effort to broach a wider range of individuals in different walks of life as this would certainly have an impact on their understanding and perceptions of the COVID-19 vaccines. Furthermore, the study was carried out early in the year when the COVID-19 vaccines were still a novelty to the world. Since then, the Malaysian government has been making considerably more efforts in using social media to disseminate accurate vaccine-related information to build public trust in the COVID-19 vaccines and this has helped with the issue of vaccine hesitancy as is evident by the current vaccination status in Malaysia that has achieved more than 78.3% of the population as of 10th November 2021 (COVIDNOW 2021). In addition, the escalating number of COVID-19 cases and fatalities due to this pandemic has spurred a majority of the general public into seeking the only therapeutic alternative available – COVID-19 vaccination. However, vaccine hesitancy is still an issue as the number of deaths due to COVID-19 in unvaccinated individuals was higher than in vaccinated individuals. Recently, it was reported in the Star newspaper that up to 70% of the COVID-19 admissions to a government hospital in Klang were unvaccinated individuals, out of which 80% of them succumbed to the disease (TheStar 2021). This makes vaccine hesitancy a very real issue with threats to the health and safety of the Malaysian public. With the emergence of the Delta variant which has driven the tremendous increase in COVID-19 positive cases and fatalities globally, it is even more crucial to be vaccinated against the COVID-19 virus. Scobie et al. (2021) reported that vaccination protected individuals from more severe illnesses due to COVID-19 including the Delta variant. Despite the tremendous efforts by the Malaysian government to achieve herd immunity among the population with the vaccination initiatives, vaccine hesitancy will continue to impede the progress of the vaccination drive and the fight against the COVID-19 pandemic.

Conclusion and Recommendations for Future Research

In conclusion, there is vaccine hesitancy toward the COVID-19 vaccines among the Malaysian population. The main issues that contribute to vaccine hesitancy could include calculation which is the misinformation regarding the COVID-19 vaccines and vaccination. Another factor is a collective responsibility, which is the lack of willingness to be vaccinated to protect others from COVID-19. Confidence is also the main issue contributing to vaccine hesitancy as many respondents did not seem to trust the speed at which the COVID-19 vaccines were produced.

This study should be carried out with a bigger sample size and should be more effectively carried out using online platforms, and perhaps collaborating with governmental agencies such as the Ministry of Health (MOH) and Ministry of Science, Technology,

and Innovation (MOSTI) as well as the mainstream newspapers such as News Straits Tines and the STAR to reach more respondents. In addition, the survey should be done in Bahasa Malaysia to encourage better participation from Malaysians from all the states in Malaysia which would help to paint a more accurate image of the current state of vaccine hesitancy against the COVID-19 vaccine in Malaysia.

Conflict of Interest Statement

The authors declare no conflict of interest. This paper has not been submitted for publication in any other journal.

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Morphotaxometry and Ultratopography of *Lytocestus haryanii* n.sp. (Caryophyllidea: Lytocestidae) from the intestine of freshwater catfish *Clarias batrachus* Linnaeus 1758 (Siluriformes: Clariidae) of river Yamuna, Yamuna Nagar, Haryana, India

Babita[1], Sushil Kumar Upadhyay[1*], Hardeep Singh Tuli[1], Kuldeep Dhama[2], Deepak Chandran[3], Ranjan Kumar Mohapatra[4], Manoj Singh[1]

[1]Department of Biotechnology, Maharishi Markandeshwar (Deemed to be University), Mullana–Ambala, Haryana– 133207, India
[2]Division of Pathology, ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India – 243122
[3]Amrita School of Agricultural Sciences, Amrita Vishwa Vidyapeetham University, Coimbatore, Tamil Nadu, India – 642109
[4]Department of Chemistry, Government College of Engineering, Keonjhar, Odisha, India

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Lytocestus haryanii n. sp.

Clarias batrachus

Morphotaxometry

Ultratopography

Histology

Microtomy

ABSTRACT

The present investigation deals with the first report of newer species of caryophyllid cestodes, *Lytocestus haryanii* n.sp. (Caryophyllidea: Lytocestidae) in the freshwater catfish, *Clarias batrachus* Linnaeus 1758 (Siluriformes: Clariidae) of river Yamuna from Yamuna Nagar, Haryana, India from July 2018 to June 2020. These helminthes are the most common cestodes (endoparasites) among the fishes of fresh water, brackish water, and marine habitat worldwide. The recovered newer worms were processed through the standardized protocol for the microscopic observations and morphometry, ultratopographic study through scanning electron microscopy, and anatomical analysis by histology using microtomy techniques followed by the double staining. The findings of the present worms were substantiated and compared with the earlier reported species of the same genera from different hosts shared the common group using advanced numerical taxonomy for the taxometric validation. The present proposed newer worms shared all the common characteristics which helped in the generic diagnosis and are closely related to the species collected from the same host species inhabiting different freshwater bodies. The worms comprised several striking contrasts in the combination of distinguishing characters of taxonomic significance in special reference to shape, size, orientation, distribution, and the dimension of the body (single proglottid), scolex, neck, testes, ovary, cirrus pouch, vitellaria, eggs, and excretory pore. Based on the striking morphological, taxometric, ultratopgraphic, and histological differences summarized here can, therefore, be used to propose the worm as a new species.

* Corresponding author

E-mail: upadhyay.k.sushil@gmail.com **(**Dr Sushil Kumar Upadhyay)

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1 Introduction

The *Lytocestus* as genera was recovered and established by Cohn in 1908 with type species *L. adhaerens* from bony fish *C. fuscus* in Hong–Kong. The generic diagnosis of the worm was performed based on an elongated, flat body with a single proglottid without segmentation and bluntly tapering somehow flat anterior extremity with undifferentiated smooth, unarmed scolex followed by a short neck (Yamaguti 1959). The body proper had a divisible cortex and medulla by two layers of longitudinal muscles. The cortical part comprised dispersed vitellaria, however, the reproductive structures like testes and ovary dispersed in the medullary area intended for the placement of the worms in the order Caryophyllidea and Lytocestidae family (Yamaguti 1959). Additionally, the uterine coil and ejaculatory duct housed within a compact parenchymatous bulb and uterine glands confirmed the worms as to the genera *Lytocestus* Cohn 1908. At the outset, Woodland established the type species of this genus as *L. adhaerens* (Woodland 1923) from *Clarias fuscus* and four more species including *L. chalmersius* (Woodland 1924), and *L. cunningtoni* (Furhmann and Bear 1925), *L. indicus* (Moghe 1925, 1931; Mehra 1930; Ash et al. 2011), from *C. batrachus* in India as well as *L. filiformes* (Woodland 1926) from *Mormyrus caschive* in Sudan. Thereafter *L. javanicus* (Bovien 1926), *L. alestesi* (Lynsdale 1956), and *L. birmanicus* (Lynsdale 1956) recovered but later on the *L. alestesi* (Lynsdale 1956) was confirmed as Syn. *L. filiformis* (Woodland 1926). Murhar (1963) reported the *L. moghei*, and Ramadevi (1973) described *L. longicollis* from *C. batrachus*; however, Singh (1975) published *L. fossilis* from *Heteropneusteus fossilis* in India. Shindhe and Deshmukh (1980) re-described two different species from the Marathwada region, however, in 1988 in the Marathwada region of India Shinde and Phad (1988) started to study the cestode fauna in *C. batrachus* and recovered *L. marathwadaensis.* Afterward, Jadhav and Gavhane (1991) find out two species *L. alii* and *L. clariasae* (Sawarkar 2012; Sahay et al. 2019a,b). Later on Kadam et al. (1998) investigated *L. naldurgensis*, Kalse and Shinde (1999) reported *L. chalisgaonesis*, Shinde and Borde (1999) documented *L. kopardaensis* from the same catfish. However, Kolpuke (1999) recovered and published *L. teranaensis* from *Wallago attu* from the same region in the same year.

Subsequently, *L. govindae* (Patil and Jadhav 2002), *L. batrachusae* (Pawar and Shinde 2002) and *L. clariasae* (Shinde and Pawar 2002), *L. teranaensis*, and *L. shindae* (Khadap et al. 2004), *L. nagapurensis* (Lakhe et al. 2004) recovered, documented and reported from catfish *C. batrachus* as well. Meanwhile, Shomendra et al. (2003) reported *L. bishnupurensis* from *Mystus seenghala* which was critically reevaluated by Singh et al. (2018). Tandon et al. (2005) reported 4 newer species including *L. attenuateus, L. assamensis*, and *L. clariae* from *C. batrachus* and *L. heteropneustii* (Ash et al. 2012) from *H. fossilis* that was later re-described by Sahay et al. (2017) as valid species under the genus *Lytocestus*. Two new species *L. bokaroensis* and *L. mujumdari* (Poonam 2007) and *L. paithanensis* (Shelke 2007) were reported from *C. batrachus*. However, Tripathi et al. (2007) recovered *L. jagtai* from *H. fossilis* in the same year which was further described in detail by Sahay and Ekka (2019). In the next consecutive year Jawalikar et al. (2008), Jadhav et al. (2008), Kaul et al. (2010), Bhure et al. (2010), and Surayawanshi et al. (2010) isolated *L. subhapradhi, L. punensis, L. murharii, L. osmanabadensis,* and *L. follicularae*, and *L. shindei* from *C. batrachus* respectively. Further, Jawale and Borde (2011), Pawar and Hiware (2011) successfully reported new species *i.e. L. khami, L. purnensis,* and *L. vyasaei* from the same catfish, while, Kadam and Dhole (2011) described *L. gariepinusae* from *Clarias gariepinus* of Marathwada region in the same year. Sawarkar and Kale (2012), and Solunke et al. (2012) added *L. thapari* and *L. manjaraensis* from *C. batrachus* respectively; however, Nimbalkar et al. (2012) contributed *L. rekhaensis* from *H. fossilis* that was further critically studied by Sahay and Khalkho (2017). Deshmukh et al. (2015), Pawar and Dandwate (2013), Kale (2017), and Kankale (2017) recovered and reported *L. indica, L. godavariensis, L. paithanensis, L. ambe* from the *C. batrachus* respectively. Further critical studies of many of these species were carried out by Sahay et al. (2018a,b). Meanwhile, a new species *L. mastacembellusi* (Pardeshi 2016) was reported from *Mastacembelus armatus* that was further re-described by Sahay et al. (2019b). Barshe et al. (2018), Dandawate (2018), and Patil (2018) reported *L. elongates, L. mulaansis, L. bharatae* from *C. batrachus* respectively, and further thoroughly studied by Sahay et al. (2018a,b). Recently, Bhavsar et al. (2020), Narayan and Srivastav (2020) reported *L. sahayi* from *C. batrachus* and *L. chhaviensis* from *H. fossils* respectively. The present study aims to morphometrically describe a new species of *Lytocestus* that is characterized and validated through numerical taxometric analysis, ultratopography, and histology.

2 Materials and Methods

The worms were extracted from the freshwater fish, *C. batrachus* Linnaeus 1758 (Siluriformes: Clariidae) from river Yamuna, Yamuna Nagar, Haryana, India. The worms were thoroughly washed in lukewarm water to remove debris and observed under a dissection microscope to identify the group of parasitic helminthes in the Zoology Laboratory, Department of Biotechnology, M.M. (Deemed to be University), Mullana–Ambala (Haryana), India. Specimens were fixed in hot 4% formaldehyde for 20–30 minutes. The fixatives were washed properly under running tap water, stained in an aqueous solution of Mayer's Haemalum, dehydrated using a series of alcohols, and cleared in xylol (dehyadrated ethanol: xylene:: 1:1 *v/v*) followed by xylene and mounted in Canada balsam. The line diagrams were drawn with the aid of camera lucida (SIPCON SP–14), and all the measurements were

recorded in millimeters (mm). The microphotographs were captured using Image Analyzer unit "MOTIC" through Biovis Image Plus software and Nikon Trinocular Computerized microphotography unit. Polythetic Divisive Classificatory System (Malhotra et al. 1981a,b,c) was applied to conduct taxometric analysis to establish various newer taxa (Upadhyay et al. 2009; Kumar et al. 2011; Upadhyay 2012; Upadhyay et al. 2013; Upadhyay 2019) based on Coefficient of Dissimilarity (Bray and Curtis 1957), Coefficient of Divergence (Klauber 1940), Mean Character Difference (Cain and Harrison 1958) and Coefficient of Similarity (Dixit et al. 1979). Some specimens were processed for ultratopography through Scanning Electron Microscopy, SAIF (DST), Department of Anatomy, AIIMS, New Delhi as per the standardized laboratory protocols (Wisse et al. 2010; Malhotra et al. 2012; Upadhyay 2012; Al–Shehadat et al. 2018). Some specific parts of freshly collected worms were processed for the histological studies as per the standardized laboratory protocol (Pearse 1968; Upadhyay 2017, 2018; Upadhyay and Nanware 2020).

3 Results and Discussion

3.1 Description of *Lytocestus haryanii* n. sp.

The description of *L. haryanii* has been illustrated in Figures 1–6. The body of recovered worms is made of a single proglottid with 11.52–13.0 x 0.575–1.185 mm in size and undifferentiated, smooth, unarmed, bluntly or pseudo–pointed tapering scolex measured

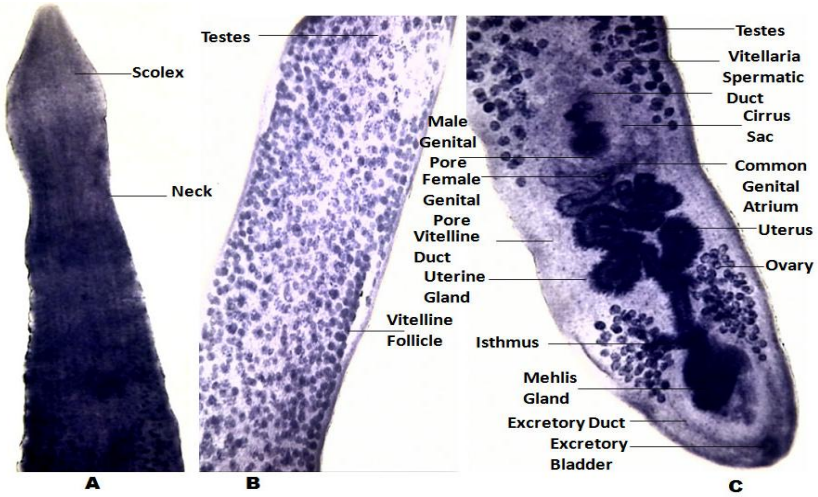

Figure 1 Microphotographs of *L. haryanii* n.sp. in *C. batrachus* from river Yamuna at Yamuna Nagar, (scale bar: 50X); A - Anterior end with pseudo–blunt scolex and an undifferentiated short neck; B - Middle region of mature worm showing the progottid filled with testes and vitellaria; C - Posterior end of mature worms showing male and female reproductive system, terminal excretory bladder.

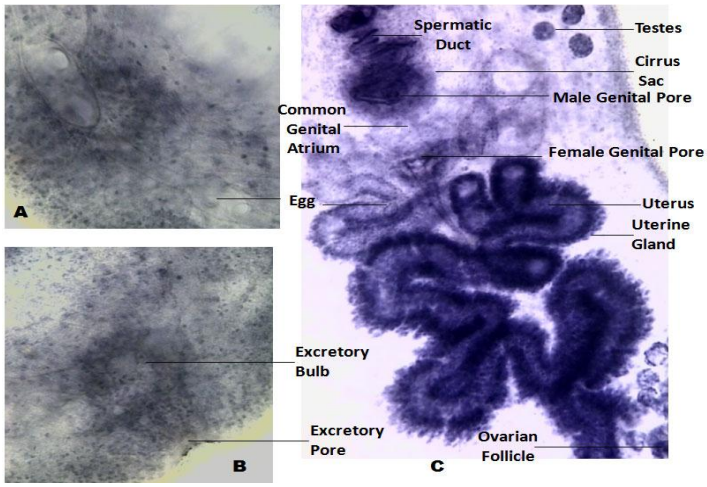

Figure 2 Microphotographs of *L. haryanii* n.sp. in *C. batrachus* from river Yamuna at Yamuna Nagar, (scale bar: 225X); A - Eggs in Uterus; B - Terminal excretory bladder, excretory bulb and excretory pore; C - Posterior end of mature worms showing uterus with uterine glands and cirrus sac with spermatic duct.

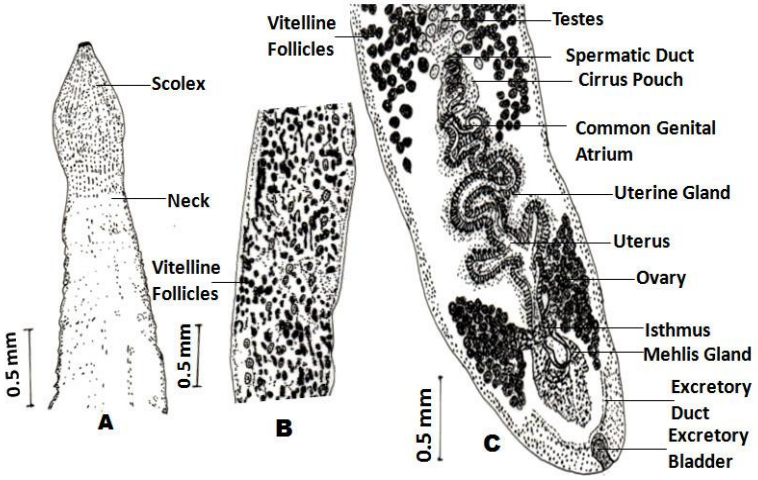

Figure 3 Camera lucida illustrations of *L. haryanii* n.sp. in *C. batrachus* from river Yamuna at Yamuna Nagar, (scale bar: 0.5mm); A - Anterior end with pseudo–blunt scolex and undifferentiated short neck; B - Middle region of mature worm carrying vitelline follicles; C - Posterior end of mature worms showing male and female reproductive system.

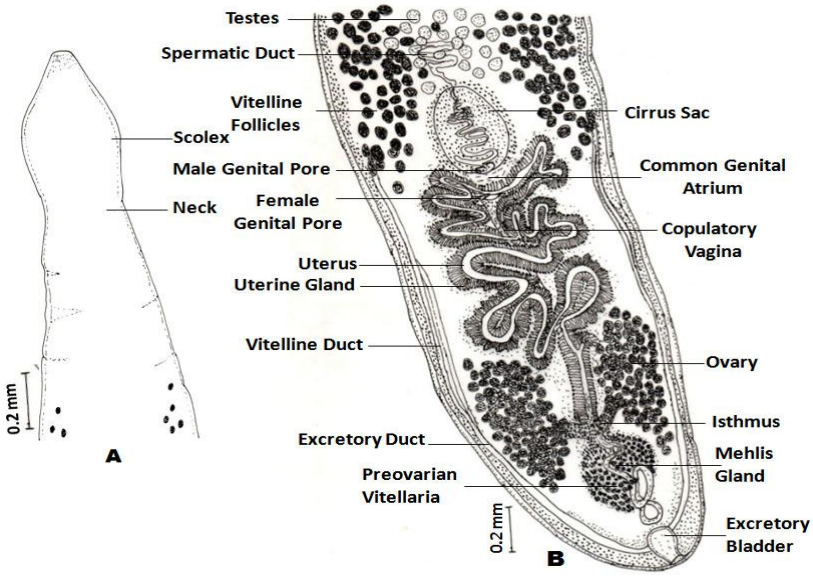

Figure 4 Camera lucida illustrations of *L. haryanii* n.sp. in *C. batrachus* from river Yamuna at Yamuna Nagar, (scale bar: 0.2mm); A - Anterior end with pseudo–blunt scolex and undifferentiated short neck; B - Posterior end of mature worms showing male and female reproductive system, vitelline duct, excretory duct and terminal excretory bladder.

1.176–1.532 x 0.441–0.747 mm. The scolex was followed by a short neck of 0.470–0.952 x 0.529–0.667mm that did not comprise reproductive structures. More than 2/3 of the posterior half of the body comprised numerous testes with the size of 0.032–0.089 x 0.065–0.138 mm that were ovoid, larger than vitelline follicles, and occupied the medullary region, extending from just behind anterior-most vitelline follicles to cirrus sac posteriorly. The cirrus sac is compact, bulbous 0.235–0.380 x 0.148–0.257 mm; the ejaculatory duct opens close to the female genital pore into a shallow common genital atrium 0.190–0.285 x 0.117–0.177 mm.

The ovary is bilobed, follicular, predominantly butterfly–shaped and have a size of 1.115–1.137 x 0.915–1.117, ovarian follicles extending posteriorly beyond Mehlis' gland, ovarian lobes cortical and joined to each other by medullary ovarian isthmus 0.135–0.238 x 0.088–0.118 mm before Mehlis' gland 0.194–0.263 x 0.166–0.205 mm. The uterus was sacular and glandular and had a size of 2.150–3.380 x 0.058–0.127 mm, extending in front of the ovarian isthmus up to the cirrus sac. The conducting vagina with a diameter of 0.030–0.045 mm joined the uterus at a little distal end, however, the copulatory vagina with a diameter of 0.040–0.067 opened together at the shallow atrium.

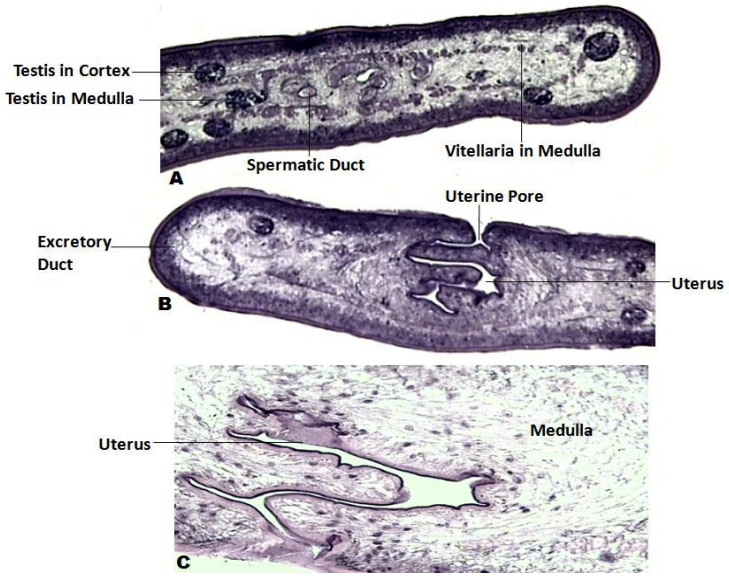

Figure 5 Microphotographs of histology section of mature proglottids of *L. haryanii* n.sp. in *C. batrachus* from river Yamuna at Yamuna Nagar; A - Testes in medullary and cortical region (scale bar: 50X); B - Lateral excretory duct and uterine pore (scale bar: 50X); C - Immense medullary region innervated with huge uterus lined by uterine glands (scale bar: 225X).

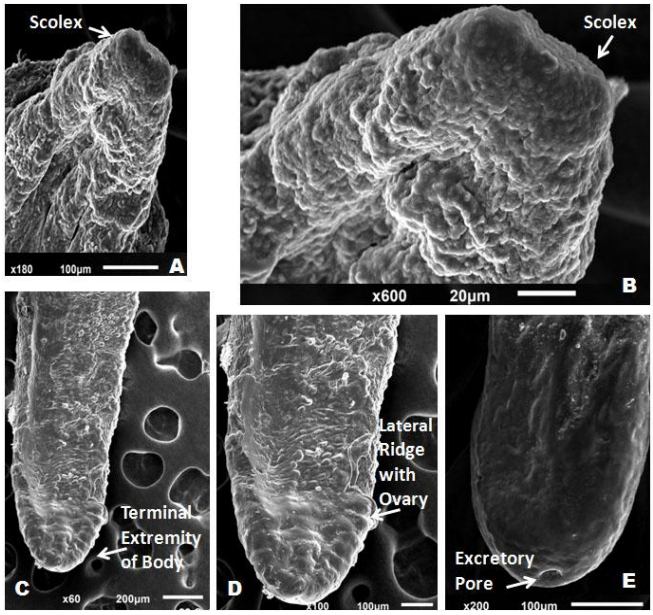

Figure 6 Scanning Electron Microphotographs showing ultratopography *L. haryanii* n.sp. in *C. batrachus* from river Yamuna at Yamuna Nagar; A - Anterior end with pseudo–blunt scolex (scale bar: 100µm; 180X); B - Anterior end with broad scolex (scale bar: 20µm; 600X); C - Posterior end of mature worms showing terminal extremity (scale bar: 200µm; 60X); D - Posterior end of mature worms showing lateral ridge with ovary (scale bar: 100µm; 100X); E - Posterior extremity of mature worms showing terminal excretory pore (scale bar: 100µm; 200X).

The vitelline follicles were ovoid to subovoid 0.027–0.078 and 0.032–0.079 mm in size and emerged a little distance anterior to the testes and traveled up to the level of the cirrus sac. The vitelline ducts diameter was 0.035–0.055 mm and the excretory ducts diameter was reported 0.025–0.040 mm running laterally connected to vitelline glands and excretory bladder 0.125–0.240 x 0.050–0.150 mm respectively. The excretory bladder carries a short excretory bulb-like structure with a 0.080–0.149 mm diameter and followed by a terminal excretory pore with a size of 0.045–0.095 mm through which it opens outside. Eggs were numerous, oval, rough 0.253–0.350 x 0.095–0.125 mm in size, and somewhat operculate in the high magnification microscopic observations.

Systematic Summary

Phylum	–	Platyhelminthes Gegenbaur 1859
Class	–	Cestoda Yamaguti 1961
Order	–	Caryophyllidea Woodland 1926
Family	–	Lytocestidae Wardle and McLeod 1952
Subfamily	–	Lytocestinae Hunter 1927
Genus	–	*Lytocestus* Cohn 1908
Species	–	*haryanii* n. sp.

The present newer worm under investigation comprised a single proglottid body with spatulate, elongated, blunt-ended flat terminal, undifferentiated scolex, short undifferentiated neck, and numerous testes in the medullary region with sparse distribution in the cortical region confirmed through the histological investigation. The follicular vitellaria running more than two third of the body and found to be suspended in the cortical region and reflecting its quite closer similarity to typical *Lytocestus* species including *L. adhaerens* (Cohn 1908), *L. filiformis* (Woodland 1923), *L. chalisgaonensis* (Kalse and Shinde 1999), *L. govindae* (Patil and Jadhav 2002), *L. shindae* (Khadap et al. 2004), *L. clariae*, and *L. assamensis* (Tandon et al. 2005), *L. paithanensis* (Shelke 2007), *L. punensis* (Jadhav et al. 2008), *L. murharii* (Kaul et al. 2010), *L. follicularae* (Bhure et al. 2010), *L. gariepinusae* (Kadam and Dhole 2011), *L. khami* (Jawale and Borde 2011), *L. manjaraensis* (Solunke et al. 2012), *L. godavariensis* (Pawar and Dandwate 2013), *L. mastacembellusi* (Pardeshi 2016), *L. mulaansis* (Dandawate 2018), *L. bharatae* (Patil 2018), *L. chaaviensis* (Narayan and Srivastav 2020), *L. heteropnuestii* (Ash et al. 2012), *L. vyasaei* (Pawar and Hiware 2011), *L. longicolis* (Ramadevi 1973), *L. purnensis* (Jawale and Borde 2011), *L. attenuates* (Tandon et al. 2005), *L. lativitellarium* (Furtado and Kim–Low 1973), *L. purvulus* (Furtado 1963), *L. indicus* (Moghe 1925, 1931; Ash et al. 2011) and *L. sahayi* (Bhavsar et al. 2020). The present worm differs from *L. sahayi* (Bhavsar et al. 2020) in the shape of scolex (undifferentiated vs. differentiated), from *L. shindei* (bluntly differentiated vs. tapering anteriorly), from *L. manjaraensis* (Solunke et al. 2012) (bluntly differentiated vs. cylindrical); and similar to *L. adhaerens* (Woodland 1923), *L. filiformis* (Woodland 1926), *L. assamensis* (Tandon et al. 2005), *L. paithanensis* (Shelke 2007) in shape and size of an undifferentiated, short neck. The present tapeworm is quite similar in appearance of scolex to *L. chalisgaonensis* (Kalse and Shinde 1999), *L. shindae* (Khadap et al. 2004), *L. clariae* (Tandon et al. 2005), *L. murharii* (Kaul et al. 2010) and *L. nagapurensis* (Lakhe et al. 2004) in the shape of the scolex (blunt-ended, spatulate, elongated, elyptical and somehow flat at anterior-most extremity) confirmed through the ultratopography using scanning electron micrography. The present form, differs from *L. assamensis* (Tandon et al. 2005) in the shape of ovary (butterfly shaped vs. inverted 'A' shaped), and differs from *L. paithanensis* (Shelke 2007) in the shape of the cirrus pouch (oval vs. cylindrical), differs in the orientation of cirrus pouch from

Table 1 Polythetic divisive classificatory system based on taxometric analysis from observations of *L. mulaansis* (Dandawate 2018), *L. khami* (Jawale and Borde 2011), *L. chaaviensis* (Narayan and Srivastav 2020) *vis–a`–vis L. haryanii* n.sp.

Worm Character	Organ dimension	*L. mulaansis*				*L. khami*				*L. chaaviensis*			
		C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.
Body	L	2.499	0.944	0.561	0.439	2.292*	0.086*	0.091*	0.909*	2.126*	0.198*	0.225*	0.775*
	W	2.517	0.604	0.370	0.630	2.638	0.373	0.363	0.637	2.128*	0.796	0.323	0.677
Scolex	L	2.838	0.201	0.559	0.441	2.760	0.465	0.307	0.693	2.758	0.204*	0.235*	0.765*
	W	2.735	0.589	0.848	0.152	2.770	0.554	0.390	0.610	2.476	0.595	0.861	0.139
Testes	L	2.345	0.527	0.385	0.615	2.317	0.370	0.458	0.542	2.484	0.349	0.445	0.555
	W	2.262*	0.276*	0.263*	0.737*	2.167*	0.189*	0.198*	0.802*	2.401	0.412	0.519	0.481
Ovary	L	2.307	0.540	0.748	0.252	2.505	0.421	0.552	0.448	2.556	0.387	0.513	0.487
	W	2.372	0.761	0.339	0.661	2.540	0.623	0.563	0.437	2.349	0.518	0.504	0.496
Cirrus Pouch	L	2.495	0.344	0.397	0.603	2.160*	0.129*	0.139*	0.861*	2.155*	0.082*	0.932	0.068
	W	2.775	0.333	0.407	0.593	2.115*	0.318	0.318	0.682	2.452	0.349	0.358	0.642
Egg	L	2.319	0.177*	0.193*	0.807*	–	–	–	–	2.679	0.322	0.251*	0.749*
	W	2.123*	0.212*	0.248*	0.752*	–	–	–	–	2.461	0.380	0.329	0.671

C.D. - Coefficient of divergence; M.C.D. - Mean character difference; C.Dis. -Coefficient of dissimilarity; C.S. - Coefficient of similarity;
* Non–significant; – Observations not available

Table 2 Polythetic divisive classificatory system based on taxometric analysis from observations of *L. clariae* (Tandon et al. 2005), *L. heteropnuestii* (Ash et al. 2012), *L. assamensis* (Tandon et al. 2005) *vis–a`–vis L. haryanii* n.sp.

Worm Character	Organ dimension	*L. clariae*				*L. heteropnuestii*				*L. assamensis*			
		C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.
Body	L	2.072*	0.172*	0.061*	0.939*	2.167*	0.557	0.394	0.606	2.518	0.554	0.392	0.608
	W	2.200*	0.320	0.412	0.588	2.336	0.782	0.499	0.501	2.539	0.613	0.900	0.100
Testes	L	2.250*	0.739	0.586	0.414	–	–	–	–	2.728	0.781	0.642	0.358
	W	2.895	0.752	0.617	0.383	–	–	–	–	2.408	0.734	0.598	0.402
Ovary	L	2.584	0.619	0.900	0.100	2.774	0.619	0.904	0.096	2.830	0.629	0.927	0.073
	W	2.667	0.819	0.731	0.269	2.737	0.646	0.579	0.421	2.806	0.844	0.515	0.485
Vitellaria	L	2.303	0.925	0.867	0.133	2.105*	0.691	0.577	0.423	2.395	0.460	0.627	0.373
	W	2.398	0.411	0.583	0.417	2.222*	0.208*	0.955	0.045	2.322	0.584	0.833	0.167
Excretory Pore	L	2.498	0.141*	0.153*	0.847	–	–	–	–	–	–	–	–
	W	2.894	0.320	0.608	0.392	–	–	–	–	–	–	–	–
Egg	L	2.266*	0.700	0.393	0.607	2.125*	0.250*	0.286*	0.714*	2.375	0.325	0.393	0.607
	W	2.335	0.630	0.400	0.600	2.333	0.467	0.629	0.371	2.381	0.333	0.400	0.600

C.D. - Coefficient of divergence; M.C.D. - Mean character difference; C.Dis. - Coefficient of dissimilarity; C.S. - Coefficient of similarity; * Non–significant; – Observations not available

Table 3 Polythetic divisive classificatory system based on taxometric analysis from observations of *L. vyasaei* (Pawar and Hiware 2011), *L. longicolis* (Ramadevi 1973), *L. purnensis* (Jawale and Borde 2011) *vis–a`–vis L. haryanii* n.sp.

Worm Character	Organ dimension	*L. vyasaei*				*L. longicolis*				*L. purnensis*			
		C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.
Body	L	2.224*	0.189*	0.094*	0.904*	2.274*	0.168*	0.185*	0.815*	2.092*	0.137*	0.148*	0.852*
	W	2.384	0.374	0.478	0.522	2.389	0.456	0.595	0.405	2.576	0.506	0.678	0.322
Scolex	L	2.333	0.254*	0.299*	0.701*	–	–	–	–	2.635	0.250*	0.288*	0.712*
	W	2.526	0.374	0.539	0.461	–	–	–	–	2.572	0.712	0.605	0.395
Testes	L	2.113*	0.114*	0.121*	0.879*	2.280*	0.336	0.427	0.573	2.584	0.616	0.380	0.620
	W	2.357	0.520	0.714	0.286	2.505	0.347	0.441	0.559	2.404	0.511	0.581	0.419
Ovary	L	2.723	0.345	0.458	0.542	2.278*	0.375	0.494	0.506	2.651	0.582	0.518	0.482
	W	2.647	0.736	0.648	0.352	2.642	0.531	0.104*	0.896*	2.594	0.854	0.769	0.231
Vitellaria	L	–	–	–	–	2.391	0.711	0.594	0.406	–	–	–	–
	W	–	–	–	–	2.515	0.501	0.700	0.300	–	–	–	–
Cirrus Pouch	L	2.547	0.549	0.879	0.121	2.341	0.165*	0.184*	0.816*	2.689	0.839	0.409	0.591
	W	2.879	0.266*	0.203*	0.797*	2.438	0.312	0.379	0.621	2.705	0.517	0.485	0.515
Excretory pore	L	–	–	–	–	2.755	0.665	0.898	0.102	–	–	–	–
	W	–	–	–	–	2.200*	0.289*	0.453	0.547	–	–	–	–
Egg	L	2.838	0.560	0.969	0.031	–	–	–	–	2.029*	0.181*	0.201*	0.799*
	W	2.756	0.557	0.787	0.213	–	–	–	–	2.260*	0.098*	0.017*	0.983*

C.D. - Coefficient of divergence; M.C.D. - Mean character difference; C.Dis. - Coefficient of dissimilarity; C.S. - Coefficient of similarity; * Non–significant; – Observations not available

Table 4 Polythetic divisive classificatory system based on taxometric analysis from observations of *L. attenuates* (Tandon et al. 2005), *L. lativitellarium* (Furtado and Kim–Low 1973), *L. purvulus* (Furtado 1963) *vis–a`–vis L. haryanii* n.sp.

Worm Character	Organ dimension	*L. attenuates*				*L. lativitellarium*				*L. purvulus*			
		C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.
Body	L	2.046*	0.389	0.558	0.442	2.198*	0.154*	0.168*	0.832*	2.446	0.526	0.328	0.672
	W	2.011*	0.477	0.627	0.373	2.399	0.885	0.526	0.474	2.693	0.623	0.924	0.076
Testes	L	2.422	0.653	0.985	0.015	2.375	0.714	0.556	0.444	2.462	0.558	0.800	0.200
	W	2.118*	0.414	0.707	0.293	2.320	0.767	0.743	0.257	2.690	0.592	0.550	0.450
Ovary	L	2.532	0.605	0.871	0.129	–	–	–	–	–	–	–	–
	W	2.609	0.706	0.624	0.276	–	–	–	–	–	–	–	–
Vitellaria	L	2.405	0.814	0.699	0.301	2.778	0.571	0.878	0.122	–	–	–	–
	W	2.545	0.733	0.584	0.416	2.469	0.534	0.750	0.250	–	–	–	–
Egg	L	2.353	0.305	0.343	0.657	2.433	0.312	0.592	0.408	0.845	0.321	0.362	0.638
	W	2.408	0.333	0.400	0.600	2.387	0.322	0.476	0.524	0.761	0.655	0.524	0.476

C.D. - Coefficient of divergence; M.C.D. - Mean character difference; C.Dis. - Coefficient of dissimilarity; C.S. - Coefficient of similarity;
* Non–significant; – Observations not available

L. punensis (Jadhav et al. 2008) (vertically placed vs. transversely placed), *L. thapari* (Sawarkar and Kale 2012) (vertically placed vs. obliquely placed), *L. godavariensis* (Pawar and Dandwate 2013) (vertically *vs.* transverse orientation) and differs from *L. murharii* (Kaul et al. 2010) in the vagina (coiled *vs.* slightly curved). The present form is quite similar to *L. gariepinusae* (Kadam and Dhole 2011) in the shape of the scolex, and presence of the neck but differed in the number of testes. However, the *L. khami* (Jawale and Borde 2011) and *L. thapari* (Sawarkar and Kale 2012) shared a similar shape of receptacle seminalis (present), vitellaria (follicular) and differed in the eggs (undifferentially operculated *vs.* nonoperculated). The present worm corroborated the vitellaria shape (follicular) with *L. indicus* (Moghe 1925, 1931; Ash et al. 2011), *L. birmanicus* (Lynsdale 1956), *L. moghei* (Murhar 1963), *L. longicollis* (Ramadevi 1973), *L. fossilis* (Singh 1975), *L. marathawadaensis* (Shinde and Phad 1988), *L. alii* (Sawarkar 2012), *L. clariasae* (Kadam et al. 1998; Shinde and Pawar 2002), *L. kopardaensis* (Shinde and Borde 1999), *L. teranaensis* (Kolpuke 1999), *L. batrachusae* (Pawar and Shinde 2002), *L. subhapradhi* (Jawalikar et al. 2008), *L. osamnabadensis* (Bhure et al. 2010), *L. vyasaei* (Pawar and Hiware 2011), *L. purnensis* (Jawale and Borde 2011), *L. ambe* (Kankale 2017) and *L. elongates* (Barshe et al. 2018). The present worm showed close resemblance with *Lytocestus indicus* (Moghe 1925) Woodland 1926 redescribed by Ash et al. (2011) in generic diagnostic characters, shape of neck, excretory duct, numerous medullary testes and vitelline follicle predominantly in cortex region, spherical to oval cirrus sac; however carried several striking contrasts including body size (smaller and narrower vs. larger and obese), shape and size of scloex (smaller blunt pseudoscloex with somewhat flat anterior extremity vs. larger, digitiform with rounded anterior terminal), cirrus pouch (smaller vs. larger), ovary follicular (ovarian follicles loose, broad 'H' or butterfly shaped vs. compact, dumbbell or butterfly shaped), uterus fantastically looped (dense and larger uterine glands vs. numerous but comparative smaller uterine glands) and well marked excretory bladder comprised tiny tube and opened outside through shallow excretory pore.

The present work was also evaluated and validated through the numerical morphotaxometry using a polythetic divisive classificatory system (C.D., M.C.D., C.Dis., C.S.) with special reference to taxonomically significant characters based on the earlier reported observations of *L. mulaansis* (Dandawate 2018), *L. khami* (Jawale and Borde 2011), *L. chaaviensis* (Narayan and Srivastav 2020), *L. clariae* (Tandon et al. 2005), *L. heteropnuestii* (Ash et al. 2012), *L. assamensis* (Tandon et al. 2005), *L. vyasaei* (Pawar and Hiware 2011), *L. longicolis* (Ramadevi 1973), *L. purnensis* (Jawale and Borde 2011), *L. attenuates* (Tandon et al. 2005), *L. lativitellarium* (Furtado and Kim–Low 1973), *L. purvulus* (Furtado 1963), *L. indicus* (Moghe 1925, 1931; Ash et al. 2011), *L. sahayi* (Bhavsar et al. 2020), *L. mastacembellusi* (Pardeshi 2016) *vis–a–vis* present newer worm that showed significant contrast characters in term of the statistical dimension and size (Tables 1 to 5). Based on the observed, compared, and discussed favored similarities to evaluate the generic diagnosis and striking contrasts for the substantiation and validation of the newer worm, the authors suppose to propose the present worms of the discussion as a new species i.e. *L. haryanii* n.sp.

Taxonomic Summary

Type host : *Clarias batrachus* Linnaeus 1758

Type habitat : River Yamuna

Table 5 Polythetic divisive classificatory system based on taxometric analysis from observations of *L. indicus* (Moghe 1925, 1931; Ash et al. 2011), *L. mastacembellusi* (Pardeshi 2016), *L. sahayi* (Bhavsar et al. 2020) *vis–a`–vis L. haryanii* n.sp.

Worm Character	Organ dimension	*L. indicus*				*L. mastacembellusi*				*L. sahayi*			
		C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.	C.D.	M.C.D.	C.Dis.	C.S.
Body	L	2.578	0.398	0.402	0.598	2.409	–	–	–	2.424	0.404	0.484	0.516
	W	2.476	0.423	0.545	0.455	2.459	0.305	0.395	0.605	2.843	0.382	0.488	0.512
Testes	L	2.620	0.323	0.556	0.444	2.462	0.171*	0.186*	0.814*	2.756	0.309	0.432	0.568
	W	2.400	0.767	0.743	0.257	2.368	0.362	0.453	0.547	2.568	0.619	0.601	0.399
Ovary	L	–	–	–	–	2.341	0.287*	0.396	0.604	2.579	0.312	0.422	0.578
	W	–	–	–	–	2.319	0.567	0.573	0.427	2.553	0.573	0.532	0.468
Vitellaria	L	2.577	0.571	0.782	0.218	–	–	–	–	2.302	0.508	0.849	0.151
	W	2.650	0.534	0.750	0.250	–	–	–	–	2.349	0.431	0.607	0.393
Egg	L	2.774	0.127*	0.238*	0.762*	–	–	–	–	2.813	–	–	–
	W	2.381	0.560	0.432	0.568	–	–	–	–	2.718	–	–	–
Cirrus Pouch	L	–	–	–	–	2.107*	–	–	–	–	–	–	–
	W	–	–	–	–	2.317	0.442	0.465	0.535	–	–	–	–

C.D. - Coefficient of divergence; M.C.D. - Mean character difference; C.Dis. - Coefficient of dissimilarity; C.S. - Coefficient of similarity; * Non–significant; – Observations not available

Type microhabitat : Intestine

Type locality : Yamuna Nagar, Haryana, India

Deposition of specimens : Holotype (BZPLC 105), Paratypes (BZPLC 106) in Zoology Laboratory, MM(DU), Haryana, India.

Etymology : The species name of newer worms proposed after the locality State, Haryana.

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Conflicts of Interest

The authors declare no conflict of interest.

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Characterization of Calcium Phosphate Chitosan Nanocomposite as Plant Growth Promoter

L.S. Niranjana[1], M. Mathankumar[2], D. Karthikkumar[1], R. Ranjithkumar[1], B. Chandar Shekar[2*], Ling Shing Wong[3], Sinouvassane Djearamane[4*]

[1]Viyen Biotech LLP, Coimbatore, Tamil Nadu - 641 031, India.
[2]Department of Physics, Kongunadu Arts and Science College, Coimbatore, 641 029, India.
[3]Life Science Division, Faculty of Health and Life Sciences, INTI International University, Nilai, 71800, Malaysia
[4]Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Kampar, 31900, Malaysia

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ABSTRACT

In this study, calcium phosphate-chitosan nanocomposite (CaP-CS NC) was prepared by a convenient and affordable co-precipitation method, and the prepared NC was tested for agriculture application. Physico-chemicals analyses of the CaP-CS NC were conducted by X-ray diffraction (XRD), scanning electron microscope with energy dispersive X-ray spectroscopy (SEM-EDS), Fourier transform infrared spectroscopy (FTIR), and ultraviolet-visible spectroscopy (UV-Vis) instruments to determine the structural characteristics, surface topology, chemical composition, function group, and optical properties. The XRD pattern of CaP-CS NC revealed that the average crystallite size was 43 nm. The SEM images showed agglomeration of the CaP-CS NC with a rod-like shape. The EDS spectrum of the CaP-CS NC indicated the presence of Ca, P, O, and N elements. FTIR displayed vibrational peaks for the active functional group such as carboxylic (C=O), amines (N-H), hydroxyl (O-H), and alkyne (C-H). Furthermore, the spectrum of CaP-CS NC showed the bending mode of phosphates at 588.37 cm^{-1} and 508.45 cm^{-1}. The UV-Vis-NIR spectrum of the prepared nanocomposite indicates the anti-reflection properties, which might be useful in solar cell applications to increase the efficiency of the solar cell. In addition, the prepared CaP-CS NC was tested for the plant growth stimulator properties at the lab scale level, wherein it exhibited substantial growth. Accordingly, the current study suggests that the prepared CaP-CS NC could be used as a plant growth promoter.

* Corresponding author

E-mail: chandar.bellan@gmail.com (Dr. B. Chandar Shekar);
sinouvassane@utar.edu.my (Dr. Sinouvassane Djearamane)

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1 Introduction

Modern agriculture must adapt to ongoing climate change as well as rising food demand due to population growth (Shahrajabian et al. 2021). The exponentially expanding population, which is expected to reach 9.6 billion by 2050 with limited resources at present, compels the need for the development of sustainable agriculture. Increasing food production by 50% or more is urgently needed to meet the demands of an ever-growing population (Mittal et al. 2020; Yu et al. 2021; Husen 2021). The development of nanomaterials-based fertilizers paves way for positive effects such as regulated nutrient depletion rate, yield enhancement, crop protection, and minimizing post-harvest loss. On the other hand, nanocomposites are known as important materials that are made up of at least two components to maximize the benefits of each component. The shape and composition of the nanocomposites were improved with the chemical, physical, optical and biological characteristics of the primary matrix material in the first place (Ates et al. 2017; Salama 2017). This subsequently broaden the application areas of newly produced nanocomposites over single-applied nanomaterials (Esumi et al. 2003; Huang et al. 2004). Nanocomposite materials that emerged from polysaccharides have more advantages as they possess unique features such as biodegradability, biocompatibility, and bioactivity (Salama 2016; Salama et al. 2020).

Combining organic polymers like chitosan with inorganic minerals such as calcium phosphate is a viable strategy for creating novel composites with improved mechanical properties. Furthermore, porous scaffolds made from chitosan and inorganic minerals have been demonstrated and employed in a variety of sectors including agriculture (Salama et al. 2016). Chitosan solely has the potential to promote plant development in various crops and it boosts plant production while protecting them from diseases (Cita et al. 2018). It also has a considerable impact on root, shoot, flowering, and flower growth rates (Pandey et al. 2018). Calcium (Ca) and phosphate (P) are the key minerals for plants, since they aid in the metabolic process such as nutrient intake, promoting plant cell elongation, strengthening cell walls, and participating in enzymatic and hormonal processes (Upadhyaya et al. 2017).

However, the incorporation of chitosan oligosaccharide with key minerals like calcium phosphate to synthesize hybrid nanocomposite is with the expectance to exhibit growth-promoting ability. As chitosan and calcium both possess growth-promoting property individually. The application of nanocomposite in agriculture will bring sustainable agriculture systems (Kamle et al. 2020). As a result, it could open up new possibilities for producing new nanomaterials-based products (Husen and Siddiqi 2014). Based on its unique structural and biological properties, chitosan-calcium phosphate has gained a lot of attention among the explored sustainable biocomposite materials. Overall, this study aimed to highlight the efficient properties of prepared hybrid CaP-CS NC as a potential plant growth promoter to provide an effective solution to increase crop production sustainably.

2 Materials and Methods

2.1 Materials and synthesis of CaP-CS NC

Chitosan (CS), isopropyl alcohol, and acetic acid were purchased from Himedia, Mumbai, India. Calcium Phosphate nanoparticles(CaP NPs) were obtained from Nanotechnology Research Lab, Kongunadu Arts and Science College, India. The simple and cost-effective coprecipitation technique was used to formulate the calcium phosphate-chitosan nanocomposite (CaP-CS NC). About, 2 g of chitosan was dissolved in 50 mL of acetic acid, and the reaction vessel was stirred for 30 minutes, till it dissolves completely to get a perfectly transparent solution. Consequently, 4.5 g of previously developed CaP NPs were dissolved in 50 mL of isopropyl alcohol and stirred for 30 minutes. After that, the two solutions were mixed well and again stirred for 2 hours. The obtained white curdy solution of calcium phosphate nanoparticle was washed with deionized water two times and the prepared powder was dried at 100°C for 3 hours in a hot oven to remove water content. Later, it was calcined at 120°C for 5 hours to eliminate impurity in the prepared nanocomposite (Figure 1).

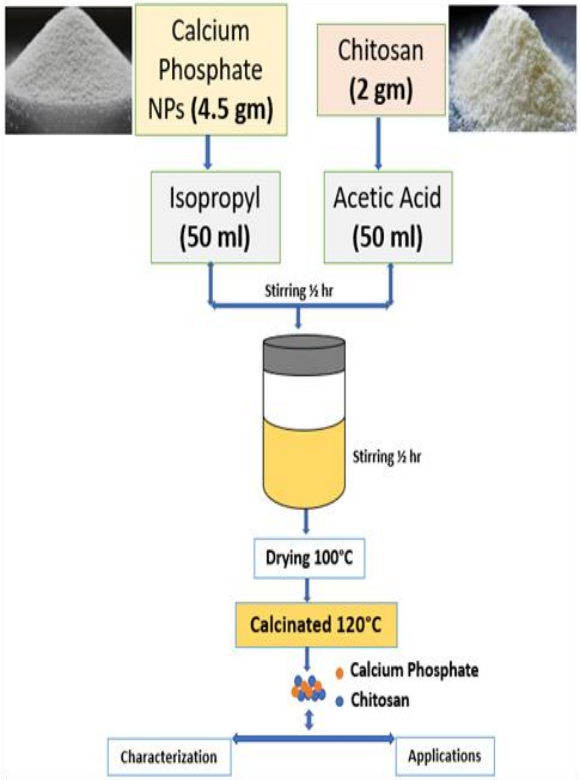

Figure 1 Schematic illustration of CaP-CS NC preparation

2.2 Physicochemical Characterizations of CaP-CS NC

To characterize CaP-CS NC, several physicochemical techniques including X-ray diffraction meter (XRD), Ultraviolet-visible spectroscopy (UV-Vis-NIR), Scanning electron microscope (SEM), and Energy dispersive X-ray spectroscopy (EDS) were used. The diffraction pattern of CaP-CS NC was analyzed using XRD (PAN analytics-BV) in the diffraction angle 2θ and range of 10°-80°. The surface topology image of CaP-CS NC was analyzed by SEM instrumentation (HITACHI SU-6000) and the elemental composition of CaP-CS NC was studied through EDX spectrum attached with SEM. The bonding linkage and functional group of prepared CaP-CS NC was studied by the FTIR spectrum. The optical characteristic of the prepared hybrid nanocomposite was analyzed through the UV-VIS spectrum (JASCO-V670, Japan).

2.3 Effect of CaP-CS NC on Black-eyed bean seed germination

About 100 mg of prepared CaP-CS NC was dissolved in 1000 mL of water. The prepared CaP-CS NC solution was used as a plant growth promoter. The black-eyed bean seeds (*Vigna unguiculata*) were purchased from the local market nearby the college campus. Collected seeds were washed with distilled water before being used. The normal plastic glasses (10 cm) were filled with about 10 g of soil (the soil sample collected from college gardening areas) and collected seeds were placed. The experiment was set with two different dosages (100 mL/day and 200 mL/day) of CaP-CS NC with control. The control experimental pot received only water and the other two experimental pots received CaP-CS NC solution of 100 mL and 200 mL per day. The whole experiment was set at the lab scale level. The CaP-CS NC treated and untreated seeds growth were measured and demonstrated. Figure 2 illustrates the schematic representation of nanocomposite treated and untreated seed germination.

3 Results and Discussion

3.1 XRD

The structure characteristic of CaP-CS NC was examined by XRD and measurements were carried out over the diffraction angle 2θ =10°- 90°(Figure 3).

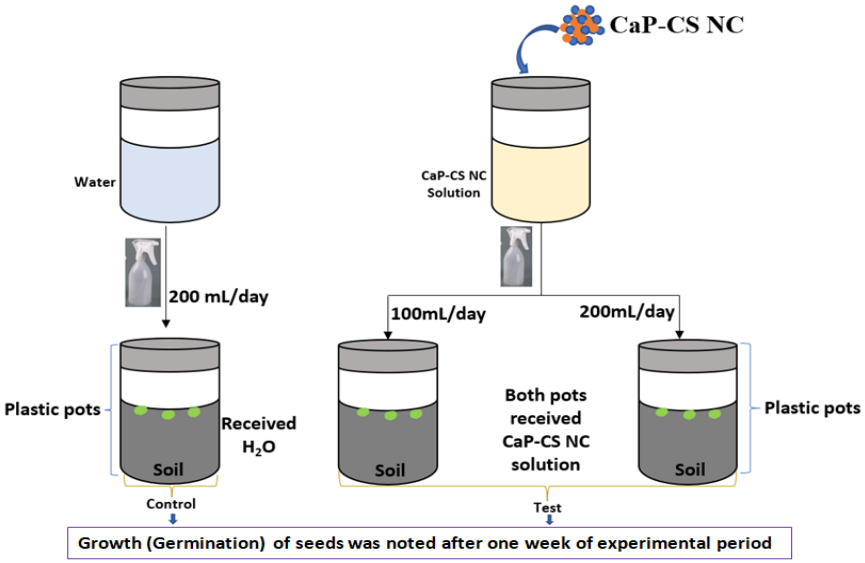

Figure 2 Schematic representation of CaP-CS NC treated and untreated seed germination

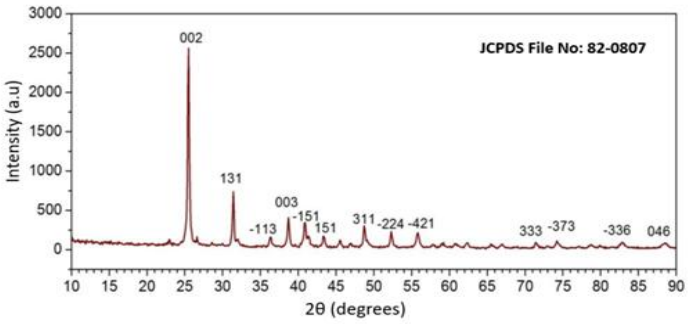

Figure 3 XRD spectrum of CaP-CS NC

Table 1 Structural parameters of CaP-CS NC

Hkl	2 θ (deg)	FWHM	Crystallite Size (D) (nm)	Dislocation Density Lines/meter
002	25.45	0.32	43.81	5.21
131	31.38	0.29	47.81	4.37
003	38.66	0.36	37.69	7.04

The diffraction peaks of CaP-CS NC were observed at 2θ values of 25.5°, 31.4°, and 38.70° corresponding to (002), (131), (003) orientation planes respectively, which indicates the hexagonal structure of the CaP-CS NC and it is in close agreement with the standard JCPDS file no 82-0807 which in turn implies that the prepared sample was free from impurities. The crystallite size of CaP-CS NC was estimated using Scherrer's formula (Manikantan et al. 2017). Table 1 shows the estimated structural characteristic of CaP-CS NC.

The average crystallite size of the CaP-CS NC was found to be 43 nm. In addition, the observed XRD characteristic peaks confirmed the crystalline nature of the prepared sample.

3.2 SEM-EDS

The surface topology of the CaP-CS NC sample was studied under SEM analysis (Figure 4). The SEM images showed the rod-like shape grains with a thickness of a few hundred nanometers of

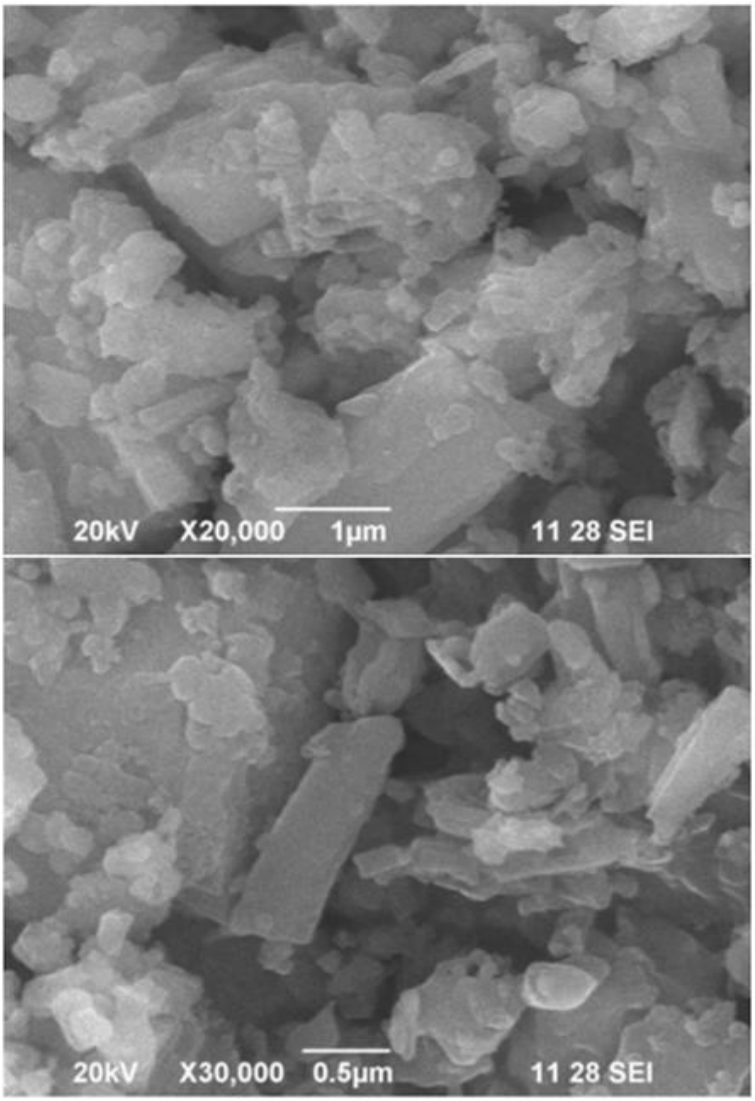

Figure 4 Surface topology of CaP-CS NC (different magnification level)

agglomeration of CaP-CS NC. The calcium phosphate nanoparticles reported by Milon and Tarakdas (2014) were about 45 nm in size with a spherical shape, and they have been utilized for biomedical applications.

The chemical composition of the prepared sample was carried out by EDAX to find out the presence of elements and their composition. It is obvious from the peaks that the product is composed of Ca, P, O, and N elements (Figure 5). The very weak P peak may be originated from the oxidation of the product exposed to the atmosphere air. The estimated elemental percentage of the prepared CaP-CS NC was 27.41 % (Ca), 11.86% (P), 56.31 % (O) and 4.42 % (N). Further, the EDAX spectrum revealed the absence of impurities in the synthesized nanocomposite (Peipei et al. 2010; Jun et al. 2013).

3.3 FTIR

FTIR spectrum of CaP-CS NC showed the characteristic peaks at various wavenumber regions revealing the presence and nature of vibration of elements in the nanocomposite. The FTIR spectrum showed the peaks at 3458.49 cm^{-1}, 2927.32 cm^{-1}, 2859.11 cm^{-1}, 2292.80 cm^{-1}, 1626.60cm^{-1},1153.30cm^{-1}, 676.55cm^{-1}, 622.13cm^{-1}, 588.37cm^{-1} and 508.45cm^{-1} respectively (Figure 6).

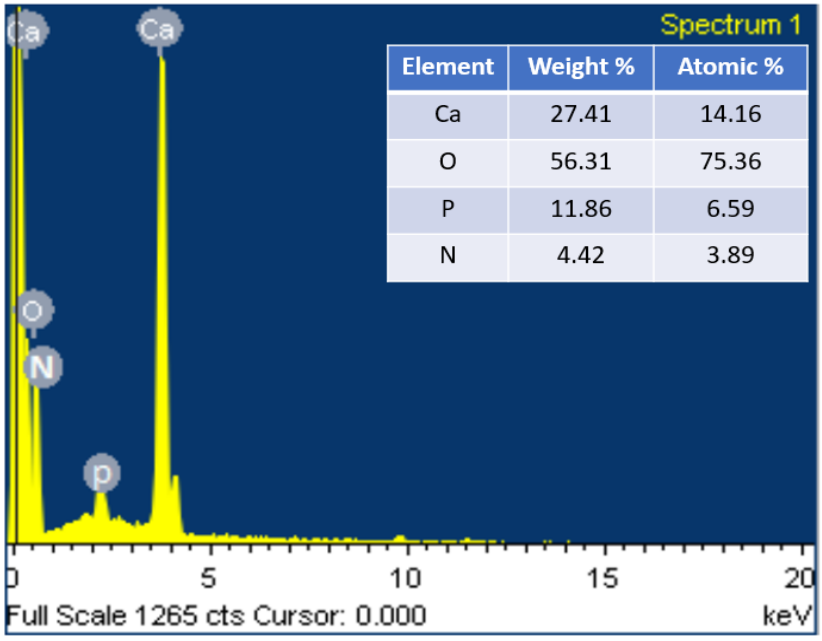

Figure 5 EDS spectrum of CaP-CS NC

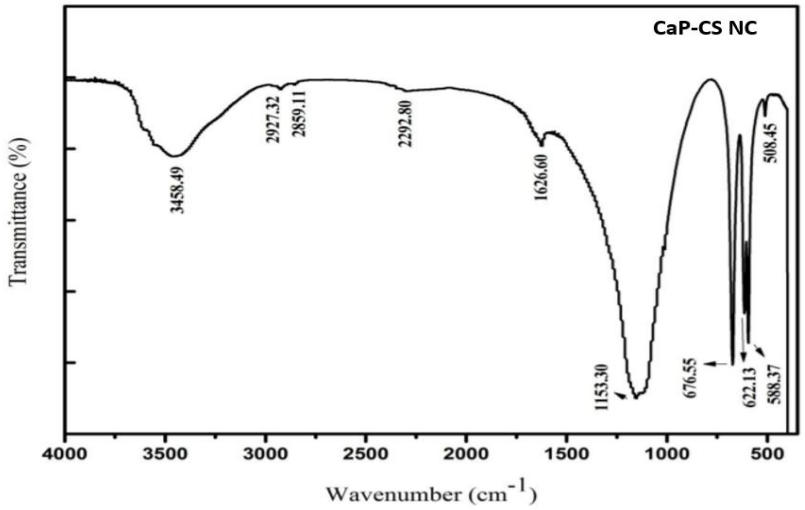

Figure 6 FTIR spectrum of CaP-CS NC

The peaks vibration at 3458.49 cm^{-1}, 1153.30 $cm^{-1}$and 622.13 cm^{-1} belong to the bending and stretching vibration of a hydroxyl group (O-H) of water molecules. This may be due to the hydroxyl atom present in the prepared sample or adsorption of atmosphere water molecules on the surface of a sample while recording the spectra analysis (Lesiak et al. 2019). The bands at 2927.32 cm^{-1}, 2859.11 cm^{-1}, and 2292.80 cm^{-1} are due to the occurrence of primary and secondary amines (N-H) and carboxylic group (C=O, O-H). The characteristic band at 1626.60cm^{-1} is a resultant of N-H stretching the function group of amines, which shows the presence of chitosan in the prepared nanocomposite (Cita et al. 2018). The peak at 622.13cm^{-1} is attributed to the bending of C-H due to alkyne group vibrations. The peaks at 588.37 cm^{-1} and 508.45cm^{-1} are due to bending modes of phosphates (Asep et al. 2019).

3.4 UV-Vis-NIR spectrum analysis

The reflectance spectrum of the prepared CaP-CS nanocomposite is presented in Figure 7.

The obtained spectrum showed that the reflectance first decreases with wavelength up to 400 nm and then it increases with an increase in wavelength. The average reflectance of about 60 % was obtained around 2000 nm. The observed high reflectance at higher wavelength regions indicates that these prepared CaP-CS NC might be applied as an anti-reflection layer in solar cells to enhance the efficiency of solar application (Huang et al. 2015). The optical properties of nanoparticles and nanocomposites such as transmission, absorption, light emission, and reflection are dynamic and may differ significantly from properties compared to the same kind of bulk material. The extensive range of optical effects of the nanomaterials may be produced for novel applications in the field of biomedical, electrical, food industry, cosmetic industry, solar, and agriculture sector by simply manipulating their size, shape, and surface functionality (Smith and Nie 2010; Daniel et al. 2012).

3.5 Effect of CaP-CS NC on Black-eyed bean seed germination

Nanotechnology can be used in agriculture crop production, animal feed, food processing, food contact materials, and food additives. In the agriculture sector, currently, researchers focus more attention on the development of novel nanofertilizers, nanopesticides, and growth biostimulators for improving productivity and suitability. In this study, an attempt has been made to utilize prepared CaP-CS NC as a seed germinator to develop a plant growth promoter to increase agricultural crop production. Figure 8 shows the seed germination of the black-eyed bean with the treatment of CaP-CS NC solution and water (control).

The present study revealed that at the end of day 7, the black-eyed bean seeds treated with CaP-CSNC solution of 100 mL/day and 200 mL/day exhibited a significant growth of shoot length of about 10 cm and 14 cm respectively. Whereas, the water-treated (control) sample showed 8 cm at the end of the experiment (7^{th} day). Further, each of the pots were sown with three seeds of the black-eyed bean; all the three seeds germinated in the CaP-CS NC solution-treated pots, while only one of the seeds germinated in the water-treated pot. In addition, the root length and root branches were found to be increased with the increasing concentration of CaP-CS NC solution (Figure 8). Thus, the present study revealed that the prepared calcium phosphate-chitosan nanocomposite (CaP-CS NC) treated seed showed significant growth as compared to untreated seed.

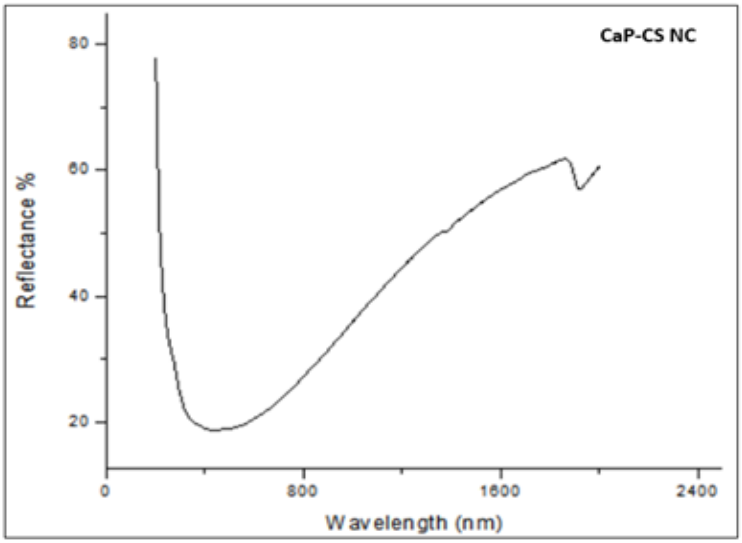

Figure 7 Reflectance spectrum of CaP-CS NC

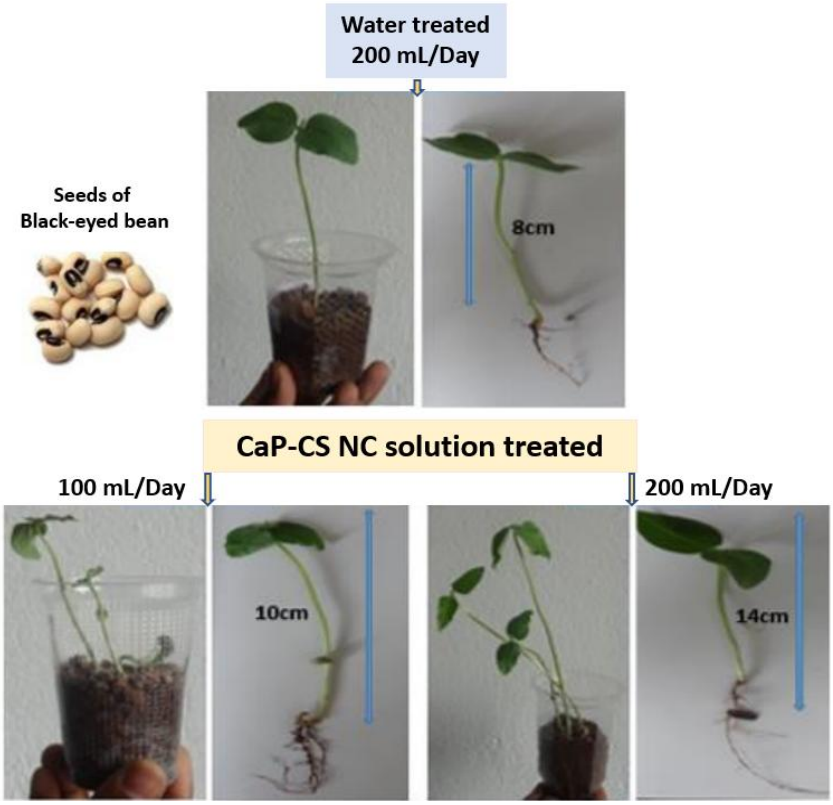

Figure 8 Effect of calcium phosphate - chitosan nanocomposite (CaP-CS NC) on Black-eyed bean seed germination (lab scale level)

Conclusion

The XRD spectrum confirmed the crystalline nature of the prepared CaP-CS NC and indicated size of 43 nm for the crystallite. The SEM images revealed agglomeration of the CaP-CS NC with a rod-like shape and EDAX instrumentation indicated the presence of elements such as Ca, P, O, and N in the prepared nanocomposite. The FTIR spectrum specified the different functional groups such as carboxyl, amine, and hydroxyl present in CaP-CS NC. UV-Vis-NIR exposed that the prepared nanocomposite might be used in solar cell applications as an anti-reflection layer to increase the efficiency of the solar cell. The prepared CaP-CS NC solution showed significant growth of black-eyed bean seeds at the lab scale level and it was observed that the growth directly depends on the concentration of the sample. Hence, the results of the present study revealed that the present nanocomposite could be used in the agriculture sector as a plant growth promoter. Further, it was revealed that calcium phosphate-chitosan nanocomposite could be used as plant growth promoters for high-yield agricultural crop production.

Conflicts of interest

The authors affirm that they do not have any conflict of interest

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Measuring Impact of Air and Agricultural Soil Pollution on Social Development in Saudi Arabia

Adel M. Ghanem[1], Khalid N. Alrwis[1], Abdulaziz M. Alduwais[1], Othman S. Alnashwan[1], Jawad E. Alhashem[1], Sahar A. Kamara[2], Sharaf Aldin B. Alaagib[1], Nageeb M.Aldawdahi[1]

[1]King Saud University, Food Security Unit, Agricultural economics department, College of food and Agricultural Sciences, PO Box 2460, Riyadh 11451.
[2]Egypt Ministry of Agriculture and Land Reclamation, Agricultural Economics Research Institute, Egypt.

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KEYWORDS

Air and agricultural soil pollution

Human development index

Social development

ABSTRACT

This research aimed to measure the impact of air and agricultural soil pollution on social development in Saudi Arabia from the period 1995–2019 by using social development indicators, concentrating on the percentages of expenditure on education and health, and the Human Development Index. In addition, this study uses multiple regressions in estimating the model to study the impact of air pollution and agricultural soil on social development. Results of the study showed that a 10% change in the number of chemical fertilizers and pesticides used in Saudi agriculture leads to a change in the total number of inpatients by 0.7% and 0.5%, respectively. It was also found that an increased percentage of health expenditure to total government spending by 10% leads to a decrease in the total number of patients in the hospital by 1.8%. An increase in air pollution, expressed as a 10% increase in CO_2 emissions, increases the total number of hospitalized patients by 11.1%. The increasing total number of patients by 10% leads to a decrease in the total productivity of the worker, as an indicator of 1.8%. Furthermore, a change of 10% in the ratio of education expenditure to total government expenditure leads to a change in the same direction of the Human Development Index by 9.6%. In light of these results, it can be recommended that the country need to reduce air pollution by expanding the use of natural gas in the industrial and transportation sectors, in addition to reducing the use of nitrogenous fertilizers and pesticides in Saudi agriculture through the expansion of clean farming and good agricultural practices.

* Corresponding author
E-mail: salaagib@ksu.edu.sa (Sharaf Aldin B. Alaagib)

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1 Introduction

Environmental pollution is one of the most severe problems that threaten human resources. It is associated with industrial, mining activities, transportation, and some agricultural practices. Among the most common pollutions, air pollution is the most common one and it is caused by desalination fumes, vehicle exhaust, industrial cities, rubbish burning, and agricultural waste in Saudi Arabia. Water pollution results from the disposal of sewage, landfills in the Red Sea, and the Arabian Gulf. Electricity and heat production are the most important sources of carbon dioxide emissions (49.2%), followed by transportation (25.9%), manufacturing and construction industries (24.1%), then buildings and services (0.8%). Further, the average per capita emissions of carbon dioxide (CO_2) increased from 10.25 tons in 1990, to 17.69 tons in 2015, and then decreased to 14.15 tons in 2020 (World Bank 2020). Like air and water pollution, agricultural soil pollution is also a major problem and is caused by residues of nitrogenous fertilizers, fungicides, and insecticides. Over the past decades, economic development plans have targeted an increase in industrial and mining activity, which has led to an increase and accumulation of pollutants and chemical and radioactive solid waste. Some governments dump this waste and bury it in the ground, which results in a negative impact on humans, animals, and plants in the long run. In light of the scarcity of water resources and the increasing population pressure, the state has tended to vertical agricultural expansion through the expansion of the use of chemical fertilizers and pesticides, which has led to the destruction of the biological life of the soil. Which confirms the excessive use of chemical fertilizers in Saudi agriculture and is an increase in the share of the land unit (ha) of fertilizers from 180.9 kg/ha in 2012 to 3500.5 kg/ha in 2019 (FAO 2020). Undoubtedly, the accumulation of chemical fertilizers (nitrogen, potassium, and phosphate) and pesticides of all kinds, had led to the pollution of soil and groundwater and an increase in their nitrogen content (Nashwan and Ghanem 2010). Due to the low environmental awareness and lack of commitment to environmental controls, the cost of environmental degradation in the Kingdom had increased to 86 billion riyals, representing 3.0% of GDP. The Kingdom of Saudi Arabia's ranking in the Environmental Performance index was 86 out of 180 countries (World Bank 2014). Al-Fraih (2010) studied the environmental pollution caused by electric power plants in the city of Riyadh. This study revealed the presence of environmental pollution inside electric power plants, which led to the emission of odors from fuel waste, and an increase in the number of workers suffering from health and hearing diseases. This study also showed the weakness of the Saudi Electricity Company in the Central Region in achieving environmental security. Kjellstrom et al. (2007) examined health risks and the relationship between social and environmental determinants in urban areas, and interventions to improve health equity through the environment that includes actions and policies that deal with risks surrounding urban areas such as providing drinking water, improving the living conditions of the poor in developing countries, reducing air pollution and limiting emissions and financial resources for emergency interventions for health equity. Khan (2011) studied the impact of environmental pollution on life and ways to treat it in Pakistani cities and reported that about 80% of the population lived in a polluted industrial environment. Pakistani cities suffer from overcrowding, deteriorating air and water quality, and waste management. The study indicated that environmental pollution is one of the main causes of human death around the world. The population increase, the expansion of industrialization, and the increased demand for energy and automobiles are among the main causes of air pollution that affected the level of public health in Pakistani cities. Azari (2014) focused on measuring the impact of environmental pollution on sustainable development, identifying the sources of pollutants, and reducing their effects on human health and the environment. This study showed that the owners of industrial activities refrained from paying the expenses of treating their waste, which led to an increase in environmental pollution and deterioration in the level of public health. Najm al-din (2016) estimated the cost of environmental degradation resulting from air pollution in the city of Kirkuk in northern Iraq, which is one of the most important oil-producing cities in Iraq. This study showed that the cost of environmental degradation in the city of Kirkuk amounted to 68.8 billion Iraqi dinars, representing 12% of the gross domestic product of the province of Kirkuk in 2013. Ministry of Environment, Water, and Agriculture in the Kingdom of Saudi Arabia (2018) prepared the National Environment Strategy. The strategy dealt with wildlife, marine and coastal environment, vegetation, air quality, climate change, water resources, waste management, chemical safety, and meteorology. On the air quality axis, it was reported that air pollution increased through the emissions of the energy and industry sector, the density of cars in large cities, dust storms, and landfills.

There is no doubt that environmental pollution harms public health, life expectancy, and the productive capacity of human resources. Due to the magnitude of the negative effects of environmental pollution, this study focused on (i) identifying the most important sources of environmental pollution in the Kingdom of Saudi Arabia, and (ii) the relative impact of air and soil pollution on the human development index as an indicator of social development. This study is distinguished from the previous studies referred to, in that it deals with measuring the impact of air and soil pollution on social development through building and measuring an econometric model, consisting of four behavioral equations that include the most important internal and external variables related to the subject of the study. This study aimed to measure the impact of air and agricultural soil pollution on social development during the period 1995-2019, by studying the (i) determine the sources of air pollution in the Kingdom of Saudi Arabia, (ii) the evolution of the use of nitrogenous, potassium and phosphate fertilizers and

pesticides in Saudi agriculture, and (iii) estimating the proposed model for studying the impact of air and agricultural soil pollution on social development.

2 Materials and Methods

In achieving its objectives, this study relied on social development indicators, the most important of which are: (1) the ratio of spending on health in total government spending, (2) the ratio of spending on education in total government spending, and (3) the Human Development Index which is a composite criterion, as it consists of three partial criteria: life expectancy criterion (health indicator), educational attainment criterion (educational indicator), and average real national income per capita criterion (economic indicator). The value of the Human Development index ranges from zero to one. The closer the human development index is to one, the more advanced the country in the field of human development, and vice versa. The United Nations classifies countries into three groups as per the Human Development Index: the first group is high-level countries, where the value of the human development index is greater than or equal to 0.8, and the second group is medium-level countries, where the value of the human development index ranges from 0.5 to less than 0.8, and the third group is low-level countries, with an HDI value of less than 0.50 (UNDP 2020).

In achieving its objectives, this study relied on estimating the proposed model to study the harmful effects of air and agricultural soil pollution on social development during 1995-2019. The proposed model consists of the following behavioral equations:

$$Y_{1t} = a_0 + a_1 X_{1t} + a_2 X_{2t} + a_3 X_{3t} + a_4 X_{4t} + e_{1t}$$

$$Y_{2t} = b_0 + b_1 \hat{Y}_{1t} + e_{2t}$$

$$Y_{3t} = c_0 + c_1 \hat{Y}_{2t} + c_2 X_{5t} + e_{3t}$$

The proposed model equations include the following variables: (i) The three endogenous variables are: the total number of inpatients in hospitals of the Ministry of Health (Y_{1t}), the average total worker productivity in thousand riyals (Y_{2t}), the human development index (Y_{3t}). (ii) The five Exogenous Variables are the total amount of chemical fertilizers used (X_{1t}), the total amount of pesticides used in Saudi agriculture (X_{2t}), the ratio of health expenditures to government spending (X_{3t}), the amount of carbon dioxide emissions generated from cars and water desalination (X_{4t}), the ratio of education spending to government spending (X_{5t}). The proposed model was estimated using the ordinary least squares method (Greene 2003).

3 Results

3.1 Main sources of air pollution in Saudi Arabia

Air pollution is the exposure of the atmosphere to chemicals, solid particles, or biological compounds that cause damage to humans,

Table 1 Total emissions (in kiloton) of air pollutants in Saudi Arabia in 2014

Sector	Sulfur oxides (SO_2)	Nitrogen oxides (NO_2)	Inhaled lingering transcriptions (IP)	Non-methane-containing volatile organic compounds ($NMVOC_s$)	Carbon monoxide (CO)
Electrical and thermal power generation and water desalination					
quantity	2120	386	52.14	33	62
%	66.46	12.03	24.45	0.10	0.40
Oil extraction and refining					
quantity	262	1122	3	358	100
%	8.21	34.96	1.41	1.04	0.65
Transportation					
quantity	52	1447	113.99	2579	15151
%	1.63	45.09	53.46	7.52	98.59
Petrochemical industrie					
quantity	0	10	0.29	0	0
%	0	0.31	0.14	0	0
Cement					
ytitnauq	83	22	3.66	2	9
%	2.60	0.69	1.72	0.01	0.06
Other sectors (iron and steel, agricultural, residential, and commercial)					
quantity	673	222	4015	31311	45
%	21.1	6.92	18.83	91.33	0.29
Total sectors					
quantity	3190	3209	213.23	34283	15367
%	100	100	100	100	100

Source Meteorological and Environmental Protection Authority and World Bank (2016); State of the Environment Report in Saudi Arabia

other organisms, and the natural environment. Air pollution is one of the most serious environmental problems resulting from industrial development, expansion of energy use, increased land, air, and sea transport, and associated emissions of carbon dioxide (CO_2), sulfur dioxide (SO_2), and nitrogen oxides (NO_X). It is clear from the data given in Table 1 that the main sources of air pollution in the Kingdom of Saudi Arabia are (i) electric and thermal power generation, and water desalination, and these are contributed about 66.46% of total sulfur dioxide and 24.45% of minute emissions; (ii) oil extraction and refining sector, responsible for about 8.21% of total sulfur dioxide and 34.96% of nitrogen oxide emissions; (iii) transport sector is the main source of air pollution, especially in major cities, where vehicles account for about 7.52% of total emissions ($NMVOC_s$), 45.09% of total NO_X, and more than 98% of total CO emissions; and (iv) the petrochemical industry sector, which contributes a small percentage to air pollution because the petrochemical industries in Jubail, Yanbu, and other economic cities depend on natural gas as input for production and source of energy. Other sources of air pollution include the cement industry, construction and other similar activities, iron and steel industry, pesticide spraying, and municipal waste incineration.

3.2 Current situation of the use of chemical fertilizers and pesticides in Saudi agriculture

Developing countries suffering from population pressure tended to increase agricultural production through the excessive and unregulated use of chemical fertilizers and pesticides. By studying the evolution of the quantities used of chemical fertilizers in Saudi agriculture during the period 2000-2019, it is clear from the data presented in Table 2 that nitrogen fertilizers were the most widely used fertilizer with a rate of 60.79% in Saudi agriculture, this was followed by phosphate and potassium fertilizers with a rate of 33.69, and 5.52% respectively during the period 2000-2019. It was also shown that the share of the land unit (ha) increased from 344.84 kg/ha in 2000 to 3500.5 kg/ha in 2019, with an annual average of about 285.55 kg/ha during the period 2000-2019.

Table 2 Evolution of the amount of chemical fertilizers used in Saudi agriculture during the period 2000–2019

Year	Nitrogen in thousand tons	Phosphate per thousand tons	Potassium per thousand tons	Total per thousand tons	Ground unit share kg/ha
2000	228.0	137.2	21.0	386.2	344.8
2001	223.1	141.7	19.0	383.8	316.7
2002	224.3	132.2	9.00	365.5	208.7
2003	235.0	138.0	9.20	382.2	361.9
2004	238.0	139.0	22.00	399.0	340.2
2005	194.0	120.0	21.70	335.7	303.3
2006	206.0	137.0	23.00	366.0	340.7
2007	221.0	151.0	22.50	394.5	367.0
2008	140.0	74.0	17.00	231.0	217.6
2009	130.0	70.0	10.00	210.0	197.9
2010	125.0	65.0	10.00	200.0	188.4
2011	127.5	63.0	10.00	200.5	188.9
2012	122.0	60.0	10.00	192.0	180.9
2013	176.6	86.5	13.50	276.6	265.6
2014	170.1	86.0	17.00	273.1	260.6
2015	202.7	98.0	17.70	318.4	306.7
2016	210.7	94.5	14.20	319.4	311.0
2017	210.7	94.5	14.20	319.4	314.0
2018	178.20	93.0	29.4	300.6	345.6
2019	178.20	93.0	29.4	300.6	350.5
average	187.06	103.68	16.99	307.73	285.55
%	60.9	33.69	5.52	100	-

Source: Compiled from:

1. Ministry of Environment, Water and Agriculture, Statistical Book, Miscellaneous Issues, 2020.
2. FAO, website, 2020.

The Kingdom of Saudi Arabia tended to import pesticides, herbicides, and fungicides, data are given in the table 3 revealed that the total imported quantities of pesticides of all kinds increased from 13.23 thousand tons in 2000 to 25.52 thousand tons in 2015, and then decreased to 11.61 thousand tons in 2019. The quantities used of pesticides in Saudi agriculture also increased from 3.02 thousand tons, representing 22.86 percent of the total amount of pesticide imports in 2000, to 10.50 thousand tons, representing 90.4% of the total amount of pesticide imports in 2019. Despite the importance of the use of chemical fertilizers and pesticides in increasing agricultural production, however, the accumulation of chemical fertilizers and pesticides led to the pollution of both soil and groundwater (renewable and non-renewable) and an increase in their nitrogen content, then this pollution is transmitted to plants, animals, and humans. The Ministry of Environment, Water, and Agriculture realized the necessity of eliminating inappropriate agricultural practices that increased environmental pollution and adopted the Good Agricultural Practices Project known as Saudi Gap (S.G.A.P.). This project aimed to rationalize the consumption of irrigation water, chemical fertilizers, and pesticides, and increase the use of organic fertilizers to improve soil properties and increase its ability to retain irrigation water (Ministry of Environment, Water and Agriculture 2020).

3.3 Estimating the proposed model for studying the impact of air and agricultural soil pollution on social development

By studying the development of the internal variables of the proposed model, the total number of inpatients in hospitals affiliated with the Ministry of Health increased from 1171.3 thousand in 1995 up to 1423.8 thousand in 2019 (Table 4). The value of productivity for the worker also increased from 95.32 thousand riyals in 1995 to 312.44 thousand riyals in 2019. Given the country's interest in human capital development, the value of the Human Development Index increased from 0.742 in 1995 to 0.854 in 2019. The internal variables of the proposed model were characterized by relative stability during the study period, evidenced by the low value of the estimated coefficients of variation, as it reached 5% for the human development index and increased to 33.47% for the value of the worker's productivity.

Table 3 Evolution of the total quantity of Saudi imports of pesticides and their use in Saudi agriculture in tons during the period 2000-2019

year	Import quantity per ton	Used quantity per ton	Percent of used quantity to the import one
2000	13229.49	3024.0	22.86
2001	16108.50	3194.0	19.83
2002	17023.85	3365.0	19.77
2003	19871.78	3535.0	17.79
2004	18124.67	3706.0	20.45
2005	21576.31	3876.0	17.96
2006	18210.68	4046.0	22.22
2007	21108.47	4217.0	19.98
2008	19644.36	4378.0	22.29
2009	17662.79	4539.0	25.70
2010	22695.24	4700.0	20.71
2011	22780.91	4861.0	21.34
2012	25493.13	5022.0	19.70
2013	23280.79	5413.0	23.25
2014	14887.59	6260.0	42.05
2015	25523.64	7107.0	27.84
2016	20634.21	7954.0	38.55
2017	12537.40	8801.0	70.20
2018	11785.93	9648.0	81.86
2019	11610.00	10496.0	90.40
average	18689.49	5407.1	28.93

Source: Food and Agriculture Organization (FAOSTAT) 2020.

Table 4 Descriptive analysis of the variables used to measure the impact of soil and air pollution on economic and social development during the period 1995–2019

Statement	Internal variables			External variables				
	Total number of patients admitted to hospitals per thousand inhabitants	Worker productivity value in thousand riyals	Human Development Index	Chemical fertilizers in thousand tons	Total pesticides in thousand tons	Ratio of health expenditure to government expenditure (%)	CO_2 emissions thousand kilotons	Expenditure on education to government expenditure (%)
1995	1171.3	95.32	0.742	284.0	994.0	6.5	235.2	21.6
2000	1312.5	110.98	0.765	386.2	3024.0	11.7	296.9	27.0
2005	1655.1	154.27	0.773	335.7	3876.0	11.6	397.6	26.4
2010	1699.4	201.42	0.815	200.0	4700.0	11.9	518.5	27.9
2015	1705.9	188.85	0.847	318.4	7107.0	13.7	601.0	28.8
2016	1648.7	180.06	0.850	319.4	7954.0	13.1	638.5	30.3
2017	1377.2	186.15	0.853	319.4	8801.0	13.5	650.0	30.5
2018	1410.1	298.88	0.857	300.6	9648.0	12.94	514.6	30.7
2019	1423.8	312.44	0.854	300.6	10496.0	17.0	582.3	30.5
Average period	1486.52	159.14	0.802	314.78	4727.2	11.47	410.45	26.59
Minimum limit	1171.30	92.91	0.742	200.0	994.0	6.50	207.70	19.80
Maximum limit	1705.90	246.37	0.853	386.2	10496.0	17.0	650.0	30.5
SD	177.07	53.26	0.04	123.37	2455.71	2.51	138.80	3.29
COV (%)	11.91	33.47	5.00	31.93	51.95	21.88	33.82	12.37

SD – Standard Deviations; COV - Coefficient of variation

Source: Compiled from:

1. Saudi Arabian Monetary Agency, Annual Statistics 2020, 31/5/2021.
2. United Nations, United Nations Development Program, Human Development Reports, 1995–2020.
3. Ministry of Environment, Water and Agriculture, Statistical Book, 2020.

About the development of the external variables of the proposed model, it is also clear that despite the decline in the cropping area, the number of chemical fertilizers used in Saudi agriculture increased to 300.6 thousand tons in 2019. There was also an overuse of pesticides, as the number of liquid pesticides used increased from 994 tons in 1995 to 10.49 thousand tons in 2019. Given the country's interest in the health sector, its relative share of government spending increased from 6.5% in 1995 to 17.0% in 2019. The education sector's share of government spending increased from 21.6% in 1995 to 30.5% in 2019. Finally, about the emission of Carbon dioxide as an indicator of air pollution, it ranged from a minimum of 207.7 thousand kilotons in 1998 to a maximum of 650.0 thousand kilotons in 2017, with an annual average of about 410.46 thousand kilotons. The external variables of the proposed model were characterized by relative stability during the study period, except for the liquid pesticides variable used, where the estimated coefficient of variation reached about 52.0% (Table 4).

The impact of agricultural soil and air pollution on social development was studied by the equations of the proposed model by successive applications of the Ordinary Least Squares (OLS) method during the period 1995-2019. It is clear from the behavioural equations of the model presented in Table 5 that: (i) the increase in the degree of agricultural soil contamination, expressed as an increase of 10% in the use of both chemical fertilizers and pesticides, leads to an increase in the total number of patients admitted to hospitals affiliated with the Ministry of Health by 0.7%, 0.5% each, respectively, (ii) an increase in the degree of air pollution, expressed as a 10% increase in carbon dioxide emissions, leads to an increase in the total number of hospitalized patients by 11.1%, (iii) an increase in the relative share of the health sector in government spending by 10% leads to an overall decrease in the number of inpatients in hospitals affiliated with the Ministry of Health increased by 1.8%, (iv) an increase in the estimated total number of patients by 10% leads to a decrease in the productivity value of the worker by 1.8%, (v) an increase of

Table 5 Equations of the proposed model for studying the impact of agricultural soil and air pollution on social development during the period 1995–2019

Statement	Equation
Number of inpatients	$Ln\,\hat{Y}_1 = 7.434 + 0.07\,\mathrm{Ln}\,X_1 + 0.05\,\mathrm{Ln}\,X_2 - 0.18\,\mathrm{Ln}\,X_3 + 1.11\,\mathrm{Ln}\,X_4 + 0.72AR(1)$ $(11.67)^{**}(1.96)^{*}(2.09)^{*}(-2.13)^{*}(3.22)^{**}(5.27)^{**}$ $R^2 = 0.85 \quad F = 124.48 \quad D.W = 1.96$ $LM\ test = 0.95 \quad Arch\ test = 0.25$
Worker productivity value	$\mathrm{Ln}\,\hat{Y}_2 = 5.333 - 0.18\,\mathrm{Ln}\,\hat{Y}_1 + 0.91\,AR(1)$ $(1.97)^{*}(-2.01)^{*}(11.32)^{**}$ $R^2 = 0.88 \quad F = 66.84 \quad D.W = 1.88$ $LM\ test = 0.88 \quad Arch\ test = 0.29$
Human Development Index	$\mathrm{Ln}\,\hat{Y}_3 = -0.511 + 0.05\,\mathrm{Ln}\,\hat{Y}_2 + 0.02\,\mathrm{Ln}\,X_5 + 0.89\,AR(1)$ $(-2.43)^{*}(1.96)^{*}(2.56)^{*}(8.15)^{**}$ $R^2 = 0.92 \quad F = 68.82 \quad D.W = 2.93$ $LM\ test = 0.11 \quad Arch\ test = 0.76$

** Significant at 1% probability level; * Significant at 5% probability level.
Source: Calculated from data in Table (4).

Table 6 Indicators for measuring the efficiency of the proposed model for measuring the impact of pollution of agricultural soils and air on social development during 1995–2019

Indicator	Behavioral equations		
	First	Second	Third
Root mean squares error R.M.S.E.	0.09	0.19	0.01
Average absolute error M.A.E.	0.08	0.16	0.01
Average percentage of absolute error M.A.P.E.	1.11	3.24	5.68
Unequal coefficient of Thiel (U)	0.006	0.02	0.03

Source: Compiled and calculated from the behavioral equations of the proposed model, listed in Table 5

10% in the estimated productivity of the worker leads to increase in the value of Human Development Index by 0.5%, (vi) The relative share of the education sector increased by 10% in government spending, which led to an increase in the value of the Human Development Index by 9.6%. Finally, the proposed model was efficient for the data used in the estimation, according to the efficiency indicators, the most important of which was the U-Theil inequality coefficient, whose value is close to zero (Table 6).

The development in the various sectors of the Saudi economy has grown fast. While the environmental dimensions don't consider, that led to an increase in the degree of air pollution in some areas where thermal and electric power generation, water desalination, and the number of cars increased, especially in major cities where concentrated. Despite the decline in the crop area to rationalize water consumption, the consumption of chemical fertilizers and pesticides in Saudi agriculture has increased, intending to increase the productivity of the various crops prevailing in the cropping structure led to an accumulation of chemical fertilizers and pesticides over many decades. The pollution of both agricultural soil and groundwater (renewable and non-renewable) increased their nitrogen content. Then this pollution was transmitted to plants and animals, and then to humans. Fingers point to the responsibility of chemical fertilizers, especially nitrogenous ones, and pesticides, for the outbreak of many incurable diseases, the most important of which are kidney failure and cancers, in addition to an imbalance in the environmental balance, which negatively affects the value of worker productivity and the human development index as an indicator of social development.

In light of the results of this study, it recommends the need to reduce air pollution by expanding the use of natural gas in the industrial and transportation sectors, in addition to reducing the use of nitrogenous fertilizers and pesticides in Saudi agriculture through the expansion of clean farming and good agricultural practices.

4 Discussion

Given the increase in environmental pollution caused by the increase in the level of carbon dioxide in the atmosphere and its link to the increase in average temperatures worldwide and global warming, the group of twenty meets annually to discuss air pollution and other components of the environment. Environmental pollution is linked to economic and social development, as reciprocal relations are established between

them, affecting the productive capacity of economic resources. It is known that economic policies in most countries focus on sustainable development. It is not possible to improve indicators of sustainable development in any country without preserving the environment and reducing pollution. In the Kingdom of Saudi Arabia, the Ministry of Environment, Water, and Agriculture has prepared the National Environment Strategy and the General Authority for Meteorology and Environmental Protection has been assigned the task of supervising its implementation. Finally, His Royal Highness, Crown Prince Mohammed bin Salman, announced on March 27, 2021, the Green Middle East Initiative, intending to address climate change by intensifying efforts and enhancing cooperation among the countries of the region. The Kingdom of Saudi Arabia has also adopted a system of good agricultural practices to preserve agricultural soil and water resources and reduce the use of chemical fertilizers and pesticides in Saudi agriculture. There is no doubt that the Kingdom of Saudi Arabia has made remarkable progress in the Human Development Index as an indicator of social development, as it increased from 0.744 in 2000 to 0.854 in 2020, due to the increase in the percentage of spending on health and education from 38.75% in 2000 to 43.59% in 2020 (The World Bank, 2020), in addition to the regulations and legislation issued to preserve the environment.

Conclusion

Air pollution has increased in Saudi Arabia because of a boom in thermal and electric power generation, water desalination, and car numbers, especially in major cities. Despite the decrease in crop area, the average per-unit share of total chemical fertilizers increased from 180.9 kg/ha in 2012 to 350.5 kg/ha in 2019. The accumulation of chemical fertilizers and pesticides leads to contamination of both soil and groundwater (renewable and non-renewable) with nitrogen content. This pollution is then transmitted to plant and animal life, and then to humans. Thus, chemical fertilizers and pesticides are seen as responsible for the spread of many incurable diseases, in addition to the imbalance in the environment, which affects overall productivity and human development as an indicator of social development in Saudi Arabia. Through successful international experiences in preserving the environment, it has been found that air pollution can be reduced, through the expansion of the use of clean energy (natural gas) in economic sectors. Also, developed countries turned to organic agriculture intending to produce healthy food, by reducing the use of nitrogenous fertilizers and pesticides on the one hand and increasing organic fertilizers on the other hand. In Saudi agriculture, there has been an expansion in clean farming and agricultural practices. The Saudi GAP project leads to saving and improving the consumption of irrigation water in agriculture through the use of modern irrigation methods, rationalizing the use of pesticides and chemical fertilizers, and increasing the use of organic fertilizers, which improves the properties and fertility of the soil, and then improves its ability to retain with irrigation water. Therefore, preserving and maintaining the environment and protecting it from deterioration and pollution is an essential pillar to ensure the continuity of life.

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Influence of Hand Anthropometry and Nutrient Intake on Hand Grip Strength: A Correlational Study Among Young Indian Badminton Players

Shreyashi Saha[1*], Prajakta Nande[1], Ravi Nimonkar[2]

[1]Department of Home Science, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India.
[2]Station Health Organization and Armed Forces Medical College, Pune, Maharashtra, India.

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ABSTRACT

Badminton is a fast shuttle-racquet game, which requires adequate endurance and agility for hitting shots. For consistent and superior performances, players need to develop decent nutritional status and tremendous physical fitness. The present study concerns with the effect of anthropometric indices and nutritional profiles on arm strength for racquet gripping. Adolescent male (N=100) and female (N=100) badminton players aged 10 to 15 years were selected from Nagpur, India, and arm anthropometric indices and skeletal muscles of the players were determined by tape and bioelectrical impedance analyzer respectively. Muscle growing macronutrient (protein) and skeletal developing micronutrients (calcium and phosphorus) were calculated from dietary data for consecutive 3 days by the 24-hour dietary recall method. Arm strength was appraised from the hand grip strength test. Statistically, the assessed data were tested at 1% and 5% significance levels. Pearson correlation coefficients were derived. All the age groups possessed substantially shorter arm lengths (2.41-15.43%) than reference standards. Older groups appeared to have greater arm circumferences (1.00-3.92 cm) than younger groups. Overall, boys showed elevated skeletal muscles (6.69% and 8.29%) than girls. Dietary protein and phosphorus ingestion were significantly higher (45.42-90.88% and 16.18-40.62%) than recommended dietary allowances (RDAs). Calcium intake (23.26-28.48%) was below the RDA. Older male players performed under excellent grade (38%) in the hand grip strength test, depicting masculine supremacy. Positive correlations (r= 0.0710 to 0.5947) between arm anthropometry and nutrient intake with grip strength proved their affirmative effects on delivering various explosive shots, which can enhance the performance level of emerging young players.

* Corresponding author

E-mail: shreyashirimpi12@gmail.com (Shreyashi Saha)

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1 Introduction

Badminton is an indoor fast-paced sporting exertion, having extensive, high-intensity activities interspersed with rest intervals (Guven et al. 2017). Players require immense physical capability especially dexterity, aerobic capacity, and explosive power, as it involves a great amount of running, jumping, and swinging, which actively utilizes all the major muscle groups (Dogra 2021). The role of anthropometry in badminton is the most crucial factor for optimum performance as "the physique, body composition, physical growth, and one's motor development are of fundamental importance in developing the criteria of talent selection and development in sports" (Mishra 2016). "Early adolescence is characterized by rapid changes in physical growth and motor skills, as well as the emergence of special skills and talents" (Brown et al. 2017). It is the most significant period in human growth and maturation; wherein, unique changes and many adult patterns are established (Patil et al. 2015).

"Nutrition is an important part of sports performance for young athletes, in addition to allowing for optimal growth and development" (Purcell 2013). A composed diet comprising suitable quantities of macronutrients and micronutrients are essential to deliver enough fuel for growth and activities (Purcell 2013). It helps to improve athletic performance by dwindling fatigue as well as disease and injury threats. Besides, it also empowers the athletes to enhance practice and speedy recovery (Hoch et al. 2008). Further, poor nutritional status can lead to growth failure or poor body dimensions in young players, which can also lead to dismal performance.

Muscle endurance is one of the most decisive factors for successful performance in individual and team sports (Newton and Kraemer 1994). The relation between active muscle strength and some specific movement performance are frequently interpreted as external validity of muscle strength tests (Wagh et al. 2017). The grip can be explained as a "forceful act resulting in flexion at all the joints of the fingers along with thumb when used as a stabilizer to the object being held between the finger and the palm" (Bohannon 1997). Grip strength is stated as a reliable and effective factor to assess the functional integrity of the hand, considering the crucial part of the musculoskeletal structure (Jones 1989). It has an imperative role in delivering effectiveness and efficiency throughout routine work and sports activities (Joseph et al. 2021). Badminton, being a racket sport, immensely requires a resilient hand grasping strength as it reflects comprehensive strength and overall health status of players (Massy-Westropp et al. 2004), hand and forearm muscles activities (Nwuga 1975), and somatic performance (Samson et al. 2000). Being a physiological parameter, it is influenced by several factors such as age, gender, and arm anthropometries (Massy-Westropp et al. 2004; Koley and Singh 2010; Kubota and Demura 2011). Significant correlation of total arm, upper arm, forearm lengths, palm length-width, hand span, and mid-upper arm, forearm, and wrist circumferences (Hager-Ross and Rosblad 2002; Koley and Srikanth 2016; Alahmari et al. 2019; Aydogmus and Ozcan 2020) with the grip strength were reported earlier. Furthermore, it is also considered to be a functional index of nutritional status (Casanova and Grunert 1989; Koley and Srikanth 2016; Nakandala et al. 2019).

Nowadays badminton emerges as the second-most played sport in India as 51% of respondents favored badminton as the sport they played most regularly (Sukumar 2021). But in comparison with South-East Asian and European countries, very few Indian players appear recurrently in international competitions. Unfortunately, due to limited sports science literatures, there is a lack of descriptive data on the physical and nutritional profiles of young Indian badminton players. So, considering the significance of the adolescent period for developing sports skills and for the aforementioned factors, it is an indispensable concern to assess the dietary intake as well as anthropometric indices and to understand their effect on the physical fitness of early adolescent badminton players of 10-15 years age for the enhanced performance level. Therefore, the present study appraises selected anthropometric indices; especially hand measures, body composition through bioelectrical impedance analysis, and particular macro and micronutrient intake, essential for musculo-skeletal build-up. Additionally, the paper concerns delineating the impact of all these parameters on arm strength, which is essential for engendering explosive power for performing shots in badminton.

Hypothesis testing was done by z test with formulating null hypothesis (H_0) and non-directional alternate hypothesis (H_1). The differences between recorded data and standards as well as recorded data between two age groups were assessed at 5% and 1% levels of significance for acceptance of H_0 or H_1.

2 Materials and Methods

2.1 Selection of subjects

For the present research, a total of 200 healthy and injury-free female and male players from 10-12 (N=100, Males: 50 and Females: 50) and 13-15 (N=100, Males: 50 and Females: 50) years age groups, involved in regular badminton game were purposively selected as sample population. The sample size determination was based on the availability of professional players enrolled in leading badminton training academies, clubs, and institutes from Nagpur city, Maharashtra, India. Only those players having at least 1.5 years of playing experience, regularly participating in various club, school, city, district, and national level competitions, and willing for assessment were considered for the present study. Players having major or minor injuries in the last three months, any serious health issues viz. diabetes, asthma, arthritis, etc., having irregular practice schedules, or furthermore not willing to take part in the assessment were kept out from the present study.

2.2 Ethical clearance

The research work was duly approved by the Central Drugs Standard Control Organization (Govt. of India) registered Institutional Ethics Committee, Arneja Heart and Multispecialty Hospital, Nagpur, and also was sanctioned by the Research and Recognition Committee of Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India. Prior consent was taken from the coaches, players, and their guardians to perform assessments. All the assessments were done keeping in the view of COVID-19 safety measure protocols by strictly using mask, face shield, gloves, and sanitized instruments.

2.3 Anthropometric measurements

Anthropometry in sports science is perhaps one of the most decisive factors for finest performance. So considering its importance, measurements of selected arm related anthropometric variables like total arm length (TAL), upper arm length (UAL), lower arm length (LAL), hand span (HS), palm length (PL), palm width (PW) and arm circumferences, like mid-upper arm circumference (MUAC), Forearm circumference (FAC) and Wrist circumference (WC) of badminton players were recorded in centimeters. The measurements were taken by using a steel anthropometer and non-stretchable plastic tape. All the measurements were done at the closest 0.1 cm error margin due to minimal clothing thickness.

2.4 Body composition analysis

Body composition is an essential kinematics for the fitness of athletes. So, in this research study, under body composition analysis, arm skeletal muscle (SMA) percentage was measured through a digital bioelectrical impedance analyzer. Bioelectrical impedance analysis is a useful tool for estimating body composition. It is based on the subcutaneous fat (fat mass) and skeletal muscle (fat-free mass) which are related to two component (2C) body composition model (Lee and Gallagher 2008). It uses equations that can explain the statistical associations based on biological relations for a certain population. Also, the equations are convenient for only those subjects that have a proximate matching of physical outline with the reference population (Duren et al. 2008).

2.5 Nutrient intake assessment

Proper nutrition is indispensable for refining the performance of athletes (Fink et al. 2011). So, the implementation of proper dietary principles is very important in daily life. To determine players' nutrient intake, '24 hour dietary recalls method' for successive three days was followed (Hausswirth and Mujika 2013). Separate sheets of tables were provided to the players for recording each and every diet in their daily meals. Protein, calcium, and phosphorous content of diets consumed by the players were estimated by using a standard Indian food composition table (Gopalan et al. 2012; Longvah et al. 2017).

2.6 Physical strength test

As a racket sport, grip strength is very important in the game of badminton as players use several grips such as continental (handshake grip), eastern, and western (usually for forehand grips) grips for delivering shots during the game. The majority of players prefer to change grips for different shots selection in the match (Koley and Srikanth 2016). The hand grip strength (HGS) in kgs of the dominant hand of every player was measured through Camry electronic hand dynamometer. The equipment was held in the dominant hand with the extended and flexed forearm and elbow positioned at right angles. The players were directed to grip the dynamometer in such a way that the second phalanx is against the inner stirrup (Wagh et al. 2017). The subjects were instructed to give utmost force to the dynamometer for consecutive three times with a recovery interval of 30 seconds (Koley and Srikanth 2016). The average of three readings was recorded. Camry electronic hand dynamometer has excellent reliability to test grip strength among all age groups (Mani et al. 2019).

2.7 Statistical analysis

The acquired data was compiled, synthesized, and classified according to gender and age groups. The mean, standard deviation, range, and percentage of excess/deficit compared with corresponding references were calculated. The data were compared with age and gender-related reference values and RDAs using two-tailed z test (Nande and Vali 2010; Koley and Srikanth 2016; NCHS 2016; Aydogmus and Ozcan 2020; ICMR 2020). The conclusion was derived at 5% (α=0.05) and 1% (α=0.01) level of significance ($z < 1.960$; H_0 accepted at α=0.05 and 0.01, $1.960 < z < 2.576$; H_1 accepted at α=0.05 and H_0 accepted at α=0.01; $z > 2.576$; H_1 accepted at α=0.05 and 0.01). The correlation of physical fitness with anthropometric profile as well as nutritional intake was derived by using Pearson's product-moment coefficient of correlation.

3 Results

The study represents a compiled data set of various assessed parameters of 200 badminton players. The descriptive statistics of the dataset suggested that it is well-modeled by a normal distribution.

3.1 Baseline characteristics

Sports specialization can be described as intense training of a single sport, barring all other sports round the year (Malina 2010; Jayanthi et al. 2011). So, the players of 10-12 years (Girls: 11.10 ± 0.86 years,

Boys: 11.03 ± 0.84 years) and 13-15 years (Girls: 14.02 ± 0.83 years, Boys: 13.90 ± 0.86 years) who were meticulously engaged in the game of badminton for at least 1.5 years were considered as professionals in badminton and were of the main choice of the present study. The sports information of the subjects (Figure 1) specified that with the increasing age their involvement in the game practice was also increased. Elder groups showed >3 years of badminton practice (60% and 64%, for girls and boys, respectively).

3.2 Hand anthropometry

Badminton, being a racket sport needs a strong hand anthropometric profile for hitting effective shots. For assessing the arm musculoskeletal trait, selected anthropometric variables were measured and displayed in Table 1.

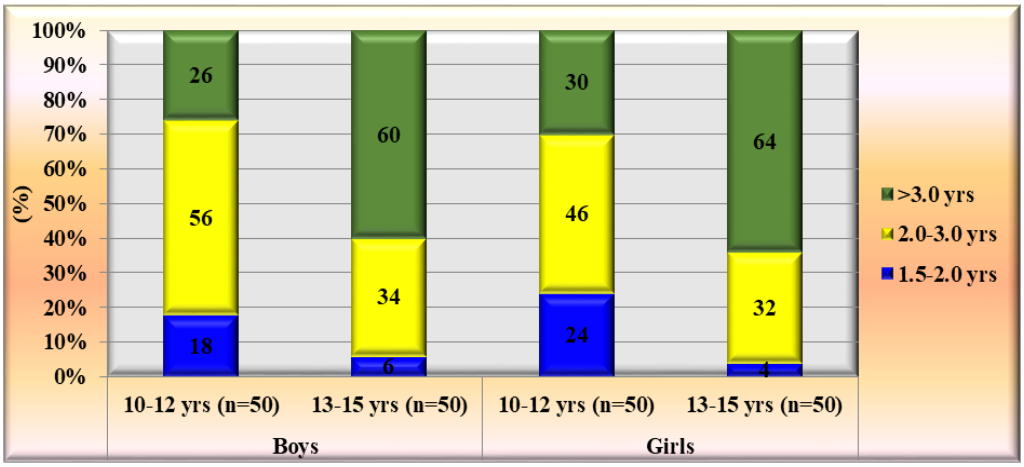

Figure 1 Percentage Distribution of Players from Different Age Groups based on Years of Game Practice

Table 1 Data on Hand Anthropometry of Badminton Players

	Parameters	Girls (N=100)			Boys (N=100)		
		10-12 years (n=50)	13-15 years (n=50)	z Values#	10-12 years (n=50)	13-15 years (n=50)	z Values#
1.	Total Arm Length (TAL) (cm)						
i.	Mean±SD	61.42±4.36	66.58±3.48		60.75±4.53	70.10±4.65	
ii.	Range	53.70-71.70	58.30-74.70		53.00-70.00	59.50-80.00	
iii.	Standard	68.36		6.54*	71.83		10.18*
iv.	z values§	11.26*	3.62*		17.30*	2.63**	
v	% Deficit	-10.15	-2.60		-15.43	-2.41	
2.	Upper Arm Length (UAL) (cm)						
i.	Mean±SD	22.89±2.00	25.31±1.62		22.52±2.02	26.18±2.04	
ii.	Range	19.00-28.00	20.60-28.50		19.20-27.00	21.40-31.00	
iii.	Standard	25.48		6.65*	26.12		9.01*
iv.	z values§	9.16*	0.74		12.60*	0.21	
v	% Deficit/Excess	-10.16	-0.67		-13.78	+0.23	
3.	Lower Arm Length (LAL) (cm)						
i.	Mean±SD	22.58±1.77	24.00±1.69		22.51±2.09	25.72±1.91	
ii.	Range	19.00-25.70	20.90-28.40		18.80-27.40	22.50-29.90	
iii.	Standard	21.36		4.10*	22.79		8.02*
iv.	z Values§	4.87*	11.05*		0.95	10.85*	
v	% Excess/Deficit	+5.71	+12.36		-1.23	+12.86	

	Parameters	Girls (N=100)			Boys (N=100)		
		10-12 years (n=50)	13-15 years (n=50)	z Values#	10-12 years (n=50)	13-15 years (n=50)	z Values#
4.	Hand Span (HS) (cm)						
i.	Mean±SD	18.80±1.44	20.27±1.18		18.11±1.75	21.22±1.43	
ii.	Range	15.50-22.00	17.70-23.50		15.20-21.40	17.00-23.80	
iii.	Standard	18.7		5.58*	21.0		9.73*
iv.	z Values§	0.49	9.41*		11.68*	1.09	
v	% Excess/Deficit	+0.53	+8.40		-13.76	+1.05	
5.	Palm Length (PL) (cm)						
i.	Mean±SD	15.96±1.07	17.27±0.87		15.66±1.15	18.41±1.30	
ii.	Range	13.70-18.50	15.70-19.20		13.80-18.00	15.20-20.50	
iii.	Standard	16.53		6.72*	17.61		11.20*
iv.	z Values§	3.77*	6.01*		11.99*	4.35*	
v	% Deficit/Excess	-3.45	+4.48		-11.07	+4.54	
6.	Palm Width (PW) (cm)						
i.	Mean±SD	6.89±0.42	7.20±0.38		6.75±0.57	7.72±0.59	
ii.	Range	6.00-8.00	6.30-7.90		5.60-8.00	6.20-9.00	
iii.	Standard	6.56		3.87*	6.58		8.36*
iv.	z values§	5.56*	11.91*		2.11**	13.66*	
v	% Excess	+5.03	+9.76		+2.58	+17.33	
7.	Mid Upper Arm Circumference (MUAC) (cm)						
i.	Mean±SD	20.69±3.28	23.32±2.27		20.23±2.87	24.15±4.13	
ii.	Range	15.00-29.00	18.00-32.00		15.50-27.00	15.50-33.00	
iii.	Standard	24.8	27.2	4.66*	24.4	28.5	5.51*
iv.	z Values§	8.86*	12.09*		10.27*	7.45*	
v	% Deficit	-16.57	-14.26		-17.09	-15.26	
8.	Forearm Circumference (FAC) (cm)						
i.	Mean±SD	19.67±2.08	21.73±1.65		19.59±1.92	22.94±3.20	
ii.	Range	15.00-25.00	19.00-30.00		15.50-24.00	16.50-35.00	
iii.	Standard	19.10	20.47	5.49*	19.10	20.47	6.35*
iv.	z Values§	1.94	5.40*		1.80	5.46*	
v	% Excess	+2.98	+6.16		+2.57	+12.07	
9.	Wrist Circumference (WC) (cm)						
i.	Mean±SD	13.49±1.18	14.49±1.24		13.59±1.08	15.32±1.32	
ii.	Range	11.00-16.00	12.70-20.50		11.00-15.50	12.00-18.00	
iii.	Standard	14.40	15.30	4.13*	14.60	16.00	7.17*
iv.	z Values§	5.45*	4.62*		6.61*	3.64*	
v	% Deficit	-6.32	-5.29		-6.92	-4.25	

z values# comparisons between younger and older groups; z values§ comparison between data of subjects and standards; Values with * indicate significant difference at both 5% and 1% levels ($p<0.01$), ** for the significant difference at 5% level but insignificant difference at 1% level ($0.01<p<0.05$) and with no mark indicate insignificant difference at both 5% and 1% levels ($p>0.05$).

3.2.1 Arm lengths

It was observed that younger boys had shorter total arm length (0.67 cm) than girls; whereas, elder boys had longer arms (3.52 cm) as compared with girl players. Both younger (Girls: 10.15%; z=11.26, p<0.01 and Boys: 15.43%; z= 17.30, p<0.01) and elder (Girls: 2.60%; z=3.62, p<0.01 and Boys: 2.41%; z= 2.63, p<0.01) players were unable to meet the standard arm lengths of Indian badminton players as per Koley and Srikanth (2016). In the present study, with an increment of age, the arm length development of boys (9.35 cm; z=10.18, p<0.01) was predominant over girls (5.16 cm; z=6.54, p<0.01). Mean UAL for younger groups depicted a significant deficit (Girls: 10.16%; z=9.16, p<0.01 and Boys: 13.78%; z=12.60, p<0.01) as compared to standard values (Koley and Srikanth 2016). On the contrary, older girls and boys exhibited trivial deficit of 0.67% (z=0.74, p>0.05) and surplus of 0.23% (z=0.21, p>0.05) than standards (Koley and Srikanth 2016). Like TAL, higher increments in UAL were also recorded throughout the ages in boys (3.66 cm; z=9.01, p<0.01) over girls (2.42 cm; z=6.65, p<0.01). Differences in LAL between elder and younger players were computed as 1.42 cm (z=4.10, p<0.01) for girls and 3.21 cm (z=8.02, p<0.01) for boys respectively. Except younger boys with a deficit of 1.23% (z=0.95, p>0.05) for all other players exceeded the corresponding reference standard values of badminton players [10-12 years girls: 5.71% (z=4.87, p<0.01), 13-15 years girls: 12.36% (z=11.05, p<0.01) and 13-15 years boys: 12.86% (z=10.85, p<0.01)] (Koley and Srikanth 2016).

3.2.2 Hand measures

The HS measures demonstrate that younger and older girls, as well as older boys had mean HS above (0.53%; z=0.49, p>0.05, 8.40%; z=9.41, p<0.01 and 1.05%; z=1.09, p>0.05) the standard values (Ruiz et al. 2006) whereas younger boys expressed considerable deficient mean value of 13.76% (z=11.68, p<0.01). Conspicuous differences of 1.47 cm (z=5.58, p<0.01) and 3.11 cm (z=9.73, p<0.01) were perceived between both the age groups for female and male players. The percentage excess for mean PL of older girls and boys was of 4.48% (z=6.01, p<0.01) and 4.54% (z=4.35, p<0.01), and younger girls and boys had shorter mean palms by 3.45% (z=3.77, p<0.01) and 11.07% (z=11.99, p<0.01) as compared with standards (Koley and Srikanth 2016). For PW, younger and elder gender had mean values far exceeded than standards (Koley and Srikanth 2016) with % excess of 5.03 (z=5.56, p<0.01), 2.58 (z=2.11, 0.05>p>0.01) and 9.76 (z=11.91, p<0.01) and 17.33 (z=13.66, p<0.01), for girls and boys respectively.

3.2.3 Arm circumferences

Assessing the parameters for arm circumference variables, it was noticed that the mean MUAC values for the elder girls and boys were larger than for younger groups (2.63 cm; z=4.66 and 3.92 cm; z=5.51, p<0.01). However, none of them were able to meet the corresponding age group standards for MUAC (NCHS 2016) by the deficit of 16.57% and 14.26% (z=8.86 and 12.09, p<0.01) for girls and 17.09% and 15.26% (z=10.27 and 7.45, p<0.01) for boys, respectively. Older badminton players (females and males) showed significantly higher mean FAC than standards (6.16%; z=5.40 and 12.07%; z=5.46, respectively, p<0.01) (Paswan 2020). Girls and boys aged between 10 and 12 years were marginally able to meet the standards for FAC (2.98%; z=1.94 and 2.57%; z=1.80, respectively, p>0.05) (Aydogmus and Ozcan 2020). Older players from both genders had significantly greater mean FAC than younger players (2.06 cm; z=5.49 and 3.35 cm; z=6.35, for older vs. younger girls and for older vs. younger boys, respectively). WC signified considerable deficits in girls (10-12 years: 6.32%; z=5.45, p<0.01 and 13-15 years: 5.29%; z=4.62, p<0.01) and boys (10-12 years: 6.92%; z=6.61, p<0.01 and 13-15 years: 4.25%; z=3.64, p<0.01) as compared to age and gender-specific standard values (Ozturk et al. 2017). Differences among WC of younger and older girls and boys were found to be 1.00cm (z=4.13, p<0.01) and 1.73 cm (z=7.17, p<0.01), respectively.

3.3 Body composition

Underbody composition variables arm skeletal muscle (SMA) was analyzed by bio- electrical impedance technique using arm–to-foot body composition analyzer.

3.3.1 Arm skeletal muscle

The SMA in Table 2 reflects that older girls possessed lesser skeletal muscle arms. However, trivial difference was recorded (0.70%; z=0.85, p>0.05). With increasing age, boys showed a gain in skeletal arm muscles (0.90%; z=1.21, p>0.05). Large individual variations were noted in SMA for girls and boys.

Table 2 Data on Skeletal Muscle-Arm of Badminton Players

	Parameters	Girls (N=100)			Boys (N=100)		
		10-12 years (n=50)	13-15 years (n=50)	z Values#	10-12 years (n=50)	13-15 years (n=50)	z Values#
1.	Skeletal Muscle-Arm (SMA) (%)						
i.	Mean±SD	34.49±4.18	33.79±4.04		41.18±4.59	42.08±2.60	
ii.	Range	24.70-43.80	24.40-42.80	0.85	30.70-48.90	36.40-46.90	1.21

z values# comparisons between younger and older groups

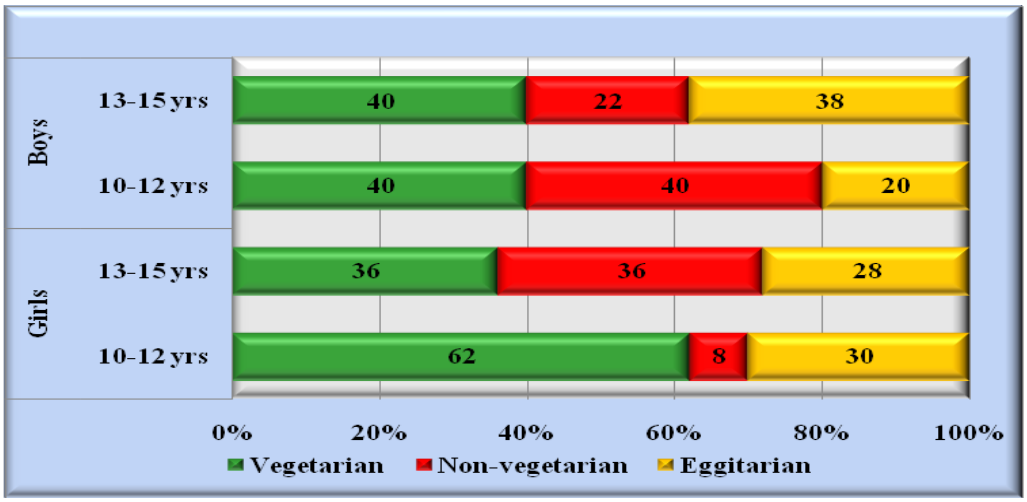

Figure 2 Food Habits of Subjects

Table 3 Data on Daily Intake of Protein, Calcium, and Phosphorus Intake by Players

	Parameters	Girls (N=100)			Boys (N=100)		
		10-12 years (n=50)	13-15 years (n=50)	z Values #	10-12 years (n=50)	13-15 years (n=50)	z Values #
1.	Protein (g/day)						
i.	Mean±SD	55.01±6.34	62.82±5.32		60.70±4.54	66.56±5.51	
ii.	Range	43.06-75.01	49.98-75.88		51.05-77.09	57.00-75.92	
iii.	RDAs	32.8	43.2	6.67*	31.8	44.9	5.80*
iv.	z Values§	24.77*	26.08*		45.01*	27.80*	
v	% Excess	+67.71	+45.42		+90.88	+48.24	
2.	Calcium (mg/day)						
i.	Mean±SD	622.06±172.59	715.19±176.58		652.29±128.98	764.09±185.71	
ii.	Range	307.04-1154.88	313.73-1260.19		416.19-906.78	436.55-1250.55	
iii.	RDAs	850	1000	2.67*	850	1000	3.50*
iv.	z Values§	9.34*	11.40*		10.84*	8.98*	
v	% Deficit	-26.82	-28.48		-23.26	-23.59	
3.	Phosphorous (mg/day)						
i.	Mean±SD	1161.79±178.44	1344.94±152.46		1305.83±130.40	1406.18±172.15	
ii.	Range	606.10-1623.13	1090.55-1616.52		889.77-1530.59	834.15-1726.75	
iii.	RDAs	1000		5.52*	1000		3.29*
iv.	z Values§	6.41*	16.00*		16.58*	16.68*	
v	% Excess	+16.18	+34.49		+30.58	+40.62	

z values# comparisons between younger and older groups; z values§ comparison between data of subjects and standards; Values with * indicate significant difference at both 5% and 1% levels ($p<0.01$), ** for significant difference at 5% level but insignificant difference at 1% level ($0.01<p<0.05$) and with no mark indicate insignificant difference at both 5% and 1% levels ($p>0.05$).

3.4 Nutrient intake

Figure 2 and Table 3 demonstrate the data on food habits and nutrient intake of players. From the nutritional assessment, it was observed that subjects had a wide variety of food choices from vegetarian, non-vegetarian, and eggetarian (Figure 2). A majority (62%) of younger female badminton players were found to be vegetarians. An equal number (40% and 36%, respectively) of boys aged between 10 to 12 years and girls aged between 13 to 15 years were found to have vegetarian and non-vegetarian food habits.

3.4.1 Protein

The mean protein intake of younger (Girls: 67.71%; z=24.77, p<0.01 and Boys: 90.88%; z=45.01, p<0.01) and older players (Girls: 45.42%; z=26.08%, p<0.01 and Boys: 48.24%; z=27.80, p<0.01) was found to be significantly higher than recommended dietary allowances (RDAs) (ICMR 2020). Overall girls (7.81 g/day; z=6.67, p<0.01) depicted a higher increment in protein intake than boys (5.86 g/day; z=5.80, p<0.01) through the ages.

3.4.2 Calcium

Calcium intake of players (Table 3) exposed significantly lesser means values for younger girls and boys as well as older girls and boys with a deficit of 26.82% (z=9.34, p<0.01), 23.26% (z=10.84, p<0.01), 28.48% (z=11.40, p<0.01) and 23.59% (z=8.98, p<0.01) as compared to RDAs, respectively. Although increment in dietary calcium intake among girls (93.13 mg/day, z=2.67, p<0.01) and boys (111.80 mg/day; z=3.50, p<0.01) with progressive ages attributed to the growth of skeletal traits during the puberty period.

3.4.3 Phosphorous

Phosphorous intake of players is displayed in Table 2. The mean intake of phosphorus by girls (10-12 years, 16.18%; z=6.41, p<0.01 and 13-15 years, 34.49%; z=16.00, p<0.01) and boys (10-12 years, 30.58%; z=16.58, p<0.01 and 13-15 years, 40.62%; z=16.68, p<0.01) from both age groups exceeded over the RDAs. Older groups of girls and boys consumed considerably higher quantities of phosphorus as compared to younger ones (183.15 mg/day; z=5.52 and 100.35 mg/day; z=3.29, respectively, p<0.01).

3.5 Physical fitness

Physical fitness is the major mantra today and players around the world are conscious of their fitness.

3.5.1 Hand grip strength

The hand grip strength (Table 4) of younger and older girls and boys demonstrates that both older girls and boys had significantly higher HGS (4.33 kg; z=5.83, p<0.01 and 7.48 kg; z=6.41, p<0.01)

Table 4 Data of Hand Grip Strength (HGS) for Players

	Parameters	Girls (N=100)			Boys (N=100)		
		10-12 years (n=50)	13-15 years (n=50)	z Values #	10-12 years (n=50)	13-15 years (n=50)	z Values #
1.	Hand Grip Strength (HGS) (kg)						
i.	Mean±SD	24.01±4.47	28.34±2.75		25.86±5.68	33.34±5.99	
ii.	Range	15.20-35.00	23.50-36.00		16.20-36.20	21.80-44.30	
iii.	Standard	20.72		5.83*	26.43		6.41*
iv.	z Values§	5.20*	19.59*		0.71	8.16*	
v	% Excess/Deficit	+15.88	+36.78		-2.16	+26.14	

z values# comparisons between younger and older groups; z values§ comparison between data of subjects and standards; Values with * indicate significant difference at both 5% and 1% levels (p<0.01), ** for significant difference at 5% level but insignificant difference at 1% level (0.01<p<0.05) and with no mark indicate insignificant difference at both 5% and 1% levels (p>0.05).

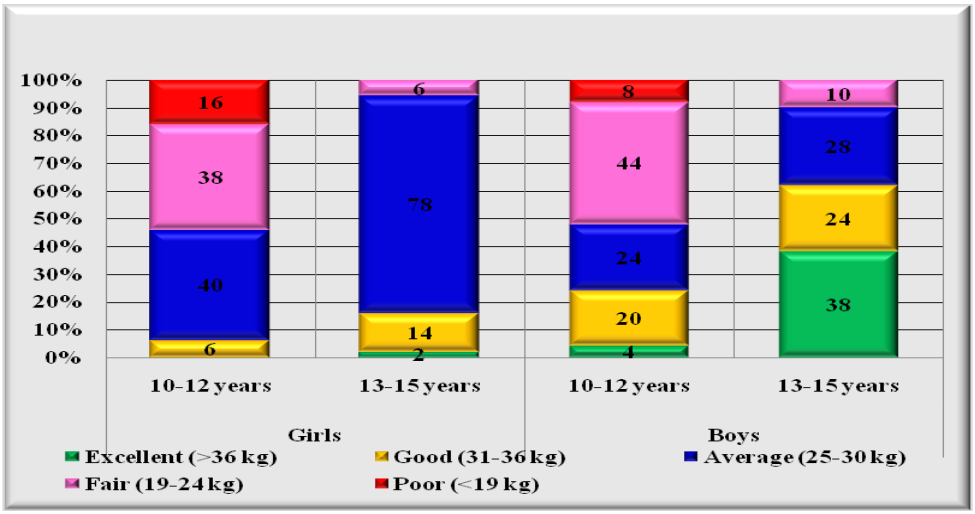

Figure 3 Distribution of Subjects based on Performance of Hand Grip Strength

than younger ones. Other than younger boys with a 2.16% (z=0.71, p>0.05) deficit in HGS, younger girls along with older girls and boys showed 15.88% (z=5.20, p<0.01), 36.78% (z=19.59, p<0.01) and 26.14% (z=8.16, p<0.01) excess of HGS.

HGS test involves the grip strength which can influence the angle of the racquet face at the time of hitting shots (Koley and Srikanth 2016). Figure 3 shows that 38% of boys aged between 13 and 15 years had excellent hand grip power. The majority (78%) of older female badminton players showed average hand grip power. Long duration of practicing, regular sports/fitness training, and upholding musculoskeletal feasibility over the years, the older players, especially the males had substantial explosive power with strong arm muscle endurance and resilient hand grip.

3.6 Coefficient of correlation between various parameters

Table 5 depicts the data on the coefficient of correlation of hand grip strength of players with anthropometric indices, body composition, and nutrient intake for players from all four age groups. From the correlation study, it was revealed that total, upper, and lower arm lengths among younger badminton players (girls and boys) reflected significant positive correlations with hand grip strength (r=0.3022, 0.05>p>0.01 to 0.5744, p<0.01). However, even though direct, these correlations were low among girls and boys of older age (r=0.1189 p>0.05 to 0.3575, 0.05>p>0.01). The larger the span of the hand, the longer and wider the palms, powerful was the hand grip among girls and boys from both age groups (r=0.1426, p>0.05 to 0.5221, p<0.01).

The hand grip strength of players was found to be directly related to arm skeletal muscle [r=0.4029, 0.5947, 0.2139, and 0.2068, respectively for girls aged 10-12 years (p<0.01), girls aged 13-15 years (p<0.01), boys aged 10-12 years (p>0.05) and boys aged 13-15 years (p>0.05)] (Table 5 and Figure 4).

Nutrition profile expressed enhanced positive correlations of protein (r= 0.1346, p>0.05 to r =0.3047, 0.05>p>0.01), calcium (r=0.1202, p>0.05 to 0.3329, 0.05>p>0.01) and phosphorus intake (r=0.0710 to 0.2211, p>0.05) with hand grip strength among all age groups of players. However, these correlations were low to moderate (Table 5 and Figures 5 - 7).

Table 5 Coefficient of Correlation of Hand Grip Strength (HGS) of Players with Different Variables

	Variables		r Values			
			Girls (N=1000)		Boys (N=100)	
			10-12 years (n=50)	13-15 years (n=50)	10-12 years (n=50)	13-15 years (n=50)
1	Hand Grip Strength vs. Anthropometric Indices	Total Arm Length	0.4556*	0.2371	0.5744*	0.2526
2		Upper Arm Length	0.4735*	0.1189	0.4731*	0.3575**
3		Lower Arm Length	0.3022**	0.1623	0.4768*	0.1325
4		Hand Span	0.4637*	0.2325	0.5099*	0.2497
5		Palm Length	0.4718*	0.4139*	0.5221*	0.2169
6		Palm Width	0.3552**	0.2267	0.4213*	0.1426
7		MUAC	0.1148	0.2044	0.4209*	0.2081
8		Fore Arm Circumference	0.1271	0.2011	0.4301*	0.1715
9		Wrist Circumference	0.2789	0.1247	0.4348*	0.1103
10	Hand Grip Strength vs. Body Composition	Arm Skeletal Muscle	0.4029*	0.5947*	0.2139	0.2068
11	Hand Grip Strength vs. Dietary Nutrient Intake	Protein Intake	0.1761	0.1378	0.3047**	0.1346
12		Calcium Intake	0.1202	0.3329**	0.1920	0.1517
13		Phosphorous Intake	0.0710	0.1823	0.2211	0.1290

Values with * indicate significant difference at both 5% and 1% levels (p<0.01), ** for significant difference at 5% level but insignificant difference at 1% level (0.01<p<0.05) and with no mark indicate insignificant difference at both 5% and 1% levels (p>0.05).

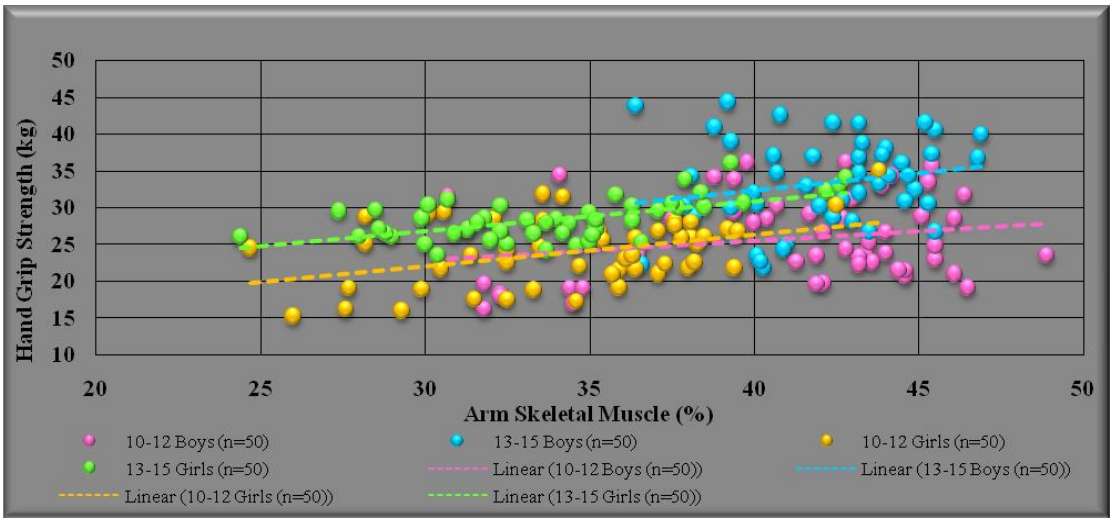

Figure 4 Scatter Graph for Correlation between Hand Grip Strength and Arm Skeletal Muscle among Subjects

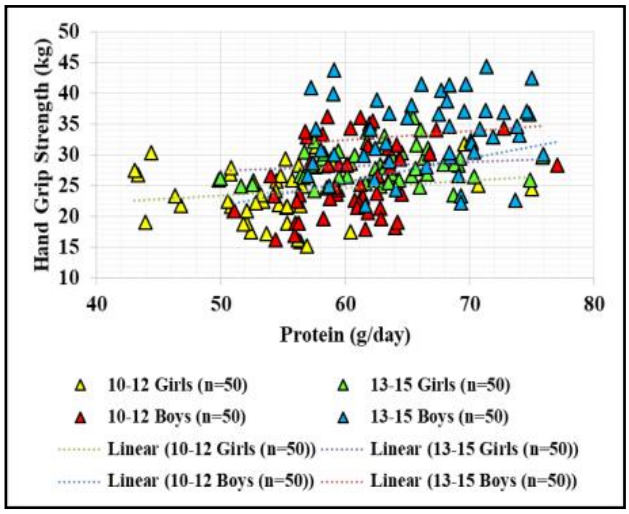

Figure 5 Binary Plot of Dietary Protein vs. Hand Grip Strength

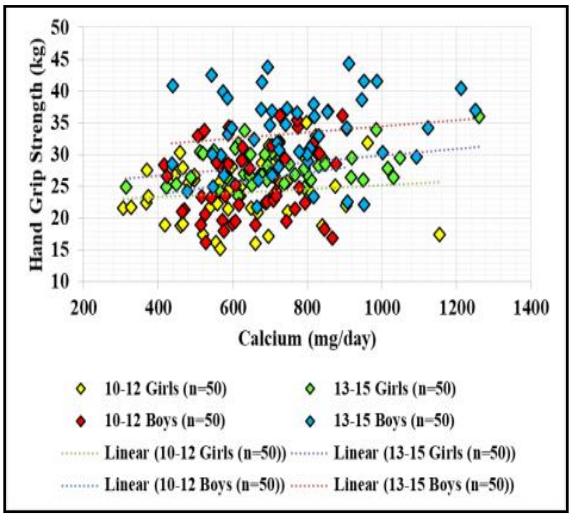

Figure 6 Binary Plot of Dietary Calcium vs. Hand Grip Strength

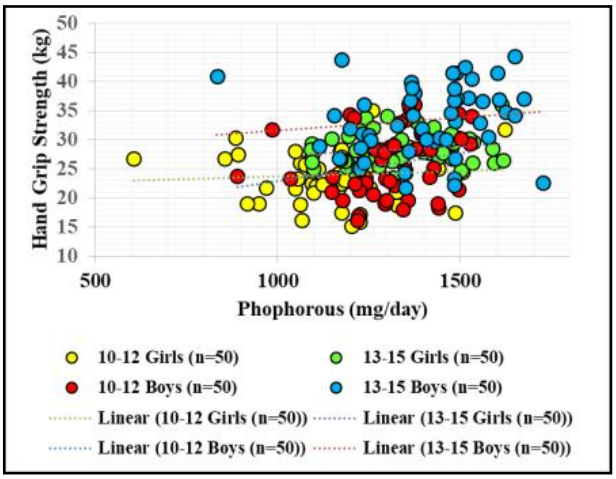

Figure 7 Binary Plot of Dietary Phosphorous vs. Hand Grip Strength

4 Discussion

The present study results have provided an exceptional insight into the impact of hand anthropometry, arm muscle mass, and nutritional profile on grip strength from the perspective of young Indian badminton athletes. However, few qualitative studies for badminton players as well as other athletes in Indian and Global scenarios were explored to complement the current literature.

4.1 Hand anthropometry

4.1.1 Arm lengths

The TAL of badminton players in the present study were longer than the gymnasts (57.08 cm; n=60) as studied by Khan and Srinivasa (2015) and basketballers (Girls: 56.30 cm, 61.84 cm, and Boys: 54.61 cm, 64.08 cm; n=400) as reported by Agrawal and Nande (2020) for the same age group. Additionally, Mishra (2016) also reported similar results while comparing the TAL between badminton (73.35 cm, n=30) and table tennis players (72.74 cm, n=30). Considering the standards as per NCHS (2016) (Girls: 32.3 cm, 34.9 cm, and Boys: 32.1 cm, 36.6 cm), all the age groups had significantly shorter UAL than the standards. However, a similar trend of higher increment in UAL through the ages in boys confirmed masculine dominance in early puberty skeletal development. Mean LAL of badminton players were lesser than Muqarram (2015) reported intervarsity (26.15 cm; n=227), national (26.57 cm; n=131), and state level (26.48 cm; n=42) Indian long-distance runners between 18 and 25 years of age and Fallahi and Jadidian (2011) recorded Iranian national and collegian grip athletes (26.86 cm; n=40) between 19 to 29 years of age.

4.1.2 Hand measures

Both PL (Girls: 1.31 cm; $z=6.72$, $p<0.01$ and Boys: 2.75 cm; $z=11.20$, $p<0.01$) and PW (Girls: 0.31 cm; $z=3.87$, $p<0.01$ and Boys: 0.97 cm; $z=8.36$, $p<0.01$) measurements revealed visible growth among both the genders as increase in age. Agrawal and Nande (2020) also reported similar increment for PL (Girls: 0.88 cm and Boys: 1.68 cm) and PW (Girls: 0.28 cm and Boys: 0.61 cm) among male (n=200) and female (n=200) basketballers whereas, Fallahi and Jadidian (2011) measured wider HS (21.24 cm) and PW (9.21 cm) among Iranian athletes (n=40) (19-29 years).

4.1.3 Arm circumferences

Among the 10-12 age group, as compared to Turkish badminton athletes (11.8 ± 0.1 years, n=72), Aydogmus and Ozcan (2020) measured higher MUAC (22.00 cm) and lower FAC (19.10 cm) than with Indian players of the present study, although Marwat et al. (2021) recorded higher MUAC (25.61 cm) and FAC (25.01 cm) among Pakistani school level badminton players as compared to Indian adolescent players. Raschka and Schmidt (2013) also assessed higher MUAC (Male: 29.2 cm and Female: 26.8 cm) and FAC (Male: 28.2 cm and Female: 25.2 cm) for senior German professional badminton players (Male: 22.70 years; n=20 and Female: 24.00 years; n=20). Considering world-class badminton players, Hume et al. (2008) observed significantly higher readings of MUAC, FAC and WC among singles (Male; 26.1 years: 30.1 cm, 27.6 cm and 16.6 cm; n=18 and Female; 25.1 years: 27.6 cm, 24.1 cm and 14.8 cm; n=20) and doubles (Male; 26.5 years: 30.9 cm, 27.3 cm and 16.3 cm; n=35 and Female; 24.5 years: 27.9 cm, 24.1 cm and 14.7 cm; n=36) players.

4.2 Body composition

4.2.1 Arm skeletal muscle

Like the present study for both the age groups, Sule and More (2020) analyzed superior higher skeletal muscle (10.75%) among elite male (n=20) and female (n=10) badminton players selected from various parts of the state of Maharashtra. Even Abian-Vicen et al. (2012) also assessed higher body muscle percentage of men's singles players (50.2 ± 1.3 %, n=46, age 22.7 ± 4.2 years) over women's singles players (46.5 ± 2.0 %, n=24, age 23.0 ± 5.7 years) at National Spanish badminton championship. However, the Spanish players were found to have significantly higher body muscles as compared to Indian players in the present study.

4.3 Nutrient intake

4.3.1 Protein

Protein is the foremost macronutrient for skeletal muscle growth. Masculine pre-eminence in skeletal muscle was also supported by higher protein intake observed among players. Deshpande and Nande (2018) assessed the protein intake of females (10-12 years. 48.59 ± 6.44 g/day, 13-15 years. 50.05 ± 5.83 g/day) and male (10-12 years. 53.84 ± 11.78 g/day, 13-15 years. 58.11 ± 11.72 g/day) Indian gymnasts of contemporary age groups. For this study, the protein intake tendency among all the age groups was quite higher and players were found to be consuming chicken, fish, and eggs. Foods like milk, curd, paneer/cheese, pulses, whole legumes, etc. were also responsible for the higher protein intake of players.

4.3.2 Calcium

Among various micronutrients, calcium is a vital mineral for the growth, preservation, and repairing of skeletons. It plays several important roles in regularising muscle contraction, nerve conduction, and blood coagulation (Culp 2015). Calcium rich bones are usually less susceptible to be fractured. Similar to the present study, Nande et al. (2009) also revealed an analogous deficiency with 52.89% (females: 20.45 years; n=4) and 62.2% (males: 21.87; n=4) among university and/ state level badminton players as compared with RDAs (Satyanarayana 1991).

4.3.3 Phosphorous

Phosphorous is the second most profuse mineral in the body following calcium. The foremost role of phosphorous is the formation of bones and teeth. It is also a desirable nutrient for the growth, maintenance, and restoration of cells and tissues (Moshfegh 2016). It is so ubiquitous in various foods that phosphorus deficiency may take places only at near starvation conditions or the presence of any metabolic disorder (Institute of Medicine 1997). Higher intake of phosphorous as compared to RDAs in all the age groups was due to the presence of surplus phosphates in foods preservatives and beverages such as flavored synthetic juices and nowadays it is commonly observed that adolescent players are tempted to have beverages after intense practice. Further, a close association between protein and phosphorus ingestion is observed. The dietary sources of protein usually provide phosphorus (Fouque et al. 2011). However, plant protein has a 40% to 50% lower absorption rate of organic phosphorus in comparison with animal protein (Gonzalez-Parra et al. 2012). All the groups of badminton players in the present study showed excessive intake of both protein and phosphorous in their diets. Contrary to the result of the present study, Nande et al. (2009) observed a substantial deficiency in phosphorus intake with 37.24% (females: 20.45 years; n=4) and 40.07% (males: 21.87; n=4) among university and/or state level badminton players as compared with RDAs (Satyanarayana 1991).

4.4 Physical fitness

4.4.1 Hand grip strength

HGS is the imperative parameter to assess the upper flex or musculature of the forearm and hand strength of badminton players. Compared with Turkish professional players, the HGS of the present study was found to be reasonably higher than the evaluation of Guven et al. (2017) [20.43 kg; n=15 (age: 11.20 years)] among younger groups but significantly lower than the assessment by Ozgur and Hotaman (2020) [Male: 46.10 kg; n=5 and Female: 32.45 kg; n=9 (age 15.5 years)] among older groups. Moreover, Akinbiola et al. (2017) determined significantly high HGS value in male (27.86 years) [47.07 kg; n=14] and female (23.63 years) [33.06 kg; n=8] Nigerian sub- elite badminton players.

4.5 Coefficient of correlation between various parameters

Low to moderate assenting correlations confirmed that the hand anthropometry along with arm skeletal muscle had a stimulus impression on strength and flexibility of the arm for forehand and backhand shots that affects the pace, spin, and placement of shots. Koley and Srikanth (2016) established significant positive correlations (r = 0.196 to 0.688) of handgrip strength of both hands with upper arm circumference, upper, lower, and total arm lengths, hand length, and breadth among junior and senior level Indian badminton athletes (12–25 years). Aydogmus and Ozcan (2020) also experienced similar affirmative correlations (r = 0.18 to 0.34) of MUAC and FAC with HGS in Turkish 10-12 years of badminton athletes (n=72). Even, Alahmari et al. (2019) also delineated a significant positive correlation that hand dimensions viz. hand circumference, hand span, hand length, and palm length were significantly correlated with the HGS in 6-16 years school going girls (n=100) (r = 0.521 to 0.755) and boys (n=100) (r = 0.678 to 0.804) of Saudi Arabia.

The relationship between protein intake and hand grip power signified the importance of protein intake for muscle endurance for playing different explosive shots for these players. In comparison to other grip strength games like basketball, Agrawal and Nande (2020) also reflected similar positive correlations (r = 0.0168 to 0.1634) with protein intake among the players of respective age groups of the present study. Also, a positive correlation of calcium and phosphorous with hand grip strength might indicate the fact that the growth of arm bones, which gives momentous advantages to the players for choosing forehand and backhand shots.

The limitation of the present study was represented by spatial population limitation as the samples were only collected from professional badminton academies of Nagpur city, Maharashtra, India.

5 Conclusion

Overall, the anthropometric indices of female and male badminton players were well developed as age progressed, which was evident from the results. Significant increments were noticed in arm lengths, palm length and width, and hand span, all of which are crucial for enhanced performance among badminton players. Increment in arm circumferences along with skeletal muscle showed the development of muscles continued with ascending ages. Further, a significant masculine preponderance of skeletal growth was found among these players at a young age. The nutritional profile also confirmed a good intake of protein and phosphorus which are involved in bone development and arm muscle growth. In general, apart from consistent badminton practice and fitness training right from a younger age, assenting effects of all anthropometric parameters and nutrient intake can lead to enhance the successive performance in terms of arm strength.

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Conflicts of interest

The authors affirm that they do not have any conflict of interest.

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Characterization of Biochar Empty Fruit Bunches OPEFB at Various Temperatures and Burning Time

Marhani[1,2], Asmiaty Sahur[3], Sartika Laban[4], Yunus Musa[3*]

[1]Doctoral Program of Agriculture Science Graduate School, Hasanuddin University. Jl. Perintis Kemerdekaan Km. 10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia
[2]Department of Agrotechnology, Faculty of Agriculture, Tadulako University, Palu Jl.Soekarno Hatta Km 9, Tondo, Palu 94118, South Center, Indonesia.
[3]Department of Agrotechnology, Faculty of Agriculture, Hasanuddin University. Jl. Perintis Kemerdekaan Km. 10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia.
[4]Department of Soil Science, Faculty of Agriculture, Hasanuddin University. Jl. Perintis Kemerdekaan Km. 10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia.

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ABSTRACT

Oil palm waste (OPW), comprising mainly of empty fruit bunch, mesocarp fiber, frond, trunk, and palm kernel shell generated from the palm oil industry, was collected, characterized, and then pyrolyzed to evaluate their potential to be converted into biochar. Oil Palm Empty Fruit Bunches (OPEFB) are a source of organic material with abundant nutrients and are highly potentially useful as biochar. This article provides experimental data for the production of biochar at a temperature range of 100 to 300 °C at time of 4 to 8 hours. The chemical components examined are pH, CEC, C-Organic, N-total, C/N, K dd, P, Ca, Mg, and Na, using Fourier Transform Infrared Spectroscopy (FTIR). The results showed that organic C, nitrogen, and pH were highest at 200–300 °C and had a burning time of 8 hours. Furthermore, the highest concentrations of P, Ca, and Mg were recorded at 200–300°C after 5 hours, Kdd at 100–200 °C after 5 hours, and Na and CEC at 200–300 °C after 4 hours. The transmittance intensity produced by the spectrum of hydroxyl (O-H) vibrations, carbonyl stretching (C=O), alkanes (-CH), and aromatics (C=C) decreased with increasing time, while stretching alcohol (C-O) vibrations increased with time. Our results demonstrate that OPEB is a biowaste that shows exceptional promise to be transformed into high-grade biochar rather than simply disposed of by landfilling or burning.

* Corresponding author
E-mail: yunusmusa@yahoo.com (Yunus Musa)

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1 Introduction

Oil palm is one of the growing plantation commodities and has an important role in the Indonesian current economy. According to the data BPS d (2018), in 2017 the total area of oil palm plantations in Indonesia has reached 12.30 million ha with an average productivity of 2.80 tons of CPO (crude palm oil) and 0.56 tons of PKO (palm kernel oil)/ ha/year. Currently, palm oil mill residues, especially empty oil palm fruit bunches, have been produced massively.

OPEFB is a source of organic matter rich in N, P, K, and Mg nutrients. The number of empty fruit bunches is estimated at 23% of the processed fresh fruit bunches. Oil palm from one tonne of empty fruit bunches contains 1.5% N, 0.5% P, 7.3% K, and 0.9% Mg. Further, it contains 6.79% N-nutrients, 40.2-45.3% C-macronutrients, 2.4-2.7% K_2O, 0.8-1.2% N, 0.05-2.6% P_2O_5, 0.4-0.5% MgO, and C/N 45–70, microelements, such as 10 ppm B, 23 ppm Cu, and 51 ppm Zn (Razali and Kamarulzaman 2020). The chemical properties of biochar, such as nutrients, have an important role in soil fertility

Furthermore, EPEFB contains 45.9% cellulose, 46.5% hemicellulose, and 22.8% lignin (Qiao et al. 2019; Ferreira et al. 2020). Oil palm shells contain 29.4% lignin, 27.7% hemicellulose, 26.6% cellulose, 8.0% water, 0.6% ash, and 4.2% extractive components, all included in hydrocarbons (Gupta et al. 2018). The lignin and cellulose content in each raw material affects the biochar formation.

Biochar provides opportunities to store carbon (C) in soil over much longer periods compared to unpyrolyzed biomass Application of biochar affects several soil properties including electrical conductivity (EC), pH, cation exchange capacity (CEC), nutrient levels, porosity, bulk density, and microbial community structures (Shaaban et al. 2018)

Biochar is a product of pyrolysis of biomass in the absence of oxygen and has a high potential to sequester carbon into more stable soil organic carbon (OC). Despite a large number of studies on biochar and soil properties, few studies have investigated the effects of biochar in contrasting soils (Ghorbani et al. 2019)

OPEFB is the best material for biochar because of its high calorific value (Bi et al. 2021). Results of pyrolysis products dependent on temperature, time, pressure, and composition of raw materials produce differences in physical form and structure. These differences significantly affect biomass carbonization, producing biochar and nutrient content (Qiao et al. 2019). The pyrolysis process at 100 - 170 °C releases water content due to evaporation The effect of biomass addition on pyrolysis characteristics and gas emission of coal gangue by multi-component (Ferreira et al. 2020). The temperature range of 170 - 270 °C causes exothermic reactions and decreases the evaporation of CO and CO_2 gases. The carbon sequestration potential (CSP) of zeolite is arguably zero while rubberwood sawdust and rapeseed meal biochars were produced at 300 °C which is too low to carbonize cellulose and lignin (Dominguez et al. 2020). The results showed that the biochars produced at different pyrolysis temperatures had different physical characteristics which influenced their reactivity to the gasifying agent during gasification. It was found that when the pyrolysis temperature was increased from 300 to 700 °C, the HHV of the biochars increased from 23.06 to 26.77 MJ/kg, and the surface area of the biochars increased from 0.0218 to 0.2720 m2/g, the biochar yield decreased from 54.83 % to 28.37 % (Selvarajoo and Oochit 2020). FTIR revealed the presence of functional groups such as -OH, -C = O, C = C, and –CH. These play important roles in the specific surface area of the biomass (Ighalo et al. 2020).

Pyrolysis using high temperatures produces biochar with a high surface area and aromatic carbon content. The temperature and duration of combustion determine the quality of biochar. Therefore, it is necessary to know the optimal temperature and burning time. This study aimed to examine the physicochemical characteristics of OPEFB biochar. Also, it aimed to determine the optimum temperature and burning time for establishing the OPEFB biochar quality. This report is part of research on increasing the productivity of lowland rice using methanotroph bacteria and TKSS Biochar in the Morowali Regency.

2 Materials and Methods

2.1 Production of Oil Palm Empty Fruit Bunch (OPEFB) Biochar

The raw material used in this study is Oil Palm Empty Fruit Bunches (OPEFB), shown in Figure 1. It was obtained from community plantations in Witaponda District Ungkaya Village, Morowali Regency, Central Sulawesi Province. OPEFB is burned using tools and procedures easily adopted by farmers. It was dried in the sun for three days and burned at temperatures around 100–200 °C and 200–300 °C (Sukmawati et al. 2020) using a closed drum without oxygen (Figure 2). Therefore, it did not undergo a complete oxidation process that removed much carbon. After forming biochar, it was doused with water and dried, at which point the temperature was adjusted using a thermocouple. The temperatures were 100–200 °C (T1) and 200–300 °C (T2), and the burn times were 4 hours (W1), 5 hours (W2), 6 hours (W3), 7 hours (W4), and 8 hours (W5). Therefore, it obtained 10 treatment combinations of T1W1, T1W2, T1W3, T1W4, T1W5, T2W1, T2W2, T2W3, and T2W4.

Figure 1 Oil Palm Empty Fruit Bunches

Figure 2 Drum and Temperature Measuring Equipment for Making OPEFB Biochar

2.2 Chemical component analysis of OPEFB biochar

The analysis of biochar samples were carried out under the laboratory conditions and the recorded parameters were pH, H_2O, KCl (Glass Electrode), C-organic (Walkley-Balck), total N (Kjeldahl), total P, and K (25% HCl), available P (Bray or Olsen); exchangeable bases (Ca, Mg, Ca, and Na) (extraction of NH4OAc pH 7,0), exchangeable Al (extraction of 1 N KCl); saturation of Al (ratio Σ exchangeable Al/KPKNH4OAc), KPK extraction of NH4OAc-pH 7), KBNH4OAc (pH 7); Al, Fe, and Si free oxide amorphous and organic associations (extraction of Citrate-Bicarbonate- Dithionite, Ammonium Oxalic, Sodium Pyrophosphate) The digestion method referred to the method for soil samples (Pierzynski 2009)

2.3 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The chemical functional groups of biochar were examined using FTIR analysis. The presence of certain chemical functional groups helps understand the mechanism of the biochar adsorption process. Before the analysis, the sample was ground to a powder and oven-dried overnight to remove residual moisture. It was then mixed with potassium bromide (KBr) in a ratio of 1 part sample to 200 parts KBr. The sample mixtures were analyzed using an FTIR analyzer (Nicolet 5700, FTIR, Thermo Fisher Scientific, Waltham, MA, USA).

The interpretation of the FTIR spectrum refers to hydroxyl acids OH (3000–3690 cm^{-1}), CH alkanes (2927–2856 cm^{-1}, 1446–1370 cm^{-1}), aromatic rings C = C (1560–1600 cm^{-1}), carboxylic acid C = O (1560–1600 cm^{-1}), C-OH (1033 cm^{-1}) (Tippayawong et al. 2018). FTIR spectroscopy was performed on a Prestige-21 FT-IR (Shimadzu Corp) IR spectrometer, equipped with a bright ceramic light source, KBr beamsplitter, and doped L-alanine tri-glycine sulfate (DLATGS). The sample measurements were collected in the range of 4000-600 cm^{-1} and 16 additional scans. All FTIR spectra are in the transmittance unit. The magnetic study of the samples was conducted using a vibrating magnetometer (Oxford Instruments, VSM 1.2 H).

3 Results and Discussion

3.1 Analysis of the Nutrient Content of Biochar in Oil Palm Empty Fruit Bunches

The main biomass components, including hemicellulose, cellulose, and lignin, were degraded during combustion. Each biomass directly influences the yield and characteristics of the resulting

Figure 3 Oil Palm Empty Fruit Bunch Biochar

OPEFB product, containing 10.45% carbon black (Figure 3). The product was analyzed for biochar content and the results are in Tables 1 and 2.

The results of chemical components produced by each biochar are presented in Table 1 and revealed that the organic C content for all treatments ranged between 24.01%-28.12%. The highest organic C content (28.12%) was reported at 200-300 ^{o}C, with a burning time of 8 hours, while it was reported 24.01% at 100-200 ^{o}C for 5 hours. Further, the results of the C-organic content can be concluded very high because it exceeds five percent.

Further, the C/N ratio analysis for each treatment was carried out and it was classified as very high and it exceeds more than 20%. A temperature of 100-200 ℃ with a burning time of 7 hours gives a very high C/N ratio of 163.41%, while the lowest C/N ratio was produced at 200-300 ℃ for 8 hours (13.13%). These results are consistent with previous studies, where the C/N of oil palm shell biochar was 31.6%, while it was reported 57.37% for the empty fruit bunches (Sung 2016). The high C/N value is due to the loss of nitrogen during pyrolysis (Qiao et al. 2019). The P content analysis results for each treatment were generally classified as very low (less than 11%). The highest P element was produced at 200-300 ℃ for 5 hours (0.52%), while the lowest was produced at 100-200 ℃ for 7 hours (0.04%). The results obtained in this study revealed that in general biochar properties are related to the influence of low pyrolysis temperature (≤500 C) that can be a source of nutrients (Zhang et al. 2017).

The result presented in Table 2 revealed that the highest concentration of micronutrient Ca, Mg, and Na (32.25, 16.35, 41.32 cmol/kg respectively) in OPEFB biochar was reported at 200-300 ℃ with a burning time of 5 hours, while the lowest concentration (16.21, 10.23, 21.85 cmol/kg respectively) was produced at 100-200 ℃ for 5 hours of burning time. Further, the pH of each biochar sample was tested and it was reported in the

Table 1 Nutrient Analysis Results on Organic matters in Oil Palm Empty Fruit Bunch Biochar

No.	Treatment	Nutrient Analysis				
		C Organic (%)	N (%)	C/N (%)	P2O5 (%)	K (cmol / kg)
1	T1W1	24.72	1.08	22.79	0.25	18.25
2	T1W2	24.01	0.61	39.20	0.32	32.25
3	T1W3	26.82	0.70	38.32	0.22	14.75
4	T1W4	26.15	0.16	163.41	0.04	15.26
5	T1W5	26.92	0.19	139.66	0.23	10.24
6	T2W1	27.38	0.38	72.77	0.46	12.36
7	T2W2	24.42	1.01	24.15	0.52	14.25
8	T2W3	27.20	0.28	98.21	0.35	13.85
9	T2W4	26.79	0.54	49.36	0.41	16.21
10	T2W5	28.12	2.14	13.13	0.39	14.02

Table 2 Nutrient Analysis Results of Oil Palm Empty Fruit Bunch Biochar

No.	Treatment	Analysis				
		Ca (cmol / kg)	Mg (cmol / kg)	Na (cmol / kg)	pH	CEC (cmol / kg)
1	T1W1	18.35	10.22	28.25	7.30	29.39
2	T1W2	16.21	13.25	35.00	7.69	20.58
3	T1W3	28.62	14.21	21.85	8.53	18.19
4	T1W4	26.32	15.32	31.14	8.51	16.68
5	T1W5	24.36	16.32	25.34	7.71	10.20
6	T2W1	22.41	10.34	41.32	7.77	23.05
7	T2W2	32.25	16.35	32.25	7.61	12.28
8	T2W3	27.15	15.21	32.27	8.20	11.48
9	T2W4	23.36	12.36	28.63	7.96	10.82
10	T2W5	21.14	10.25	32.25	8.56	15.35

moderately alkaline category. The highest pH value of 8.56 was reported from the OPEFB biochar samples burned at 100-200 °C temperature for 6 hours, while the lowest pH value 7.30 was reported from the OPEFB biochar sample burned at 100-200 °C for 4 hours. The results are consistent with, where oil palm shells and corn cobs had a pH value of 7.3, while empty fruit bunches had 7.2 (Sukmawati et al. 2020).

The analysis of CEC values showed various results in the moderate category of 10–29 cmol/kg (Table 2). The highest CEC value (29.39 cmol/kg) was reported at 100–200 °C for 4 hours, while the lowest (10.20 cmol/kg) was reported for 8 hours at 100–200 °C. In contrast to this, Mukherjee & Zimmerman (2013) reported that the CEC of biochar from oil palm empty fruit bunches was reported 21.5 cmol/kg while the biochar produced by pyrolysis at 300–400 °C had a high CEC value of 50.52–56.88 cmol/kg. Further, CEC values for oil palm shells, empty fruit bunches, and corn cobs were reported 50.52 cmol/kg, 52.36 cmol/kg, and 56.84 cmol/kg respectively.

The highest organic C and nitrogen content (28.12%) was reported from the OPEFB biochar samples burned at 200–300 °C after 8 hours of burning, while the lowest was at 100–200 °C for 5 hours of burning. Pyrolysis enhances the thermochemical process, forming cellulose and lignin from long-to-short carbon chain structures. These results are contradictory to the findings of Sukmawati et al. (2020) who reported the lowest value of 59.85% at 300–400 °C burning time for 4 hours. Good biochar is produced at high temperatures without oxygen. High temperatures accelerate the decomposition process of organic matter biomass components into lignin, cellulose, and hemicellulose. Moreover, a higher rate of decomposition of organic matter increases the formation of nutrients. The high lignin, cellulose, and hemicellulose content in OPEFB increase the organic C content. Furthermore, the high organic C content increases the amount of organic matter in the soil, improving its physical, chemical, and biological properties. The amount of lignin and cellulose in each raw material affects the process of biochar formation, and high lignin content makes more carbon.

The high value of C/N at 100–200 °C with a burning time of 7 hours is caused by the reduced N in nitrogenous biomass. The low organic C content reduces the C/N ratio at 200–300 °C for 8 hours due to organic carbon compounds' decomposition and nitrogen compounds' changes during combustion. The C/N ratio is reduced through combustion by converting organic C into CO_2 and nitrogen loss as NH_3. A higher C/N ratio increases the time it takes to overhaul the organic matter.

The analysis regarding the highest P_2O_5 content showed that it was lowest at 200-300°C for 5 hours and at 100-200°C for 7 hours. This indicates that phosphate decomposes and stabilizes at high temperatures. Phosphorus is difficult to decompose and reacts slowly at low temperatures. Similarly, the analysis showed the highest K content at 100-200 °C with a burning time of 5 hours and the lowest at 100-200 °C and 200-300 °C with a burning time of 8 hours. A longer burning time causes more potassium content to be lost because its volatility is different from that of hard-to-decompose phosphorus. Table 2 shows that low organic C contributed to the high potassium content. This is in line with what showed that increasing pyrolysis time increases the loss of volatile components and fixed carbon value.

The analysis showed that the highest content of calcium, magnesium, and sodium was at 200–300 °C with a burning time of 4–5 hours, while the lowest was at 100–200 °C for 4, 5, and 6 hours. The results showed that higher temperatures increase the produced Ca, Mg, and Na content. This might be due to the increased pH and cations content of biochar at 200–300°C. The results indicate that the pH of each biochar was alkaline, with a moderate base category ranging from 7.30 to 8.50. The pyrolysis process decomposed three biomasses of lignin, cellulose, and hemicellulose content. Furthermore, it produces volatile substances that regulate the pH of biochar, forming a carboxyl functional group on the biochar surface. Biochar is generally alkaline with a pH of between 7.1 to 10.5 due to the presence of carboxyl groups, oxygen, and carbonate (Sukmawati et al. 2020). These components are produced by decomposing cellulose and hemicellulose into organic and phenolic acids during pyrolysis at 200-300 °C (Spokas et al. 2012)

The highest CEC analysis results were reported at 200-300 °C with a burning time of 1 hour and the lowest at 100-200 °C for 8 hours. The cation exchange capacity of biochar shows how well the nutrients bound to biochar are available for plant uptake and prevent leaching from ground and surface water. The higher the CEC represents the better quality of the biochar and this could increase soil fertility. This is because biochar holds and stores nutrients in the soil colloid, which stops them from being washed away by ground or surface water.

3.2 FTIR Analysis of Oil Palm Empty Fruit Bunch Biochar

Results presented in Table 3 show the FTIR spectrum of oil palm empty fruit bunches biochar produced by pyrolysis at 100–200 °C (T1) and 200–300 °C (T2). The burning time was 4 hours (W1), 5 hours (W2), 6 hours (W3), 7 hours (W4), and 8 hours (W5). The results of the study revealed that the ten analyses have similar FTIR spectra based on the resulting functional groups. Functional group analysis also has similarities with research that has been carried out by Sukmawati et al. (2020) and Rosli et al. (2016). Figure 4 shows the FTIR spectrum pattern of biochar absorption from oil palm empty fruit bunches. Treatments T1 and T2 show the absorption spectrum of stretching hydroxyl vibrations (OH) appearing on all biochar surfaces at wavenumbers between 3415.93 and 3446.79 cm^{-1}. The spectrum shows a shift in wavenumber with changes in time and temperature.

Table 3 FTIR Spectrum of Oil Palm Empty Fruit Bunch Biochar

Biochar	Hydroxyl	Alkanes		Carbonyl	Aromatic	Alcohol
	(O-H)	(-CH)	(C-H)	(C=O)	(C=C)	(C-O)
T1W1	3414.00	2922.16	1427.32	1693.50	1600.92	1028.06
T1W2	3415.93	2922.16	1433.11	1701.22	1591.27	1029.99
T1W3	3444.87	2922.16	1427.32	1693.50	1602.85	1026.13
T1W4	3415.93	2922.16	1433.11	1701.22	1595.13	1024.20
T1W5	3415.93	2922.16	1417.68	1693.50	1598.99	1028.06
T2W1	3417.86	2924.09	1446.61	1689.64	1606.70	1029.99
T2W2	3444.87	2922.16	1427.32	1697.36	1591.27	1018.41
T2W3	3417.86	2922.16	1419.61	1689.64	1598.99	1028.06
T2W4	3446.79	2922.16	1427.32	1693.50	1597.06	1026.13
T2W5	3421.72	2924.09	1425.40	1716.68	1610.56	1045.42

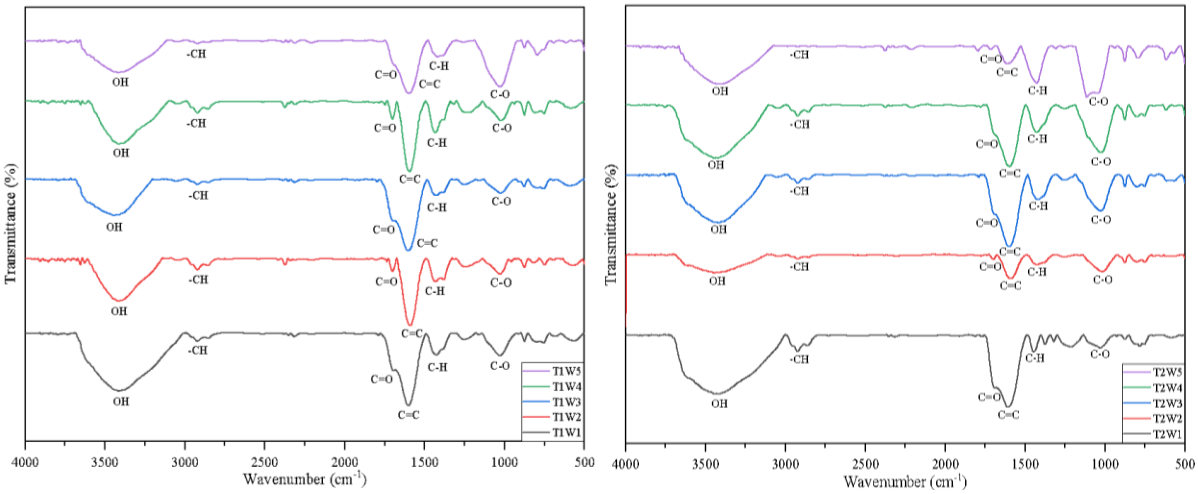

Figure 4 FTIR Spectrum of OPEFB Biochar at variations in temperature and burning time

Figures 4a and 4b show that the transmittance intensity produced by the hydroxyl (O-H) stretching vibration decreases with time. This indicates that the percentage of IR radiation transmitted decreases with combustion time. The stretching vibrational spectrum of alkanes (-CH) in the T1 and T2 treatments appears in the wavenumber of 2922.16–2924.09 cm^{-1}. The wavenumber is strengthened by bending the alkane (CH) vibration spectrum at 1417.68–1446.31 cm^{-1}. The stretching vibrational spectrum of alkanes (-CH) does not show a significant shift in wavenumber. The bending vibrational spectrum of alkanes (C-H) shows a shift in wavenumber with changes in temperature and time. Moreover, it shows that the transmittance intensity produced by the stretching vibrational spectrum of alkanes (-CH) decreases with time. In contrast, the transmittance intensity produced by the bending vibration spectrum of alkanes (C-H) increases with time. The spectrum of carbonyl (C=O) stretching vibrations in T1 and T2 treatments appeared at wavenumbers of 1689.64–1716.68 cm^{-1}, indicating a shift with changes in time and temperature.

Figures 4a and 4b also show that the transmittance intensity produced by the carbonyl (C=O) stretching vibration spectrum decreases with time. The spectrum of aromatic stretching vibrations (C = C) in T1 and T2 treatments appeared at wavenumbers of 1591.27–1610.56 cm^{-1}. This indicates a shift in wavenumbers with changes in time and temperature. Also, it showed that the transmittance intensity produced by the stretching aromatic vibration spectrum (C=C) decreased with time. The spectrum of alcohol (OH) stretching vibrations in T1 and T2 treatments appeared at a wavenumber of 1018.41–1045.42 cm^{-1}. This shows a shift in wavenumber with changes in time and temperature. The figures show that the transmittance intensity generated by the stretching alcohol (OH) vibration spectrum increases with time.

Conclusions

The choice of temperature and combustion time depends on the desired nutrients, each element required a specific temperature and time to produce the highest nutrients. The characterization results showed that organic C, nitrogen, and pH gave the highest values at a temperature of 200-300 ºC and a burning time of 8 hours. The highest content of P, Ca, and Mg is at a temperature of 200-300 ºC with a burning time of 5 hours, K dd content at a temperature of 100-200 ºC for 5 hours, and the content of Na and CEC at a temperature of 200-300 ºC with a burning time of 4 hours.

Conflicts of interest

There are no conflicts to declare.

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Evaluation of Antioxidant and Antibacterial Activities of Bubble Belly Massage Oil and their Crude Ingredients

Jeyasharlina Ganesan, Sit Nam Weng, Sangeetha Arullappan*

Department of Allied Health Sciences, Faculty of Science, Universiti Tunku Abdul Rahman, Perak Campus, Bandar Barat, Jalan Universiti, 31900 Kampar, Perak, Malaysia.

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Bubble belly massage oil

ABSTRACT

Bubble Belly massage oil is popular among Malaysians since its commercialization in 2018. The massage oil contains lemon oil, vitamin E oil, aloe vera oil, eucalyptus oil, ginger oil, black pepper, fenugreek, *Caesalpinia sappan*, *Usnea barbata*, and *Helicteres isora*. The massage oil is believed to reduce weight, cellulite, menstrual pain, body ache, and scar appearances. The study evaluated oil and its crude ingredients for antioxidant activity using DPPH and ABTS assays, antibacterial activity was evaluated by using disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. The crude ingredients soaked in the massage oil were dried and underwent aqueous extraction. Phenols, tannins, and quinones were detected qualitatively in the samples. Highest DPPH and ABTS radical scavenging of 73.1% at 0.78% (v/v), and 98.2% at 12.5% (v/v), respectively were shown by the oil. Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Bacillus cereus*, *Klebsiella pneumonia*, and *Enterococcus faecalis* were susceptible to the oil at 100% (v/v) with a zone of inhibition of 15.0 mm, 14.0 mm, 12.0 mm, 9.0 mm, and 14.0 mm, respectively. All the tested bacteria were resistant to the crude ingredients. The MIC values against *B. cereus*, MRSA, *K. pneumonia*, and *E. coli* treated with oil were in the range of 0.39 to 0.78% (v/v). Both the crude ingredients and oil showed MBC values of 12.5 mg/mL and 0.39% against *B. cereus* and MRSA, respectively. In a nutshell, the massage oil showed significant inhibitory and radical scavenging activities and thus is potential as an antibacterial and antioxidant agent.

* Corresponding author

E-mail: sangeetha@utar.edu.my (Dr. Sangeetha Arullappan)

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1 Introduction

Natural products have become a key focus of inquiry for therapeutic agents and drug discovery research. Natural therapeutic compounds are derived from numerous sources such as microbes, minerals, plants, and animals. However, plants have emerged as the most prominent source of natural medicine attributed to their structural and chemical diversity of secondary metabolites. Secondary metabolites such as phenolic, alkaloids, and terpenoids contribute significantly to a plant's survival and ability to thrive in a harsh environment (Bernhoft 2010). Many oil-based drugs isolated from plants possess antibacterial and anticancer properties. For example, camphene isolated from *Piper nigrum*, carvacrol from the essential oil of oregano and thyme, and vapor of *Litsea cubeba* seed oil were reported to inhibit bacteria (Blowman et al. 2018).

Bubble belly massage oil is a commercialized product introduced in Malaysia in early 2018 and since then, this product has become popular among women and men. Traditionally, massage oil is applied to the skin to reduce belly fat, improve circulation, reduce constipation, act as a moisturizer, lighten black marks, and tighten the skin. The massage oil is also believed to reduce cellulite, menstrual pain, body and joint pains, and scars (Natalie 2017; Debra 2019; Deepali 2019; Debra 2020; Go Outdoor 2021). The oil contains various herbs namely lemon oil, vitamin E oil, aloe vera oil, eucalyptus oil, ginger oil, black pepper, fenugreek, *C. sappan*, *U. barbata*, and *H. isora*. The oil can be applied daily to the skin and according to end users, the oil is effective in reducing pain and scar appearances (New Directions Aromatic Inc 2018; Go Outdoor 2021). The herbs found in the massage oil are reported to exhibit medicinal values such as antibacterial, anti-inflammatory, antiviral, antidiabetic activities, treating skin diseases and stomach aches (Nirmal and Anil 2014; Kalunta 2017; Takooree et al. 2019; Akhlaghi and Najafpou 2021; Pandey et al. 2021; Hwang et al. 2021; Sepahvand et al. 2021; Luo et al. 2021; Almatrodi et al. 2021). The oil can reduce anxiety and depression symptoms, relieve morning sickness, prevent acne breakouts, inhibit fungal growth, anti-aging, and remove stretch marks (Jugreet et al. 2020).

World Health Organization (2021) stated that infectious diseases are caused by pathogenic microorganisms such as bacteria, viruses, parasites, or fungi and can be spread directly or indirectly from one person to another. Infectious diseases caused by multidrug-resistant (MDR) bacteria have become a public health concern all over the world. These bacteria drastically reduced the efficacy of antibiotics, consequently, increasing the therapeutic failure and mortality rate. It was estimated that every year, around 25,000 patients die due to infections with MDR bacteria (Fankam et al. 2017). Scientists are searching for new antimicrobial substances especially from plant extracts and natural products due to their potential source of novel antibiotic prototypes (Dzotam et al. 2016). The essential oils of cinnamon, oregano, and thyme individually showed strong antimicrobial activities with MIC ≥ 0.125 µL/mL and MBC ≥ 0.25 µL/mL (Mith et al. 2014).

With the rise in multidrug-resistant bacteria and mortality rate, the present study was intended to reveal the potential of massage oil and crude extract as antibacterial agents. To date, no study was reported on the biological properties of the bubble belly massage oil conducted *in vitro* and *in vivo*, hence this study aimed to evaluate the effect of massage oil and its crude ingredients for antioxidant and antibacterial activities.

2 Materials and Methods

2.1 Aqueous extraction and lyophilization

Bubble belly massage oil was purchased from a few Indian shops located at Perak and Seremban, Malaysia. The crude ingredients of the massage oil namely black pepper, fenugreek, *C. sappan*, *U. barbata*, and *H. isora* were dried in an oven at 50°C. The completely dried ingredients were powdered and boiled using deionized water for 20 minutes thrice to obtain a higher aqueous extract yield. The filtrates were kept at -20°C overnight and lyophilized using a freeze-dryer. A stock concentration of 100 mg/mL of the crude aqueous extract was prepared using either distilled water or dimethyl sulfoxide (DMSO) based on the bioassays.

2.2 Phytochemical analysis

Both the crude aqueous extract (100 mg/mL, w/v) and oil (100%, v/v) were tested qualitatively for the presence of phenols, saponins tannins, quinones, flavonoids, and coumarins as described by Anjali and Sheetal (2013) with minor modifications, especially on the volume of the samples. The formation of color, froth, or layers were observed and compared with respective standards for the detection of classes of secondary metabolites.

2.3 Antioxidant assays

2.3.1 DPPH assay

The crude aqueous extract and oil were evaluated for antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay based on Chen et al. (2012) with minor modifications in terms of the volume of the samples. The samples were serially diluted (two-fold) at various concentrations ranging from 0.02 to 100 mg/mL (mg/mL or % v/v) in a 96-well, along with standards (ascorbic acid and α-tocopherol), and negative control (DMSO). DPPH reagent (0.2 mM) was added to each well and incubated in dark for 20 minutes at room temperature. The absorbance was measured using a microplate reader at 517 nm. The percentage of radical scavenging activity was calculated based on equation [1] (Shekhar

and Anju 2014). The values are expressed as the means of triplicate analyses.

DPPH radical scavenging activity = $[(A_{NC} - A_S) / (A_{NC})] \times 100\%$ [1]

Where A_{NC} is absorbance of negative control and A_S is absorbance of samples.

2.3.2 ABTS assay

The crude aqueous extract and oil were evaluated for 2,2′ -azino-bis-3-ethylbenzthiazoline-6 sulphonic acid (ABTS) radical scavenging activity based on Nilima and Hande (2011) with minor modifications. The crude aqueous extract (100 mg/mL) and oil (100%, v/v) were serially diluted at various concentrations in a 96-well along with standards (quercetin, α-tocopherol), and negative control (DMSO). ABTS reagent (7 mM) was added to each well and incubated in dark for 10 minutes at room temperature. The absorbance was measured using a microplate reader at 734 nm and the percentage of ABTS scavenging activity was calculated based on equation [2] (Nilima and Hande 2011). The values are expressed as the means of triplicate analyses.

ABTS radical scavenging activity = $[(A_{NC-} A_S) / (A_{NC})] \times 100\%$ [2]

Where A_{NC} is absorbance of negative control and A_S is absorbance of samples.

2.4 Antibacterial assays

Both the crude aqueous extract and oil were evaluated for antibacterial activities using disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays on *B. cereus* (ATCC11778), *E. faecalis* (ATCC29212), *E. coli* (ATCC35218), *K. pneumonia* (ATCC13883), and methicillin-resistant *S. aureus* (MRSA)(ATCC33591). The bacterial concentration of 1×10^6 CFU/mL was used in the bioassays. These bacteria were adjusted to a 0.5 McFarland standard by measuring absorbance at 625 nm. The absorbance of 0.08 to 0.1 is equivalent to 0.5 McFarland standard proportional to 10^8 CFU/mL (Carvalho et al. 2021).

2.4.1 Disc diffusion assay

Gram-positive and Gram-negative bacteria at a concentration of 1×10^6 CFU/mL were streaked onto MH agar plates in disc diffusion assay. Approximately 20 µL of various concentrations of massage oil (12.5 to 100.0%), crude aqueous extract (0.02 to 100 mg/mL), negative control (10% DMSO), and positive control (streptomycin sulfate) were impregnated onto a sterile, blank disc of 6 mm in diameter. The air-dried discs were placed on agar and incubated at 37°C in an incubator for 18 to 24 hours. The zone of inhibition was measured and recorded (Horvath et al. 2016). The assay was repeated thrice and the antibacterial activity was expressed as the mean diameter zone of inhibition (mm).

2.4.2 MIC and MBC assays

The concentration of 1×10^6 CFU/mL was used in the MIC assay using broth dilution method as described by Eloff (1998) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Crude aqueous extract (0.02 to 100 mg/mL), oil (12.5 to 100.0%), negative control (10% DMSO) and positive control (streptomycin sulfate) were two-fold diluted in a 96-well plate, followed by the addition of adjusted bacteria suspension. The plate was incubated at 37°C for 18 to 24 hours. INT dye (0.4 mg/mL) was added and incubated for 20 minutes, followed by an absorbance reading at 600 nm. Based on the color observation, pink color indicates the viable cells and yellow indicates the non-viable cells. The percentage of cell viability was calculated based on equation [3] (Eloff 1998).

Percentage of cell viability = $[A_S / A_{NC}] \times 100$ [3]

Where A_{NC} is absorbance of the negative control and A_S is absorbance of the samples

Sample well with no color changes and lower absorbance were subjected to MBC by streaking on MH agar aseptically. The agar was incubated at 37°C for 18 to 24 hours and the number of colonies in the sample was compared with positive and negative controls. The lowest concentration that inhibited the most colony formation on agar was recorded as MBC (Eloff 1998).

2.5 Data analysis

Both antioxidant and antibacterial assays were repeated thrice and the data were reported as mean±standard deviation. EC_{50} values were determined using GraphPad prism (version 9.3.1). Levels of significance for the bioassays were compared between treatments and standards using a student t-test and $P < 0.05$ was considered statistically significant unless otherwise specified.

3 Results and Discussion

3.1 Phytochemical analysis

The presence of phenols, saponins, tannins, and quinones was detected from the crude aqueous extract, while only phenols, tannins, and quinones were detected in the oil. In addition, flavonoids and coumarins were not detected in both oil and crude aqueous extract (Table 1). Even though essential oils found in the bubble belly massage oil separately are rich with flavonoids and coumarins as reported previously (Mariappan et al. 2014; Sruthi

Table 1 Determination of classes of secondary metabolites in qualitative phytochemical analysis

Class of secondary metabolites	Crude extract (mg/mL)	Massage oil (%, v/v)
Phenols	+	+
Saponins	+	-
Tannins	+	+
Quinones	+	+
Flavonoids	-	-
Coumarins	-	-

+ Presence, - Absent

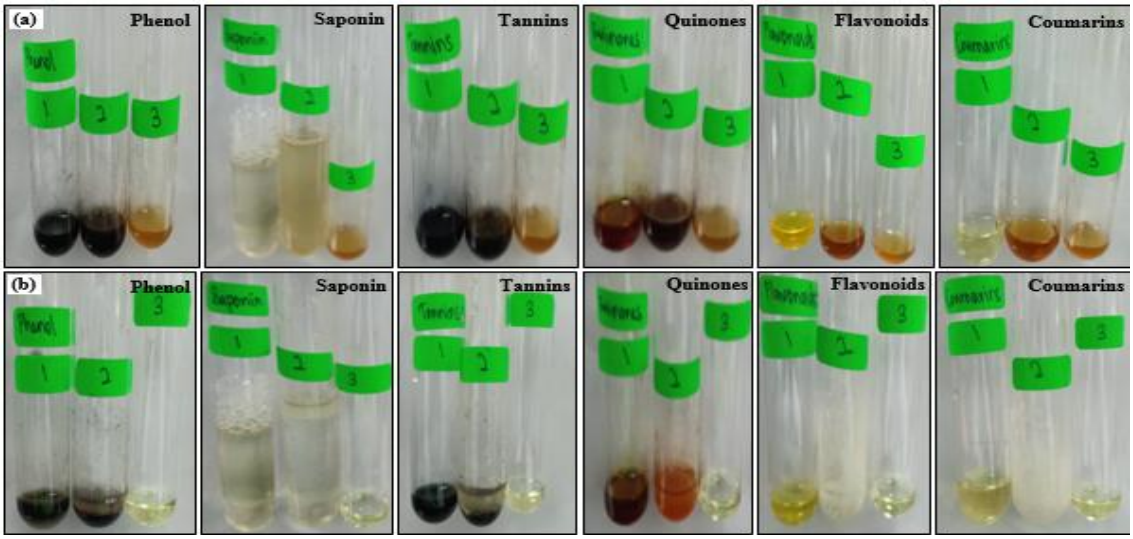

Figure 1 Qualitative observation of the classes of secondary metabolites as compared to standard in crude aqueous extract (a) and massage oil (b); (1) Standard, (2) Samples and test reagent, (3) Samples only.

and Zachariah 2017; Akhlaghi and Najafpou 2021; Manjunath and Mahurkar 2021), however, these two metabolites were not detected qualitatively in the study. The detected classes of secondary metabolites consist primarily of polar functional groups. Functional groups such as hydroxyl, carbonyl, and amines are found to enhance solubility, whilst retaining lipophilic character in both ionized and non-ionized forms by binding with its target through specific hydrogen bonding and/or formation of salt bridges (Dai and Mumper 2010; Nicholas and Graham 2019).

Flavonoids and coumarins were not detected in the study possibly due to the extraction method, which is aqueous extraction. The choice of solvent is critical in extraction because it will influence the quantity and bioactive compounds composition of the crude extract (Ncube et al. 2008). Factors to be considered when selecting an extraction solvent include polarity of the target compound of interest, low toxicity, low boiling point or high volatility for ease of removal, inability to cause structural or functional alteration to the extracted compound, and rapid diffusivity (Ncube et al. 2008; Abubakar and Haque 2020). The best solvent used to isolate flavonoids and coumarins is methanol, as water has lesser extraction efficiency. This result is in agreement with a study by Truong et al. (2019) which stated the highest levels of flavonoids were observed in methanolic extract due to higher solubility of these compounds in methanol than in the other solvents such as distilled water. In addition, quantitative analysis using liquid chromatography (LC) coupled with mass spectrometry (MS) can be used to identify the components, instead of qualitative determination.

Methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plants (Xu and Chang 2007). For example, methanol is more efficient in isolating polyphenols, while flavanols with aqueous acetone. This solvent can degrade cell membranes and simultaneously dissolves the secondary metabolites and stabilizes them (Metivar et al. 1980; Shi et al. 2005; Nirmal and Anil 2014). Water extraction is suitable for extracting heat-stable compounds and usually results in the isolation of more oil-soluble compounds (Azwanida 2015; Zhang et al. 2018). Figure 1 shows the presence and absence of the classes of secondary metabolites in both samples.

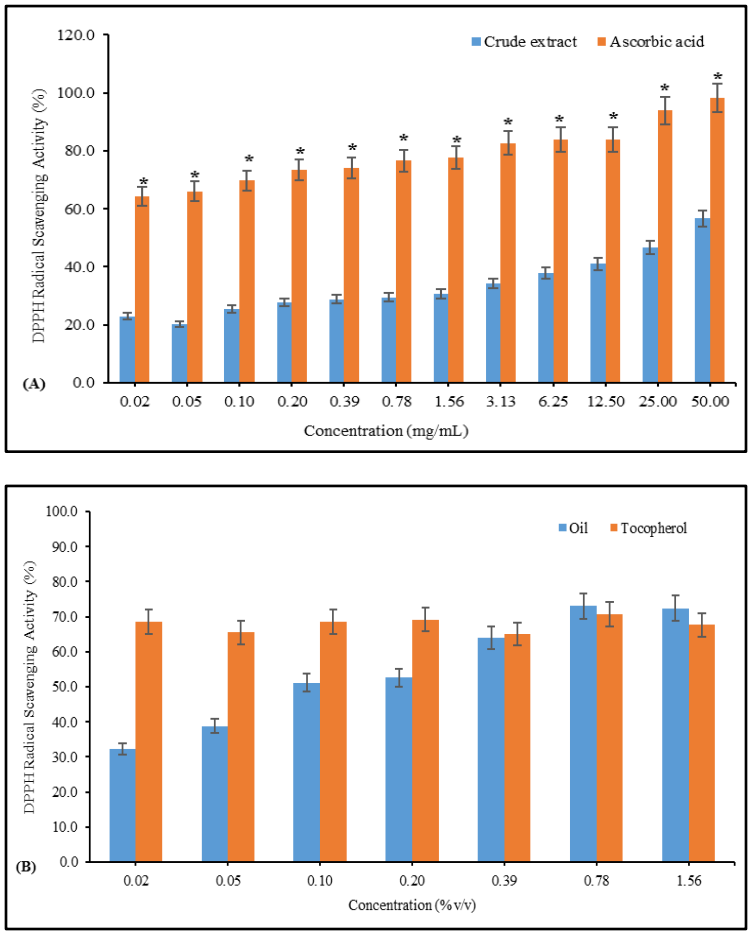

Figure 2 DPPH radical scavenging activity of crude aqueous extract (A) and massage oil (B) at various concentrations. The points represent means ± standard deviation (n=3). * Indicates P < 0.05, student t-test.

Table 2 Effective concentration at 50% (EC_{50}) showed by samples in both antioxidant assays.

Sample	EC_{50} values (mg/mL or %, v/v)	
	DPPH assay	ABTS assay
Crude extract	0.288 ± 0.030	0.221 ± 0.020
Massage oil	0.026 ± 0.019	0.117 ± 0.020
Ascorbic acid	0.003 ± 0.010	*not tested*
α-tocopherol	14.900 ± 0.080	40.620 ± 0.010
Quercetin	*not tested*	377.700 ± 0.010

3.2 Antioxidant activities

The antioxidant capabilities of the samples were determined using ABTS and DPPH radical scavenging activity based on their respective mechanism of action. DPPH radical forms a purple solution and will be reduced upon reacting with an antioxidant that can donate a hydrogen atom forming yellow colored diphenylpicrylhydrazine (Marrufo et al. 2013; Albayrak and Aksoy 2013). In comparison, ABTS will be oxidized to its radical cation ABTS+, which turns blue when reacted with potassium persulfate. The radical scavenging capacity of antioxidants is determined when the blue ABTS+ is converted back to the colorless ABTS (Ammar et al. 2012).

Both the crude aqueous extract and oil showed increasing trends in the DPPH and ABTS radical scavenging activities as the concentration increases from 0.02 to 1.56 mg/mL. The radical scavenging activity displayed a concentration-dependent manner as

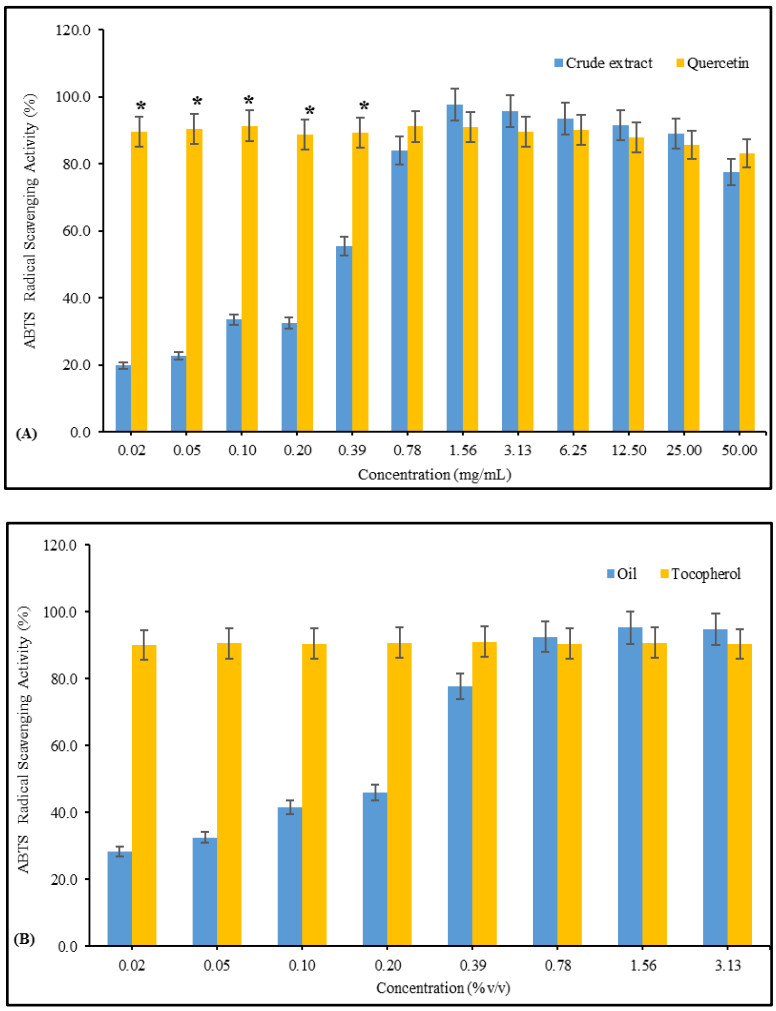

Figure 3 ABTS radical scavenging activity of crude aqueous extract (A) and massage oil (B) at various concentrations. The points represent means ± standard deviation (n=3). * Indicates P < 0.05, student t-test.

shown in Figures 2 and 3. However, it was observed that the oil exhibited higher radical scavenging activity in DPPH (73.1%) and ABTS (98.2%) as compared to crude aqueous extract. Ascorbic acid, α-tocopherol, and quercetin displayed higher radical capacity in the range of 75.0 to 99.0% in both DPPH and ABTS assays. DPPH radical scavenging activity of the crude aqueous extract and ascorbic acid were significantly different ($p<0.05$) based on the student t-test. In contrast, the radical scavenging activity of the oil was comparably good as α-tocopherol, a well-known antioxidant agent. The same trend was observed in the ABTS assay. The effectiveness of the oil and crude aqueous extract was determined using EC_{50} values. The lowest EC_{50} value of 0.026 ± 0.019% (v/v) and 0.117 ± 0.020% (v/v) was exhibited by oil in DPPH and ABTS assays, respectively as shown in Table 2.

The differences exerted by both assays could be attributed to the stereochemical structures of ABTS $^{+\cdot}$ and DPPH radicals, sample solubility, polarity, and the metal-chelating capacity of antioxidants (Shalaby and Shanab 2013). Generally, lemon oil, vitamin E oil, aloe vera oil, eucalyptus oil, and ginger oil separately were reported as good antioxidant agents (Zoran et al. 2022; Oussame et al. 2022; Chatarina et al. 2022). Previous studies on Eucalyptus essential oil showed 79.55 ± 0.82% (v/v) of DPPH radical scavenging activity (Arun et al. 2010), while lemon essential oil showed a reduction of lipid peroxidation levels and nitrile content, increased reduced glutathione levels, superoxide dismutase, catalase and glutathione peroxidase activities in mouse hippocampus model (Campêlo et al. 2011). In addition, oils isolated from a few *Eucalyptus* species had an antioxidant effect with an IC_{50} value of 4.21 ± 0.35 mg/mL for DPPH free radical scavenging due to the presence of the phenolic terpenoids, thymol, and carvacrol (Sapit et al. 2022). Hence, the massage oil showed promising antioxidant activity possibly due to the synergistic effect among the detected phenols, tannins, and quinones as a free scavenger to destroy radical molecules.

3.3 Antibacterial activities

The antibacterial properties of the crude aqueous extract and oil were performed using disc diffusion, MIC, and MBC assays. In the disc diffusion assay, it was noted that the crude aqueous extract did not inhibit all the test bacteria, while the oil inhibited all the bacteria at higher concentrations. Disc diffusion is a simple method that does not require special tools and the results can be easily interpreted as susceptible, intermediate, or resistant. The present study showed that the disc diffusion assay may not be a reliable tool for susceptibility testing due to some factors such as the absorption of secondary metabolites onto the disc and manual preparation of the disc which might introduce some errors. Since the crude aqueous extract was detected with many phytochemical constituents, perhaps these components were not able to penetrate completely onto the agar and leading to no inhibition. However, the broth microdilution method showed inhibition due to its nature as a quantitative measurement (Alizade et al. 2016).

Table 3 and Figure 4 show the diameter zone of inhibition observed in MRSA and *E. coli*, in which these bacteria displayed the highest zone of 15.0 mm and 14.0 mm, respectively at 100% (v/v). Treatment of streptomycin sulfate (25 µg/mL) to MRSA showed the largest inhibition zone of 26.0 mm indicating susceptibility. Reducing trends were observed at various concentrations of MRSA and *E. coli* indicating the death of the cells upon treatment with oil (Figure 5).

Table 3 Zone of inhibition of the respective samples and streptomycin sulfate against test microorganisms.

Sample	Diameter zone of inhibition (mm)				
	B.cereus	*E.faecalis*	*K.pneumonia*	*E.coli*	MRSA
Crude aqueous extract (mg/mL)					
12.5	-	-	-	-	-
25.0	-	-	-	-	-
50.0	-	-	-	-	-
100.0	-	-	-	-	-
Massage oil (%, v/v)					
12.5	-	-	-	-	-
25.0	-	-	-	7.0	7.0
50.0	10.0	11.0	7.0	11.0	10.0
100.0	12.0	14.0	9.0	14.0	15.0
Streptomycin sulfate (25 µg/mL)	19.0	14.0	19.0	15.0	26.0

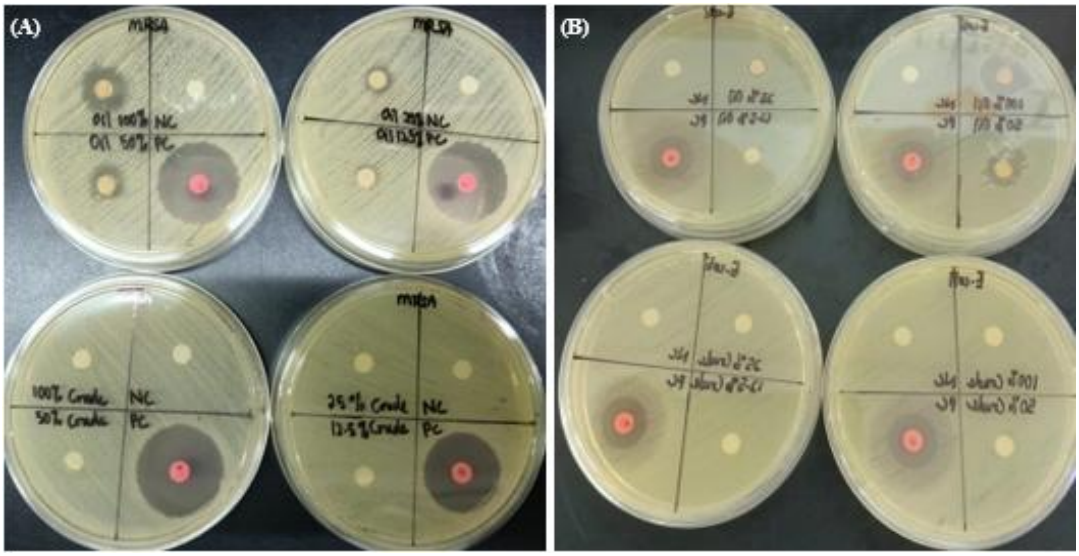

Figure 4 Diameter zone of inhibition observed using both crude aqueous extract and massage oil against (A) MRSA, (B) *E. coli* (NC = negative control, PC = positive control).

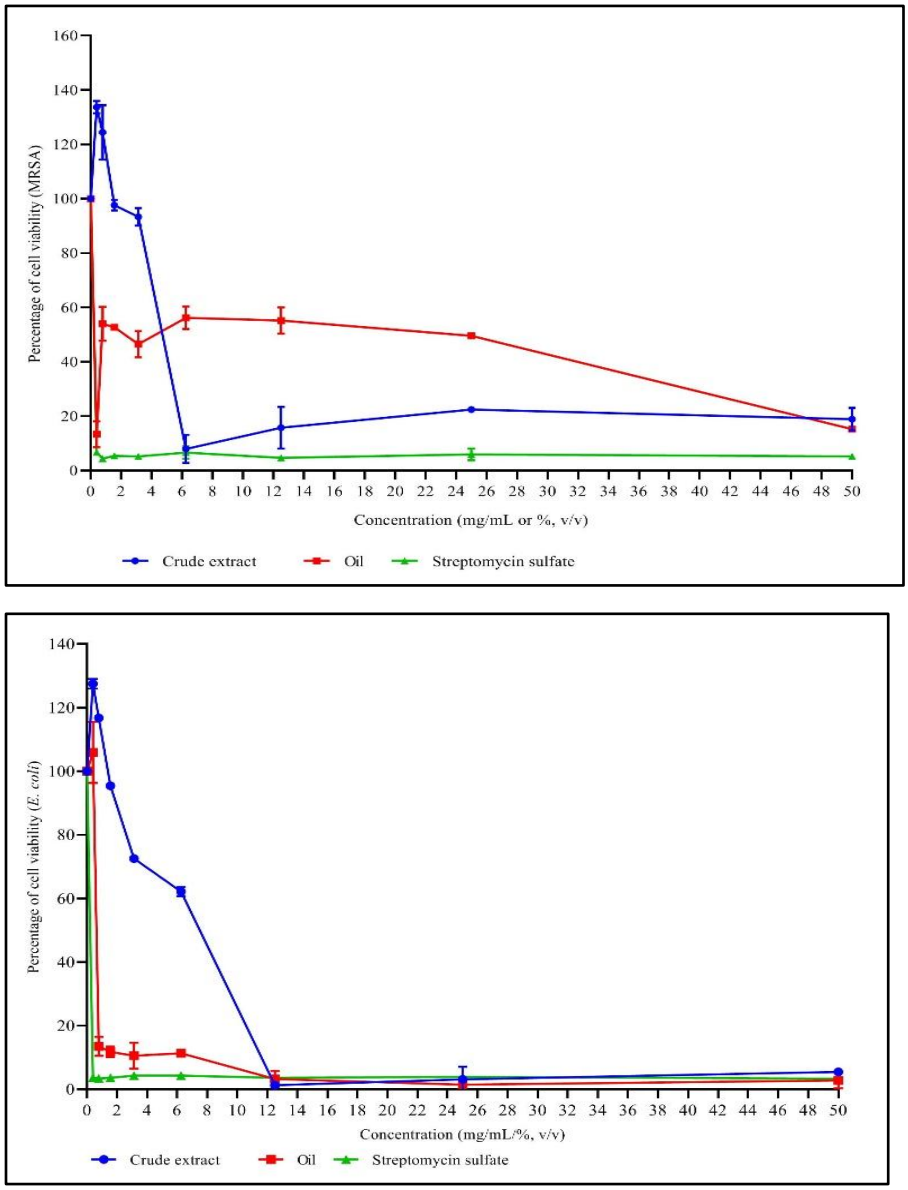

Figure 5 Percentage cell viability of MRSA and *E. coli* upon treatment with various concentrations of samples for 18 to 24 hours

Table 4 Minimum inhibition concentration values of the samples and streptomycin sulfate against test microorganisms.

Bacteria	Minimum inhibition concentration (MIC)		
	Crude aqueous extract (mg/mL)	Massage oil (%, v/v)	Streptomycin sulfate (mg/mL)
Gram-positive			
B. cereus	12.5	0.39	0.39
E. faecalis	50.0	1.56	0.39
MRSA	12.5	0.39	0.39
Gram-negative			
K. pneumonia	12.5	0.78	0.39
E. coli	12.5	0.78	0.39

Table 5 Minimum bactericidal concentration values of samples and streptomycin sulfate against test microorganisms.

Bacteria	Minimum bactericidal concentration (MBC)		
	Crude aqueous extract (mg/mL)	Massage oil (%, v/v)	Streptomycin sulfate (mg/mL)
Gram-positive			
B. cereus	12.5	0.39	0.39
E. faecalis	> 50.0	3.13	0.39
MRSA	12.5	0.39	0.39
Gram-negative			
K. pneumonia	25.0	0.78	0.39
E. coli	25.0	0.78	0.39

*MBC value > 50.0 mg/mL indicates possible activity using higher concentration of samples.

In MIC and MBC assays, both the crude aqueous extract and oil showed inhibition upon all the bacteria. However, once again oil displayed MIC and MBC values. MIC and MBC values of 0.39% (v/v) was observed on *B. cereus* and MRSA, respectively, and compared with streptomycin sulfate. Both Gram-positive bacteria were killed efficiently upon treatment with the oil. The MIC and MBC values are shown in Tables 4 and 5. *K. pneumonia* and *E. coli* showed bacteriostatic and bactericidal at a concentration of 0.78% respectively upon treatment with the massage oil. However, *E. faecalis* showed MIC of 50 mg/mL and 1.56% upon treatment with the crude aqueous extract and oil, respectively and this was the highest inhibitory concentration needed to inhibit their growth.

The possible explanation for the antibacterial activity of the oil could be due to the interaction between the secondary metabolites and bacterial cells that may lead to inhibition of enzyme activity, disruption of cell wall synthesis, plasma membrane interference, prevention of protein synthesis, and inhibition of DNA synthesis (Nicholas and Graham 2019). Gram-negative organisms tend to be less susceptible compared to Gram-positive due to the presence of an outer membrane and hydrophilic periplasmic space. This feature will possibly influence the penetration and the fate of compounds or secondary metabolites. In addition, secondary metabolites with broad spectrum activities and polarities can penetrate in both Gram types, probably passing through the biological membrane space by hydrophilic groups. Thus, the oil can kill the bacteria due to the penetration of the secondary metabolites into the biological membrane and affects the cellular processes of the target cells synergistically as described by Nicholas and Graham (2019).

Conclusion

The present study showed that massage oil can exhibit significant antioxidant and antibacterial activities as compared to crude aqueous extract. Thus, a mixture of oils found in the bubble belly massage are potential antibacterial and antioxidant agents and further investigation on other biological activities such as anticancer, antifungal, animal study, mechanistic study on the chemical modification of the functional groups, structure-activity relationship, and identification of putative molecular target will be useful in the development of the compounds as potential drugs.

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Conflicts of Interest

The authors declare no conflict of interest.

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Effect of Testosterone, Dihydrotestosterone, Estradiol and Cortisol on the Quality of Fresh and Cryopreserved Stallion Sperm

Mikhail M. Atroshchenko[1], Anna M. Shitikova[2*], Lidia V. Krokhotina[3], Mariya G. Engalycheva[2], Mariya N. Dmitrieva[2]

[1]All-Russian Research Institute for Horse Breeding (ARRIH), Ryazan Oblast, Rybnovskij District, 391105, Russia
[2]Ryazan State Medical University, 9, Vysokovol'tnayastreet, Ryazan, 390026, Russia
[3]Center Zdorovya, (LLC), 95/1, Ostrovskogo street, 390026, Russia

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ABSTRACT

The effect of steroid hormones on the quality of fresh and cryopreserve sperm has not been fully understood. This study aimed to evaluate the effect of testosterone, dihydrotestosterone, estradiol, and cortisol on the quality of fresh and cryopreserved stallion sperm. The study was conducted on 40 *Equus caballus* stallions, including Arab (n=20), Oryol trotting (n=4), Standardbred (n=4), and Soviet Heavy Draft (n=12) breeds. The average age of the experimental animals was 9.9 ± 0.7 years. We determined standard quality indicators in fresh and cryopreserved sperm and the concentration of steroid hormones in the blood plasma of stallions. Results of the study suggested a negative correlation between the level of testosterone with total (r=-0.41; p<0.01) and progressive (r=-0.44; p<0.01) sperm motility in cryopreserved sperm as well as in fresh sperm (r=-0.38; p<0.05 and r=-0.39; p<0.05 correspondingly). While the level of estradiol showed a positive correlation with survival rate in cryopreserved (r=0.35; p<0.05) and in fresh (r=0.33; p<0.05) sperm. Further, the level of cortisol in the blood plasma of stallions did not show any statistically significant correlations with the qualitative characteristics of sperm. A positive relationship was found between the concentration of dihydrotestosterone with the volume of ejaculate (r=0.37; p<0.05) and the total number of sperm in the ejaculate (r=0.43; p<0.01). Results of the study can be concluded that steroid hormones have different effects on the quality indicators of fresh and cryopreserved sperm of stallions and their concentration in the blood should be considered when selecting stallions for cryopreservation of sperm.

* Corresponding author
E-mail: anyakudlaeva@mail.ru (Anna M. Shitikova)

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1 Introduction

The expansion of assisted reproductive technologies in the horse breeding industry has facilitated the use of frozen sperm to preserve valuable genetic material. Cryopreserved sperm has several advantages, including long-term storage and long-distance transportation, rational use of semen, prevention of infection, and preservation of sperm from endangered species (Atroshchenko et al. 2017). However, freezing followed by thawing negatively affects the fertility of cryopreserved sperm compared to fresh ones (Atroshchenko et al. 2019b). The proportion of spermatozoa that survive after freezing-thawing is affected by their susceptibility to cold shock, cooling rate, diluent composition, and osmotic stress, and the functional state of spermatozoa after cryopreservation depend on the action of oxidative stress, membrane stability, membrane receptor integrity, and nuclear structure state (Watson 2000; Elkina et al. 2011). Various biologically active compounds can also influence sperm quality and cryostability, among which steroid hormones play a special role (Atroshchenko et al. 2019c; Martínez-Fresneda et al. 2019; Coloma et al. 2010).

Testosterone is an important regulator of cellular processes occurring in the stallion testes (Roser 2008), it is involved in maintaining the normal functioning of the blood-testis barrier (Meng et al. 2005), inducing meiosis (Holdcraft and Braun 2004; Larose et al. 2020) and regulating apoptosis of germ cells (Jeremy et al. 2021). The mechanism of testosterone participation in the process of spermatogenesis is known by binding to the androgen receptor, which is expressed in various types of testicular cells, such as Sertoli cells (Walker 2011), and this is realized both through classical intracellular mechanisms and through non-classical membrane mechanisms (Cooke et al. 2022). The expression of the androgen receptor varies at different stages of a horse's life and in different glands. An increased expression is observed in the vesicular gland of the fetus compared to the stallion and a lower expression in the bulbourethral gland in comparison to other glands (Ellerbrock et al. 2021).

Studies in recent decades have established the importance of estrogens as a paracrine-autocrine factor in the regulation of seminal gland function and spermatogenesis in stallions (Roser and Hughes 1992). The synthesis of estrogens from androgens is carried out with the participation of the aromatase enzyme (Robertson et al. 1999). Aromatase expression in stallion changes with age, as demonstrated by immunocytochemistry (Hess and Roser 2004), and it is significantly lower in the bulbourethral gland than in the other accessory sex glands at all horse`s life stages (Ellerbrock et al. 2021). Estrogen receptors in stallion testes are found in Leydig, Sertoli, and germ cells (Bilinska et al. 2006; Pearl et al. 2011) and both ER-α and ER-β are present in the accessory sex glands of the horse (Ellerbrock et al. 2021). The low level of estrogens in the blood of subfertile stallions is associated with a low concentration of spermatozoa in semen (Roser and Hughes 1992). The state of the endocrine function of the testis in stallions can be assessed by the concentration of testosterone and the total level of estrogens in the blood (Inoue et al. 1993). There is evidence of a positive correlation between the concentration of estradiol in the blood plasma of stallions and sperm motility (Hoffmann and Landeck 1999), and it is also known that estradiol increases the membrane transport of monosaccharides in spermatozoa (Ballesteros et al. 1983).

Dihydrotestosterone is a product of testosterone reduction under the action of 5α-reductase. Like testosterone, dihydrotestosterone also binds to androgen receptors and triggers protein synthesis in the cell, but dissociates more slowly from the receptor (Wilson 2001). The effect of dihydrotestosterone on spermatogenesis is currently being discussed. Since the concentration of dihydrotestosterone in the testes of rats is 5 % of the concentration of testosterone (Turner et al. 1984), while in humans it is only 2 % (Jarow and Zirkin 2005), some researchers believe that dihydrotestosterone does not play a significant role in the process of spermatogenesis (Kang et al. 2014). How dihydrotestosterone affects the reproductive function of stallions remains unexplored.

Glucocorticoids are steroid hormones that are produced in response to stress. Some researchers believe that glucocorticoids can inhibit reproductive function (Wiest et al. 1988; Claus et al. 2005; Rengarajan and Balasubramanian 2007). Thus the concentration of cortisol in blood plasma is higher in Japanese black beef bulls during puberty with abnormal fresh sperm (Weerakoon et al. 2020). However, a positive correlation between cortisol and testosterone levels had been reported in the blood of stallions (Villani et al. 2006). Further, in vitro effects of cortisol on bull sperm have been characterized by increased sperm motility (Cheng et al. 1980). In this regard, the question of the effect of cortisol on the quality indicators of stallions' sperm remains open and given that at the moment there is no literature data on the effect of cortisol on the quality indicators of cryopreserved sperm. Therefore, this study aimed to evaluate the effect of testosterone, dihydrotestosterone, estradiol, and cortisol on the quality of fresh and cryopreserved stallion sperm.

2 Materials and Methods

2.1 Animals and Semen Collection

The study was performed on the livestock of the All-Russian Research Institute for Horse Breeding (ARRIH, Ryazan Region, Russia) and the Tersk Stud Farm N169 (Stavropol Region, Russia), Perevozsky and Pochinkovsky studs (Nizhny Novgorod region, Russia). All manipulations with animals were carried out according to the Law of the Russian Federation on Veterinary Medicine No. 4979-1 (14 May 1993) and the “European

Convention for the protection of vertebrates used for experimental and other scientific purposes" ETS No. 123 (18 March 1986). The protocol of the present investigation was approved by the Local Ethics Committee of the All-Russian Research Institute for Horse Breeding (ARRIH), Ryazan Oblast, Russia.

Studies were conducted on 40 breeding stallions, of which 20 stallions of the Arabian breed, 12 of the Soviet Heavy-Draft, 4 of the Standardbred, and 4 of the Oryol trotting breed. The average age of the experimental animals was 9.9 ± 0.7 years.

Stallions were kept in individual stalls. The stallions received hay, oats, and granulated compound feed with added minerals under established standards and were exercised for at least 1 h daily.

All stallions were used in natural mating. Sperm from stallions was obtained during the breeding season (February-May) with an interval of 48 hours for an artificial vagina (ARRIH model, Ryazan, Russia) for a mare in heat. After a long period of sexual rest (10 days or more), three ejaculates were taken from stallions at 48-hour intervals, the first two ejaculates were not used in studies, and the third ejaculate was taken for research since the sperm characteristics in the first two ejaculates after prolonged sexual rest may differ from the sperm indicators inherent in this stallion.

2.2 Sperm Examination

Immediately after receiving the sperm, the gel was removed, and the sperm was filtered using a sterile gauze filter. This was followed by the estimation of the volume of the ejaculate, concentration of spermatozoa in 1 ml sperm, total and progressive motility, total sperm number (TNS), and the number of sperm with progressive motility (TNS PM) in the ejaculate, as well as the survival of sperm under the regime of hypothermic storage of diluted semen at +4 °C (Atroshchenko et al. 2020**)**. The ejaculate volume (in ml) after filtration was determined using a measuring cylinder while the sperm concentration was measured using the SDM1 photometer (Minitube GmbH, Tiefenbach, Germany). The Argus CASA computer analysis system (ArgusSoft LTD, Saint Petersburg, Russia) was used to assess the progressive (PM) and total (TM) sperm motility.

2.2.1 Sperm survival test

After diluting the sperm with a lactose-chelate-citrate-yolk medium (LCCY) containing 3.5% glycerin to a sperm concentration of 45-50 million/ml in samples, the sperm was placed in a refrigerator at a temperature of +4 ° C. Sperm motility was assessed at 24-hour intervals. The survival rate of spermatozoa was defined as the time during which spermatozoa retain a PM of at least 5% under the hypothermic sperm storage regime.

2.3 Freezing and thawing of sperm

Sperm was frozen in liquid nitrogen vapors in aluminum tubes with a volume of 20 ml according to the standard methodology of the All-Russian Research Institute of Horse Breeding (Atroshchenko et al. 2019a; Naumenkov and Roman'kova 1971). Cryopreserved sperm were thawed in a water bath at a temperature of +40°C for 90 seconds. After thawing, the total and progressive motility and survival of sperm were determined using the above methods.

2.4 Blood Plasma Samples

A blood sample from the jugular vein was taken twice during the sperm collection period during the breeding season from each stallion. Vacuum tubes for taking venous blood "Vacuette"(5 ml, 13×100 mm) series "Premium" with a clot activator and gel (Greiner Bio-One GmbH, Austria) were used for this purpose. For this, blood samples were taken before morning feeding and centrifuged at 400 g for 20 min and plasma was stored at -20 °C until analysis was performed.

2.5 Determination of Hormones

Determination of testosterone, estradiol, and cortisol was performed using a corresponding commercial CLIA kit (DRG Instruments, Marburg, Germany) on a chemiluminescent analyzer Immulite 2000 (Siemens Healthcare Diagnostics Inc., USA), while dihydrotestosterone was measured using a commercial ELISA kit (DRG Instruments, Marburg, Germany) on a Multiskan ELISA analyzer ("Thermo Labsystems OU", Vantaa, Finland).

2.6 Statistical Analysis

Statistical analysis was performed using the program Statistica 10 and "Microsoft Office Excel 2016" (StatSoft Inc., USA). The nonparametric Mann – Whitney U-test and the Spearman coefficient (Rs) were used to evaluating the rank correlation in the study groups. The results were presented in the format Median (Quartiles). Differences were considered statistically significant at $p < 0.05$.

3 Results and Discussion

The results of measuring the quality of stallions' sperm before and after cryopreservation are shown in Table 1. The table shows a statistically significant decrease in the total and progressive motility after cryopreservation of sperm, as well as a decrease in sperm survival at +4 °C ($p < 0.01$). Cryopreservation with subsequent thawing of sperm has a negative impact on the integrity of sperm membranes: there are damages associated with temperature shock, cell defects associated with the extracellular and intracellular formation of ice crystals, oxidative and osmotic

stress (Khan et al. 2021). This also leads to an increase in the number of ultrastructural organelles damage affecting sperm motility and survival (Atroshchenko et al. 2019b; Aurich et al. 2020; Greiser et al. 2020).

Table 1 Qualitative characteristics of stallion sperm before and after cryopreservation

Indicator	Sperm	
	Fresh	Thawed
The volume of ejaculate, ml	36.65	-
Sperm concentration, million / ml	235.70	-
TNS, billion	8.27	-
TNS PM, billion	4.64	-
Total motility, %	69.50*	40.66
Progressive motility, %	60.80*	28.32
The survival rate at T +4 ° C, hour	127.70*	72.00

n=40; Median, * p< 0.01.

The results of measuring the concentrations of hormones in the blood plasma of stallions are shown in Table 2. It is known from the literature that the concentration of testosterone in healthy stallions normally varies from 0.04 to 1.02 ng/ml (Haffner et al. 2010; Seale 2010; Hind et al. 2021; Basiru et al. 2022), while the median estradiol concentration in healthy stallions normally varies from 13.40 - 66 pg/ml (Haffner et al. 2010; Basiru et al. 2022). The normal cortisol concentration in healthy stallions varies from 19.88 - 70 ng/ml (Tamanini et al. 1983; Villani et al. 2006; Haffner et al. 2010). Results of the measuring hormone concentrations in the current study are approximately in the specified range. Therefore, the studied stallions were within the physiologically acceptable limits for the studied indicators.

The analysis of the rank correlation between the concentration of steroid hormones in the blood plasma of the studied stallions (n = 40) and the quality of fresh and thawed sperm was carried out separately for each studied hormone (Table 3). A negative correlation was found between testosterone concentration with total motility, as well as with progressive motility in fresh and thawed sperm. Further, in the case of estradiol, a positive correlation was reported between the hormone concentration with sperm survival rate in fresh and thawed sperm. The study of dihydrotestosterone correlations also showed a direct correlation between the volume of ejaculate and the total number of sperm in the ejaculate while in the case of cortisol, statistically significant results were not obtained.

Table 2 Concentration of testosterone, dihydrotestosterone, estradiol and cortisol in stallion blood plasma.

Hormone	Concentration
Testosterone (ng/ml)	0.50
Dihydrotestosterone (pg/ml)	197
Estradiol (pg/ml)	42.22
Cortisol (ng/ml)	37.34

Median, (n=40).

A positive correlation was reported between the concentration of dihydrotestosterone and testosterone in the blood plasma of stallions (r = 0.37; p = 0.018), and these results are in agreement with the findings of Hoffmann and Landeck (1999) those who reported a similar relationship in the spermoplasm of stallions. This may be because testosterone is a precursor for the synthesis of dihydrotestosterone (Wilson 2001). A positive correlation was also obtained between the concentration of estradiol and dihydrotestosterone in blood plasma (r = 0.40; p = 0.011). The findings of the current study are contradictory to the findings of Hoffmann and Landeck (1999) who did not find any correlation in stallion seminal plasma however they have not investigated these correlations in the blood plasma. Whether dihydrotestosterone affects the synthesis of estradiol or vice versa, or whether some

Table 3 Spearman's correlation coefficient (R_S) between sperm quality indicators and the blood plasma concentration of steroid hormones in stallions

Hormone	Indicators of sperm quality	R_S	p value
Testosterone	Total motility (TS)	-0.41	0.009
	Progressive motility (TS)	-0.44	0.004
	Total motility (F)	-0.38	0.016
	Progressive motility (F)	-0.39	0.012
Dihydrotestosterone	Ejaculate volume	0.37	0.018
	TNS	0.43	0.006
Estradiol	The survival rate at T +4 ° C (TS)	0.35	0.028
	The survival rate at T +4 ° C (F)	0.33	0.040

Abbreviations: TS- thawed sperm; F-fresh sperm, TNS- total number of spermatozoa

third factor simultaneously acts on the synthesis of these hormones in stallions remains unknown.

Further, statistically nonsignificant correlations were reported between the age of the studied animals and the concentration of hormones in the blood plasma, but a negative correlation between the survival rate at T +4 ° C of fresh sperm and the age of stallions ($r = -0.32$; $p = 0.042$).

Seale (2010), found a positive correlation between the concentration of testosterone in the blood plasma of stallions and progressive motility in fresh sperm, while the results of the current study suggested a negative correlation between the total and progressive motility of fresh stallion sperm and the concentration of testosterone. Similarly, negative correlations were obtained between the concentration of testosterone in blood and sperm motility in cryopreserved sperm and these results contradict with the findings of previous research. Coloma et al. (2010) revealed that high levels of testosterone in the *Iberian ibex* blood plasma were associated with a decrease in progressive sperm motility and a decrease in acrosome and membrane integrity, and this relationship is characteristic only for the frozen-thawed sperm. Bóveda et al. (2021) also found a reduction in the concentration of testosterone in the blood plasma of *Iberian ibex* and it increased with the sperm cryostability duration increases and vice versa. Moreover, these changes most likely occur at the final stage of sperm maturation in cauda epididymis and secretion of the sperm and may be associated with remodeling of the protein and lipid components of the sperm membrane. Epididiosomes (Nixon et al. 2019) and extracellular vesicles (Leahy et al. 2020) may play a key role in the changes described above. In a series of recent experiments, it was demonstrated that *in vitro* incubation of small ruminants spermatozoa in a testosterone medium leads to a decrease in the acrosome integrity in thawed sperm. The authors of the study also suggested that testosterone negatively affects the resistance of sperm to cryopreservation and its destabilizing effect directly depends on the concentration *in vivo* (Martínez-Fresneda et al. 2019).

The molecular mechanisms of testosterone's influence on the quality characteristics of sperm have long been discussed. Shivaji and Jagannadham (1992) attempted to evaluate the direct effect of testosterone on fluidity, aggregation, fusion, and leakage of human and hamster spermatozoa membranes and reported a very little effect of the testosterone on the selected indicators. Purohit et al. (2000) have observed that supraphysiological doses of testosterone administered to mice can cause oligospermia and alter the liquid-crystalline configuration of sperm membranes (Purohit et al. 2000).

Similarly, Calzada et al. (1988) found that the incubation of human sperm in testosterone changes the configuration of the plasma membrane and induces the depolarization of the membrane. Testosterone has also been shown to increase the molecular transport of carbohydrates across the sperm membrane in the *in vitro* study of Ballesteros et al. (1983). According to Cai et al. (1994), dihydrotestosterone is synthesized under the action of 5α-reductase. Analysis of the men's sperm with homozygous mutation of 5α-reductase showed that dihydrotestosterone regulates the volume and viscosity of sperm, but does not affect the total number of viable spermatozoa, however, the authors of the study do not exclude that a small concentration of dihydrotestosterone is still necessary for normal spermatogenesis. In addition, there was a decrease in the total number of viable spermatozoa, sperm concentration, and volume in healthy men with reduced levels of dihydrotestosterone due to the use of finasteride, a 5A-reductase-2 inhibitor, and these indicators were restored to normal levels when the drug was discontinued (Amory et al. 2007). This is showing positive correlations between the concentration of dihydrotestosterone with the volume of sperm ejaculates and the total number of sperm in ejaculates.

In the analysis of the correlation between blood plasma estradiol and stallion sperm quality indicators, we obtained a positive correlation between the concentration of the hormone with the survival rate of sperm in fresh and thawed sperm. Previous studies have found a positive effect of estradiol on sperm motility. Mesbah et al. (2022) found that adding 3 µM estradiol concentrations to the goat semen extender increased survival rate, sperm integrity, and progressive motility through cryopreservation, and this process seems to be calcium-dependent. Hoffmann and Landeck (1999) reported a positive correlation between the concentration of estradiol in the blood plasma of stallions and sperm motility. In addition, the role of aromatase/estrogens in the acquisition of sperm motility in men was also established (Lambard et al. 2004; Carreau et al. 2009). Reduced sperm count and sperm motility have been described in men with genetic aromatase deficiency (Rochira et al. 2005) and in knockout mice (O'Donnell et al. 2001). Previously, it was shown that estradiol enhances the molecular transport of glucose and fructose across the sperm membrane (Ballesteros et al. 1983) and since these monosaccharides are the main sources of energy for sperm (Varner et al. 2016), they can be used to provide energy for movement. This mechanism likely underlies the increase in sperm motility and, possibly, survival rate under the influence of estradiol.

The effect of cortisol on reproductive function in many studies is considered through its effect on testosterone synthesis. It was found that in some species, glucocorticoids inhibit reproductive function by inhibiting the expression of proteins involved in testosterone biosynthesis (Claus et al. 2005; Rengarajan and Balasubramanian 2007). In the case of stallions, it has also been suggested that cortisol affects the testes by inhibiting the production of testosterone (Wiest et al. 1988). In other studies, there was a positive correlation between cortisol and testosterone levels in the blood of stallions (Villani et al. 2006). The findings of

Bishop et al. (1999) and Borg et al. (1991) are contradictory to the findings of previous researchers and suggested that cortisol does not affect the synthesis of testosterone in bulls and boars. Also, Seale (2010), found no statistically significant correlations between cortisol levels and the concentration of testosterone in the blood plasma of stallions, as well as indicators of the quality of fresh sperm. Deichsel et al. (2015) showed that an increase in cortisol concentration in stallions in response to physical activity and the introduction of adrenocorticotropic hormone does not affect the quality of sperm, which indicates good protection of stallions' testes from the effects of glucocorticoids (Deichsel et al. 2015). In mature stallions, this is probably achieved by oxidizing active cortisol to inactive cortisone using the enzyme 11β-hydroxysteroid dehydrogenase (Draper and Stewart 2005). This may explain the results of this study, in which a statistically significant correlation was not reported between the concentration of cortisol and the quality of fresh and cryopreserved sperm.

Conclusions

Results of the current study suggested that the level of testosterone in the blood plasma of stallions negatively correlates with total and progressive sperm motility in fresh and cryopreserved sperm. We also obtained a positive correlation between estradiol concentration with the survival rate in fresh and cryopreserved sperm. It is known that these hormones can affect spermatozoa at the molecular level; the mechanism of their effect on sperm cryostability requires further study. Results of this study also suggested that dihydrotestosterone affects the fertility of stallions, since a positive relationship was found between the concentration of the hormone with the volume of ejaculate and the total number of spermatozoa. We did not get statistically significant results for cortisol which may be due to the good protection of the stallion testes from the action of this glucocorticoid. Results of the study can be concluded that the studied steroid hormones have different effects on the quality of stallion sperm and their concentration in the blood should be taken into account when selecting animals for cryopreservation of sperm.

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Conflicts of interest and financial disclosures

The authors declare no conflict of interest.

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Disease Complication in a Geriatric Pig-Tailed Macaque (*Macaca nemestrina*) reported from Wildlife Rescue Centre (WRC) Jogja – Case Study

Irhamna Putri Rahmawati[1], Novra A. Sandi[2,3], Andreas Bandang Hardian[*,4]

[1]Wildlife Rescue Centre (WRC) Jogja, Yayasan Konservasi Alam Yogyakarta (YKAY)
[2]Eijkman-Oxford Clinical Research Unit (EOCRU), Eijkman Institute for Molecular Biology (EIMB)
[3]Department of Paraclinical Science, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan
[4]Laboratory of Veterinary Anatomic Pathology, Faculty of Veterinary Medicine, Universitas Brawijaya

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ABSTRACT

In the current study, a geriatric male pig-tailed macaque having a mass growing up to the left shoulder for the past five months has been reported from the Wildlife Rescue Centre (WRC) Jogja. It was an approximately 4x4x2 cm, firm, hairless focal non-encapsulated mass suspected as a skin-derivative tumor. A cytological test was performed on the mass and a skin biopsy was carried out to see the tissue content of the mass. Histopathological examination confirmed that the mass was identical to papilloma. In the next step of the study, the surgery was conducted and the mass was removed. However, after surgery, the health condition of the macaque gradually deteriorated and the performed hematological tests revealed a gradual increase in the number of leukocyte cells that indicated a developing chronic inflammatory response. Further, the erythrocyte level was in the normal range but showed a declining trend which suspecting progressing anemia. One month after the surgery general muscle stiffness was reported in the macaque which is a clinical sign of tetanus and the macaque died even after the ATT serum treatments. Necropsy findings confirmed a significant chronic bilateral diffuse pneumonia. This case study showed an unpredicted sequence of disease occurrence in geriatric macaque after undergoing the initial surgery of cutaneous mass extraction. Hence, the results of the study suggest that a routine regular health screening coupled with clinical laboratory examination may assist the early detection based on the subclinical signs of disease in geriatric animals in rehabilitation centers.

* Corresponding author

E-mail: andreasbandangh@ub.ac.id (Andreas Bandang Hardian)

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1 Introduction

A male adult macaque was having more than 15 years of age was surrendered to the study center. During the routine monitoring, it was found that it often sounded 'coughing' and hiccup without any reason. In November 2017, a 4x4x2 cm, firm, hairless focal non-encapsulated mass grew up from its left shoulder, and results of this section biopsy revealed a proliferative tissue from epidermal derivative. After this abnormal growth, the health conditions of macaque was unexpectedly kept worsening during the next 6 months and the macaque was found dead after undergoing a series of spasmodic signs indicating tetanus. This study was conducted to evaluate the disease complication and sequential changes that occurred during the development of abnormal growth in a Geriatric Pig-Tailed Macaque.

2 Materials and Methods

The macaque having geriatric Pig-Tailed was sedated using a combination of ketamine-xylazine by following the common procedure applied at the research center. To rapidly assess the type of mass growing up, a chunk of mass tissue was incised and a biopsy was carried out by following the biopsy procedure proposed by Nischal et al. (2008). A series of peripheral blood collection and haematologic evaluations was also performed during the health monitoring period of this macaque. For this, 3-ml-blood was taken in each collection and analyzed by an automatic hematology analyzer under laboratory conditions.

The necropsy of the deceased macaque was performed by following the standard necropsy procedure. All suspected lesions found on the tissues during necropsy were preserved in 10% nonbuffered formalin for histopathologic examination. The tissues were processed through a common tissue processing protocol for histopathology followed by haematoxylin-eosin staining.

3 Results

During this study, a series of blood tests were performed during March, April, and May 2018. A significant increase in haematocrit and thrombocyte was observed that indicating the increase in the number of cells and possibly caused acute vascular damage. Further, a slight rising in leukocytes and a decline in haemoglobin were reported which indicated symptoms of anemia. Differential leukocyte counts represent a sharp increase in neutrophil and lymphocyte number which revealed a prolonged infection pattern (Figure 1).

The gross inspection found abdominal fat atrophy indicated by thinning of fat deposits in the abdominal omentum and mesentery. The lungs seemed very congested, dark red on the right side with diffuse, poor demarcated, white discoloration and irregular surface on both sides. The transverse section of the lung revealed 2-5 mm in diameter, well-demarcated, multifocal, irregular yellow lesions (Figure 2).

Histopathologic examination of the mass revealed that there is an extensive proliferation of epidermal layers forming elevated papillary projection. The histological architecture shows clusters of epidermal cells in almost concentric fashion and fibrovascular tissue underlying the base of epidermal growth (Figure 3). The tissue growth is invasive and infiltrates the dermal layer. High numbers of pleomorphic cells were observed with moderate mitotic figure count and haemorrhages. The histopathology of the lungs showed thickening of alveolar walls and severe haemorrhages. The thickened alveolar walls were stuffed with cells consisting of polymorphonuclear cells and lymphocytes (Figure 4).

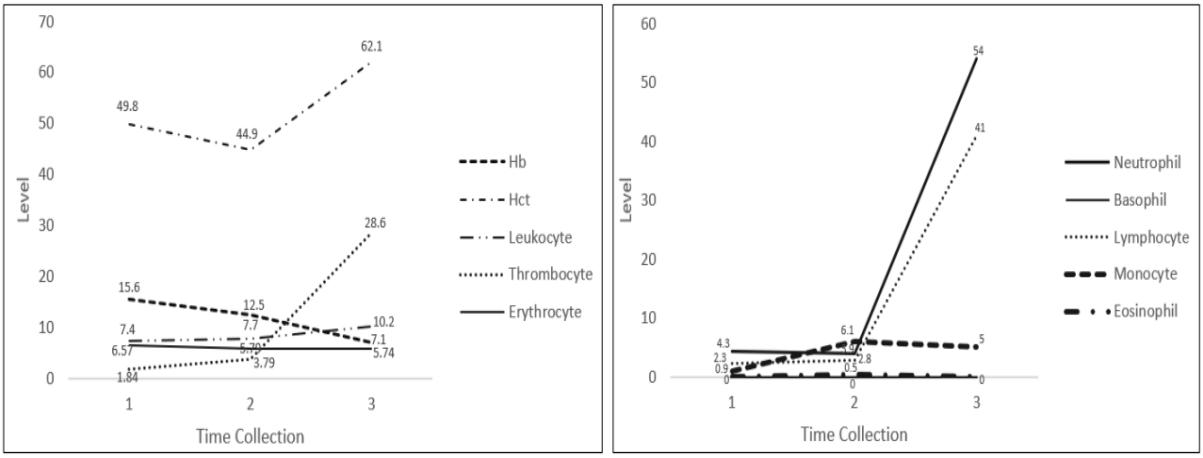

Figure 1 Line charts of macaque's haematological dynamics were compiled from three-month monitoring (left) and differential leukocyte count (right) was sharply increased to the end of the diseased event. The hypochromic anemia was observed in the second Hb check; however, the neutrophilic and lymphocytic inflammations were the predominant findings.

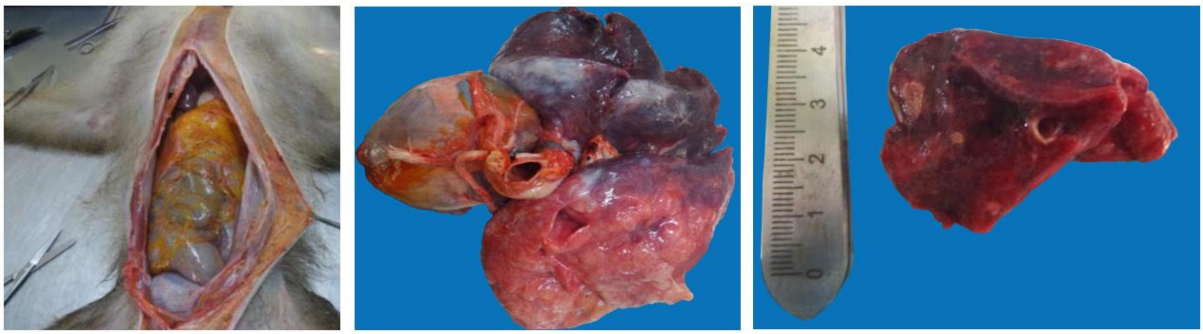

Figure 2 The gross finding showed a thin abdominal fat (left), asymmetrical enlargement of lung lobes, and white discoloration with darkening of almost 60% of the right lobe (middle). Transverse sections presented 2-5 mm in diameter multifocal lesions were observed in the parenchymal tissue of lungs (right).

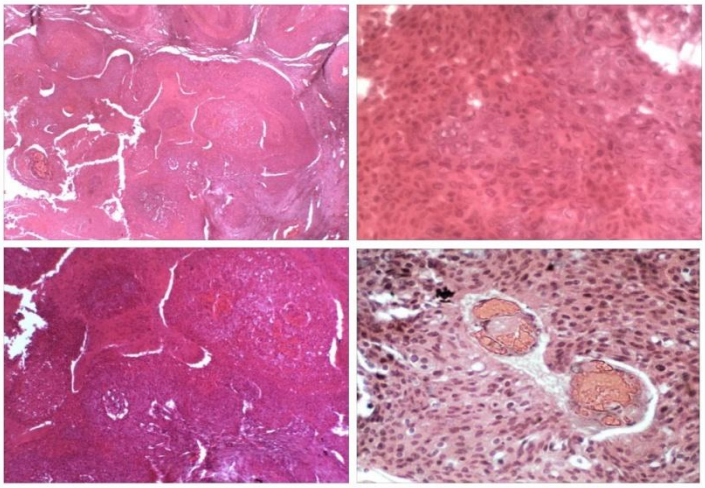

Figure 3 The histopathology of skin presents proliferative and invasive epidermal derivatives infiltrating the underneath tissue. The outward growth seems to form an irregular projection with no complete differentiation of the keratinized stratum. Anisokaryosis and haemorrhages (resembling keratin pearl) are observed (bottom right).

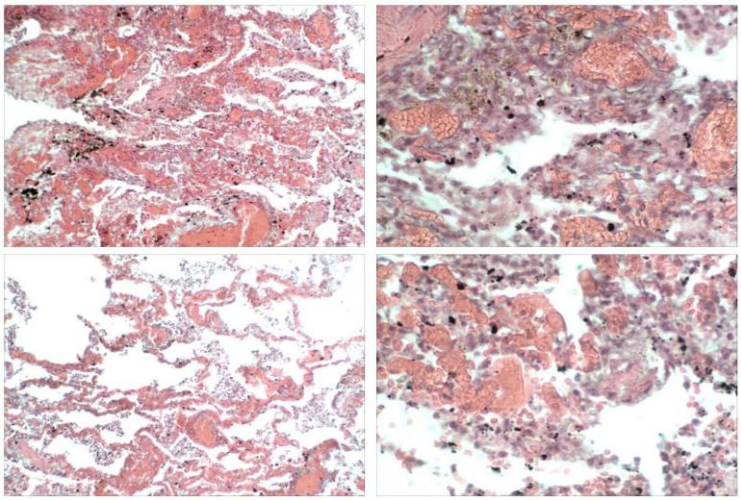

Figure 4 The histopathologic findings of diseased lungs showed thickening of alveolar walls (interstitial pneumonia), congestive capillaries, and haemorrhages. Presumptive alveolar edema might appear and induce coughing reflexes. Inflammatory cells predominantly neutrophilic and lymphocytic cells infiltrate the walls of alveoli.

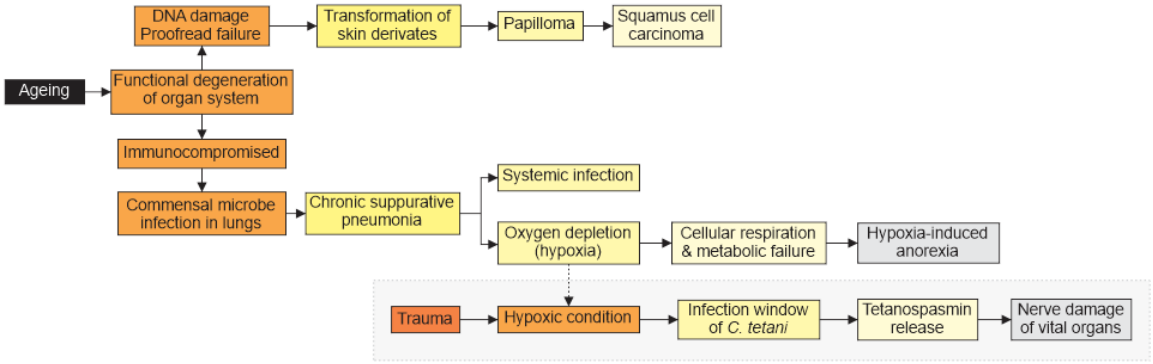

Figure 5 The deductive scheme of pathogenesis in this aging macaque. Results of this study presume that there were at least three complications that might play important role in disease development and need specific diagnosing approaches

4 Discussions and Conclusion

Results of the study deduced that the presence of several unrelated lesions in this macaque was strongly associated with multiple infection events in a time. Aging might be the most plausible etiology of these prolonged diseased events (Figure 5). These results are also supported by the findings of Day (2010) those who reported that aging degenerates the organ systems which might affect the immune system and develop immunosuppressive conditions which make the animals more susceptible to commensal infections. Further, aging is also associated with the increased error of DNA replication and proofreading which contribute to the transformation of neoplastic tissue (Maynard et al. 2015).

According to this case study, coughing and poor body condition score may indicate a chronic and compensating respiratory syndrome; however, the prognostic decision might need ancillary tests. No report existed when the macaque started exhibiting the syndrome though our daily health supervision had recorded that the coughing remained persistent for about three years. Lung histopathology revealed a classic presentation of suppurative lobar pneumonia with congestive and haemorrhagic capillary vessels. The congestion leading to microhaemorrhages induced the increase of hydrostatic pressure and stretch of endothelial gaps, allowing the plasma to excessively infiltrate the nearby tissue interstitials (Ackermann 2017). The filling of alveolar lumens with plasma and blood cells induced the reflex to expel the fluidic alveolar contents through coughing (Polverino et al. 2012). Visual gross inspection of the lung lobes showed asymmetrical enlargement in the left lobe which might be associated with compensatory adaptation of prolonged low oxygen intake or merely pulmonary exudation. Similarly, Schols and Westerterp (2002) suggested that hypoxic conditions might induce the loss of body fat leading to hypoxia-induced anorexia. Moderate intermittent hypoxic conditions may also induce body weight loss due to the release of leptin and or liver leptin which inhibits fat deposition, food, and energy intake (Ling et al. 2008).

The series of haematologic tests observed a single event of hypochromic anaemia, however neutrophilic and lymphocytic inflammations might more significantly contribute to the severity. In general, all cellular indices of haematology increased through the progression of the disease, except haemoglobin level (Hb) which gradually declined to the end of the disease. Apparently, the sharp rise of neutrophil and lymphocyte levels contributed mostly to the haematocrit level (Hct). Neutrophil rise might be a classic presentation of a cellular phase of acute inflammation in response to bacterial infection (Ackermann 2017) which might be associated with the presence of suppurative pneumonia. Lymphocytes increase in the response to viral infection and poor physiologic condition and pain might induce stress leukogram (Latimer 2011). In the current study, prolonged pneumonia and pain might contribute to the haemodynamic of the macaque during disease progression.

The mass growing upon its shoulder was diagnosed as squamous cell carcinoma (SCC) according to its architecture and the presence of neoplastic figures. Several skin derivate tumors in macaques were located predominantly in the buccal cavity and it is showing resembling with the human cases (Kollias et al. 1975; Nakamura et al. 2000; Stockinger et al. 2014). Moreover, identification of SCC subtypes has been introduced and classified (Pereira et al., 2007). Either the biopsied and processed tissue from the mass in our case presented a proliferative and inwards invasive of epidermal cells. Outwards growth contained multiple layers of epidermal stratum with minimal thickness of keratinized stratum. Based on the buccal SCC subtype, it was identical to the moderate differentiation subtype which is recognized with no complete keratinized layer of stratum corneum and no presence of keratin pearl (Pereira et al. 2007). There were inflammatory infiltrates in the underneath of invasive projection to the dermal layer; however, the significance of this lesion to the deterioration of its health is apparently low. Several aetiologies have been reported inducing the SCC formation including herpesvirus and papillomavirus infection, UV exposure, some carcinogens, and aging (Molho-Pessach and Lotem 2007;

Tsatsou et al. 2012), however, the transformation causing of epidermal cells in the current study is still unknown. This superficial mass might be misdiagnosed as skin abscess but it can be easily discriminated by using basic cytological examination such as fine needle biopsy which can assess the cellular content (Kashi et al. 2011).

Tetanus came up as the final event of this disease complication. Although no ancillary test was performed in the current study to confirm the presence of Clostridium tetani, but the clinical symptoms suggested the tonic muscle spasm of the jaw and extremities (Hassel 2013). Springer et al. (2009) suggested that tetanus is very common in those nonhuman primates in which metal circumstance is suitable for the germination of clostridial spores.

In the current case study, it was believed that routine health examination and screening will be helpful in the early detection of visible animal diseases. According to our experience, health supervision in wild animals can be tricky considering their ability to mask the pain in the wild. The animals might look healthy with no sign of abnormality, however, there will be a concealed developing disease that visibly ends up as sudden severe disease. Thus, the results of the current study strongly recommend a regular periodic health examination and this will be beneficial in significantly reducing the morbidity and mortality of animals in rehabilitation centers.

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Feline extrauterine pregnancy (EUP) in Persian cat with fetal mummification: a case study

Dian Vidiastuti[1*], Ahmad Fauzi[2], Aldila Noviatri[3]

[1]Laboratory of Surgery and Radiology, Faculty of Veterinary Medicine, Universitas Brawijaya, Kalisongo, Dau, Malang, East Java 65151, Indonesia
[2]Laboratory of Clinical Pathology, Faculty of Veterinary Medicine, Universitas Brawijaya, Kalisongo, Dau, Malang, East Java 65151, Indonesia
[3]Laboratory of Pharmacology, Faculty of Veterinary Medicine, Universitas Brawijaya, Kalisongo, Dau, Malang, East Java 65151, Indonesia

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Cat

Extrauterine Pregnancy

Ultrasound

Radiographic

HE staining

ABSTRACT

Extrauterine pregnancy (EUP) is caused by the implantation of the fetus outside the uterus. In this study, during a routine check-up of a 3-year-old non spayed female Persian cat, a mass on the abdomen was found on palpation, which later was diagnosed as an abdominal tumor. Clinical signs presented are pollakiuria and no lethargic. The radiographic examination revealed a well-defined circular mass with mineral opacity in the caudal abdomen. Ultrasound examination of this abdominal mass illustrated the presence of mummified fetus with an irregular arrangement of bones. Moreover, there was no heart movement, the fetal bones were hyperechoic. An exploratory laparotomy was performed for mass collection, and excised mass was dense. Histopathology investigation using HE staining results in bone, tongue, lung and pigmented skin. The fetal age was around 60 days old.

* Corresponding author
E-mail: vidian@ub.ac.id (Dian Vidiastuti)

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1 Introduction

Extrauterine pregnancy (EUP) or ectopic pregnancy is a pregnancy that occurs when the fetus is found outside the uterus. There are two types, namely primary and secondary. Primary EUP is a pregnancy where fertilized egg enters the abdominal cavity instead of following its path through the tubal structures. The entire pregnancy occurs outside the uterus. While in secondary EUP, the developing embryo or fetus is dislodged into the abdominal cavity due to uterine rupture. The pregnancy formed in the uterus is then continued in the extrauterine environment (Nack 2000). In humans, it is associated with pathological conditions with an incidence rate of 20.7 cases per 1000 pregnancies as reported by Van Den Eeden et al. (2005). While it is rare in animals, detailed epidemiological studies have not been conducted (Corpa 2006; Mirsepehr et al. 2015). The current case study was carried out to diagnose extrauterine pregnancy in a 3-year-old non spayed female Persian cat through abdominal radiography or ultrasonography and an exploratory laparotomy surgery was performed to explore tissue and remove the fetal mass.

2 Materials and Methods

A 3-year-old female Persian cat that was not spayed was referred to Veterinary Hospital Universitas Brawijaya, Indonesia for a routine check-up due to a mass on the abdomen and increased urination. Physical examination of the cat was carried out by inspection, palpation, and auscultation. Subsequently, an ultrasound examination was performed using a frequency of 7.5 MHz with an axial and sagittal plane (Honda-HS2200V). A study using x-rays (DR Tech®) with 60 kVP and 3 mAs on the left lateral and ventrodorsal views. Hematoxylin Eosin (HE) staining was confirmed for mass evaluation.

3 Results

Physical examination and auscultation showed no abnormalities; the cat was very active, palpation there was a mass in the caudal abdomen with a kidney-like consistency. The aim of radiographic study is to identification the location and the number of space occupying lession. Radiographs of the left lateral view (Figure 1A) and ventrodorsal view (Figure 1B) showed one mass in the cranial bladder, circular in shape, smooth and clear margins, showing the presence of multiple fetuses. The space occupying lession contain three fetuses with disorganized bone. Mass dimension are 7.25 cm in length and 4.74 cm in width. Two fetuses superimposition in one another (Figure 1C).

The sonogram (Figure 2) illustrate the formation of vertebrae; the fetal head and bones looked hyperechoic, which were protected by a thick layer of sac wall. The amniotic fluid area in fetus 1 (figure 2A) looks a mixture of hypoechoic and anechoic, while fetus 2 (figure 2B) is hypoechoic; there may be changes in amniotic fluid to solid material. In the reported fetal cardiac or extremity movements were not detected which led to the mummification. The fetus's gestational

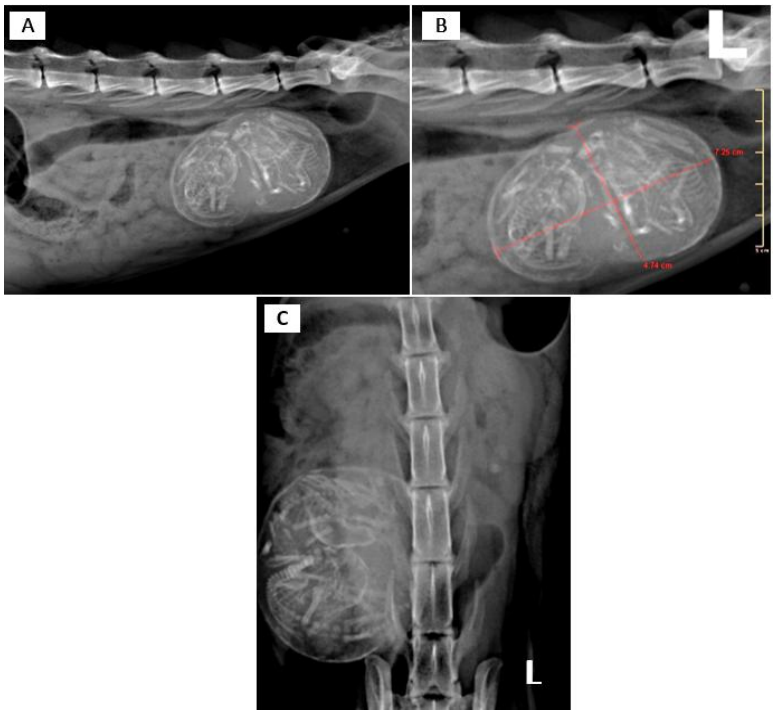

Figure 1 Radiographic examination of the abdomen. (A) left lateral view, a circular structure of the cranial bladder containing the fetus with irregular bony structure. The mass margin is clear and smooth (B) Mass dimensions are 7.25 cm long, 4.74 cm wide. (C) The ventrodorsal projection finding the mass on the right side of the abdomen and the arrangement of the fetal vertebrae

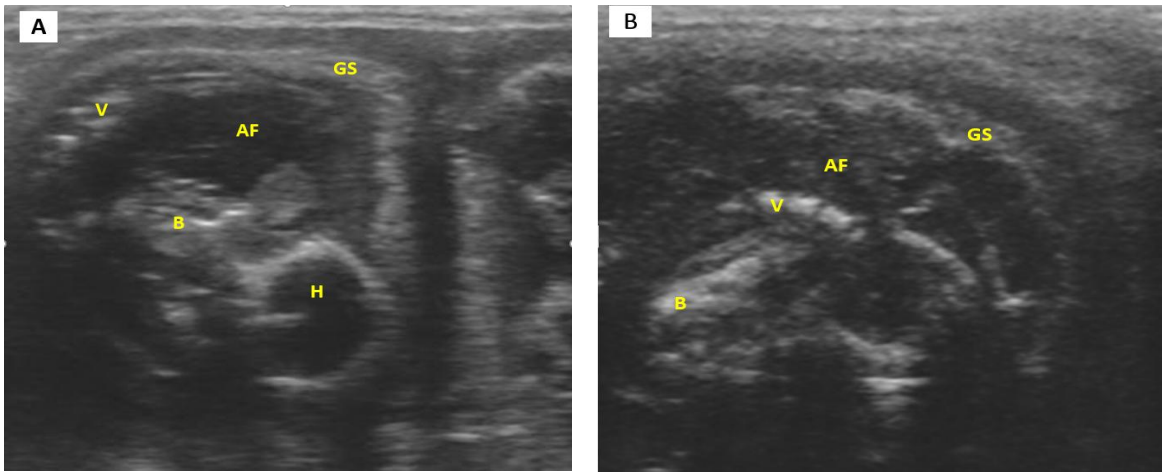

Figure 2 Ultrasound aspect of fetal structure in the sagittal plane. Hyperechoic structures were identified as vertebrae (V), bone (B), head (H), and gestational sac membrane (GS). There is a difference in the echogenicity of amniotic fluid (AF) in fetus 1 (A), a mixture of anechoic and hypoechoic, while in fetus 2 (B), it is only hypoechoic

Figure 3 Gross anatomy of a potato-like mass, round shape, smooth surface and hard consistency

age cannot be differentiated by ultrasound because the fetal's head and the body are ill-defined. Subsequently, the patient was referred for surgery. The results of exploratory laparotomy of the mass were outside the uterus and attached to the omentum. Normal uterine conditions do not show signs of rupture. Gross anatomy mass looks like a potato with a smooth surface and tough (Figure 3).

Histopathological results of HE staining revealed osteogenesis of compact bone structure formation in long bones and spinous processes (Figures 4A and 4B). The bones dominated by chondrocytes, while in the skin, there were melanin deposits and hair follicle growth (Figures 4C and 4D). The lumen of the pulmonary alveoli is cloudy with the surrounding bronchi

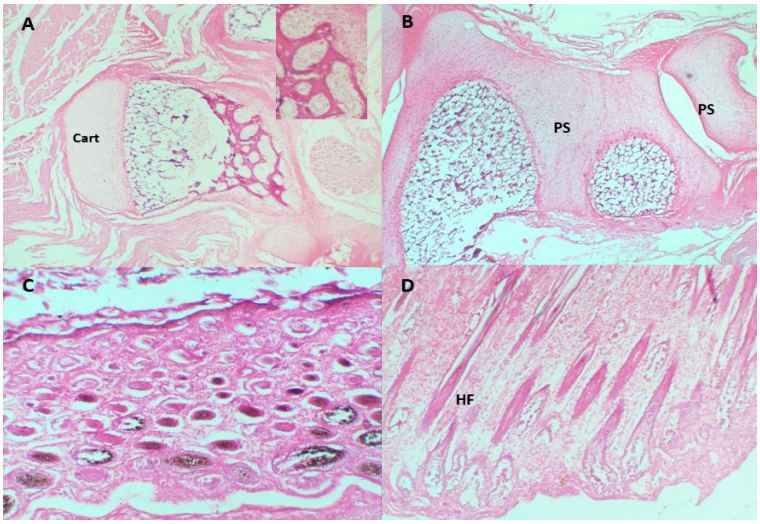

Figure 4 Histopathology of excised tissue mass staining with HE (A) Osteogenesis in long bones starting from cartilage (Cart) composed of chondrocytes magnification 40x to the formation of new bone (insert: magnification 100x); (B) Cartilage-dominated spinous process structure: magnification 40x; (C) The epidermis of the fetus is found with deposits of melanin (100x); (D) Fetal hair follicle growth (HF): magnification 100x

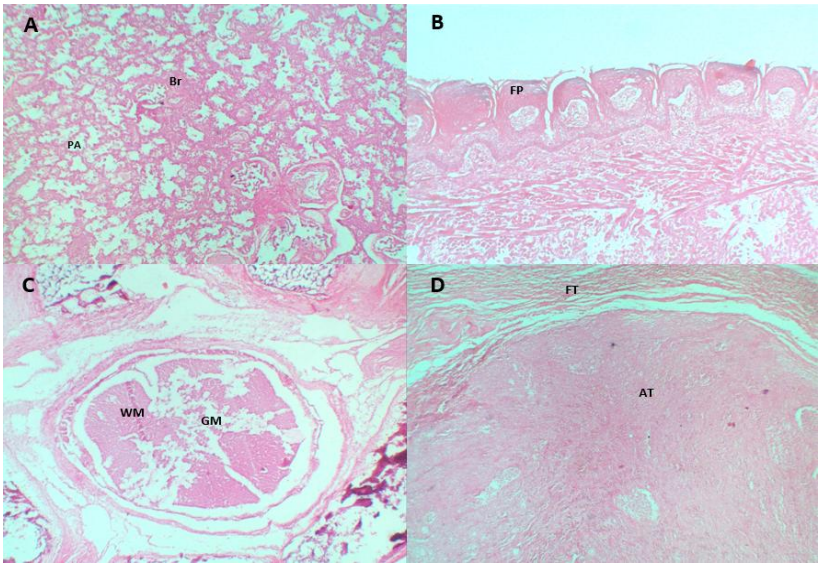

Figure 5 Microscopic examination of other fetal organ tissues (A) Partially pulmonary alveolar (PA) eosinophilic and bronchial (Br) magnification 40x; (B) Fungiform papillae (FP) fetal tongue magnification 100x; (C) The cross-sectional spinal cord shows rudimentary white matter (WM) and gray (GM) structures; (D) There is an atypical tissue (AT) resembling mesenchymal architecture encapsulated with fibrous layer (FT)

(Figure 5A). In the findings of the tongue that is seen are the fungi form papillae (Figure 5B) and spinal cord with areas of gray matter and rudimentary white matter (Figure 5C). The gestational sac is a typical tissue-lined externally with fibrous tissue (Figure 5D).

4 Discussions and Conclusion

According to the Rosset et al. (2011), primary EUP can be caused by a fertilized egg implanting outside the uterus, omentum, peritoneum, or fallopian tube while the secondary EUP is caused by the rupture of the uterine wall due to trauma and the subsequent ectopic fetal developed in the peritoneal cavity. The clinical symptoms in this case are pollakiuria leads to the FLUTD condition. Further, the presence of a hard mass in the caudal abdomen presses the bladder and correlates this with the frequent urination in the cat. These results are in agreement with the findings of Osenko and Torello (2014) those who reported high urination in the animals suffering from abdomen tumors. In general, the clinical condition of cats does not show any specific abnormalities. Extrauterine pregnancy is often an incidental finding because animals are generally asymptomatic and in good health and undetected for several months to several years (Nack 2000; Corpa 2006; Myung et al. 2016).

Radiography is one of the diagnostic tools that is used to determine the condition of ectopic pregnancy because it can see fetal bone mineralization and can calculate the number of gestational sacs (Osenko and Torello 2014; Myung et al. 2016). Furthermore, ultrasonography identifies heart rate and fetal movement (Mirsepehr et al. 2015). Ectopic pregnancy is generally accompanied by fetal death; although primary abdominal pregnancy has been reported in domestic animals, no fetoplacental guarantees successful growth outside the uterus. This type of placentation in cats does not allow the development of extrauterine pregnancy (Corpa 2006). There were no abnormalities in the uterus and ovaries in exploratory laparotomy, It was suspected that this pregnancy was not a traumatic factor. Ivanova et al. (2019) reported that ectopic pregnancy could be triggered by a long period of gestation.

In the current study, the outer layer of the gestational sac formed by fibrous tissue was autolysis inside of fetal tissue and similar types of findings were reported by Myung et al. (2016). It is suspected that the absorption of amniotic fluid into solid tissue has no clear structure (atypical). All fetuses found in the current study have bone structure and osteogenesis appears to have occurred (Mirsepehr et al. 2015; Chong 2017; Ivanova et al., 2019). Other findings such as the structure of the tongue's papillae, melanin, and hair follicles in the skin, spinal cord, and lungs indicate the stages of fetal maturation at the end of gestational age around 60 days. According to Knospe (2002), at 60 days' gestation, pigmentation is possible on the skin, hair, pigmentation, and nails. Furthermore, the brain, neuron cells and bones ossification are present during this gestation period.

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Descriptive Comparison of Gastrointestinal Tract Histology of Various Avian Species based on their Natural Diet

Dini Agusti Paramanandi[1], Irhamna Putri Rahmawati[2], Guruh Jaya Wisnuwardana[2], Warih Pulung Nugrahani[2], Andreas Bandang Hardian[3*]

[1]Laboratory of Veterinary Anatomy and Histology, Faculty of Veterinary Medicine, Universitas Brawijaya, Jl. Puncak Dieng, Kunci, Kalisongo, Dau, Malang, East Java, Indonesia, 65151
[2]Wildlife Rescue Centre (WRC) Jogja, Yayasan KonservasiAlam Yogyakarta (YKAY) Jl. Pengasih-Nanggulan, Paingan, Sendangsari, Pengasih, Kulonprogo, Special Region of Yogyakarta 55652
[3]Laboratory of Veterinary Anatomic Pathology, Faculty of Veterinary Medicine, Universitas Brawijaya, Jl. Puncak Dieng, Kunci, Kalisongo, Dau, Malang, East Java, Indonesia, 65151

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Proventriculus

ABSTRACT

Many animals species develop their gastrointestinal tube with special features to accommodate their natural diet to survive under adverse conditions including the nutrient absorption capability. Information related to the histologic description of various bird species' digestive organs based on their diet and its significance is yet limited. This study aimed to present a descriptive explanation of gastrointestinal organs of a changeable hawk-eagle (*Nisaetus cirrhatu)* and oriental honey buzzard (*Pernis ptilorhynchus)* as carnivorous, a southern cassowary *(Casuarius casuarius*) as an omnivorous, and a domestic chicken (*Gallus gallus*) as granivorous. In the current study, proventriculus (glandular stomach) and intestinal segments were microscopically examined and compared to understand the special histological features among avian species due to their important roles to digest the ingesta. The dissected specimens were preserved in 10% non-buffered formalin, then were processed through the common standard procedure of tissue processing and eventually stained with haematoxylin-eosin. Microscopic observation showed variation in shape and size of proventricular glandular architecture among raptors. The intestine muscular layer of the cassowary also showed distinct thickness among birds. These results of the study preliminary proved that variation in diet might affect the histologic features of avian gastrointestinal tracts.

* Corresponding author

E-mail: andreasbandangh@ub.ac.id (Andreas Bandang Hardian)

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1 Introduction

Around the world, there are approximately 8,600 kinds of birds (Al-Saffar and Al-Samawy 2016a), and places the second in the number of species among vertebrates (AbdElnaeem et al. 2019). The digestive organs are responsible for mechanical and chemical digestion, along with the absorption of water and foods (Hussein et al. 2020). In birds, the stomach is appraised as the most active organ in the digestive system (Al-Saffar and Al-Samawy 2016b). The bird's stomach presents the glandular and the gizzard (Umar et al. 2021). The proventriculus (glandular stomach) is characterized morphologically and functionally, which is influenced by the type of food taken (Al-Saffar and Al-Samawy 2015). Vegetable matter, animal matter, and both are the three important terminologies frequently used for classifying avian diets. According to Lopes et al. (2016), on the behalf of feeding habit, birds can also be classified into three categories i.e., granivorous, carnivorous, and omnivorous. Further, the size of the proventriculus is varied with the type of feeding habits and it was reported relatively tiny in granivorous but massive in carnivorous (Duke 1997; Al-Saffar and Al-Samawy 2015). The proventriculus also has a secretory function and releases HCl which helps in food digestion and protection from the microorganism (Zhu 2015). Further, the small intestine is the principal place for enzymatic activity and also for the absorption of amino acids, fatty acids, and carbohydrates (Igwebuike and Eze 2010; Mnati et al. 2021). It tends to be longer in granivorous and herbivorous birds as compared to the carnivorous (Duke 1997). Further, even among the various kinds of birds, there is a variation in the length and weight of the organs (Kausar et al. 2019).

Therefore, this study was designed to provide the basic histology information of the proventriculus in changeable hawk-eagle, oriental honey buzzard and domestic chicken, and intestine in southern cassowary and domestic chicken.

2 Materials & Methods

In the present study, a changeable hawk-eagle (*Nisaetus cirrhatu),* an oriental honey buzzard (*Pernis ptilorhynchus),* and a southern cassowary *(Casuarius casuarius*) were collected from Wildlife Rescue Centre (WRC) Jogja. These birds were necropsied based on the standard procedure applied in the center. At the same time, the domestic chicken (*Gallus gallus*) was obtained from the laboratory of Veterinary Histology, Faculty of Veterinary Medicine, Universitas Brawijaya, Indonesia, and proceeded through the standard procedure. During the procedure, No birds were otherwise harmed. During this study, proventriculus of changeable hawk-eagle, oriental honey buzzard, and domestic chicken were isolated and used for the histology examination.

Furthermore, from southern cassowary and domestic chicken, a segment of the intestine was obtained and proventriculus and intestine are preceded into histology. These organs were preserved by using 10% non-buffered formalin as a fixative. Then, these organs were dehydrated and cleared to pull out all the liquid from the tissue (Kusumarini et al. 2017) and sectioned in paraffin block for 5 um thick. Afterward, these organs were stained with Hematoxylin-Eosin for general identification and histology study.

3 Results

The results of the present study revealed significant differences in the proventriculus of the selected birds and these variations depending on the feeding habit (Figures 1 & 2A, B). Further, the results of the study suggested that the mucosa layer in the proventriculus of changeable hawk-eagle and oriental honey buzzard is thinner than domestic chicken (Figures 3 A & B). The shape of the submucosa gland in the submucosa layer showed irregular structure in domestic chicken, while it was oval-shaped in changeable hawk-eagle and diamond-shaped in oriental honey buzzard. Furthermore,

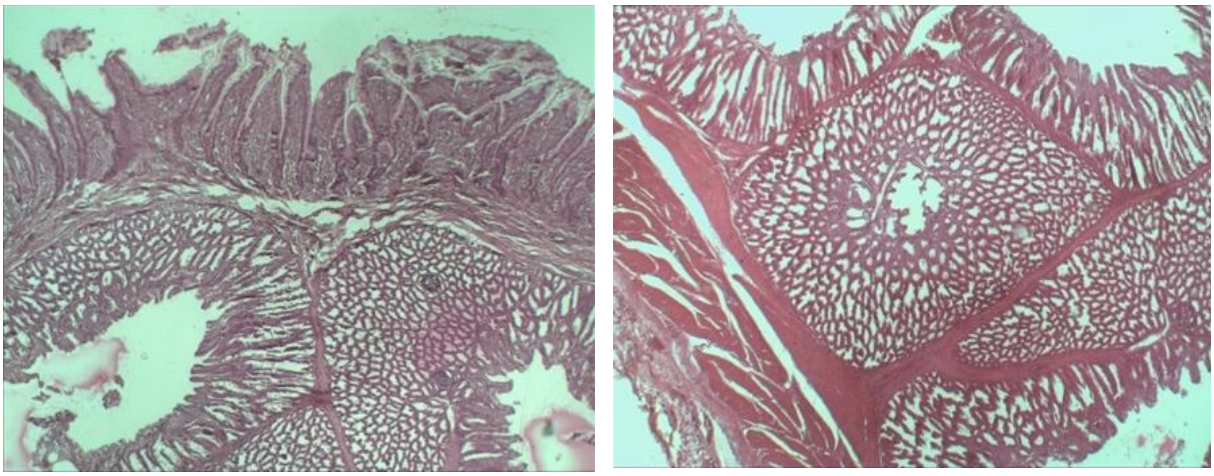

Figure 1 Thin section of the domestic Chicken Proventriculus (40x)

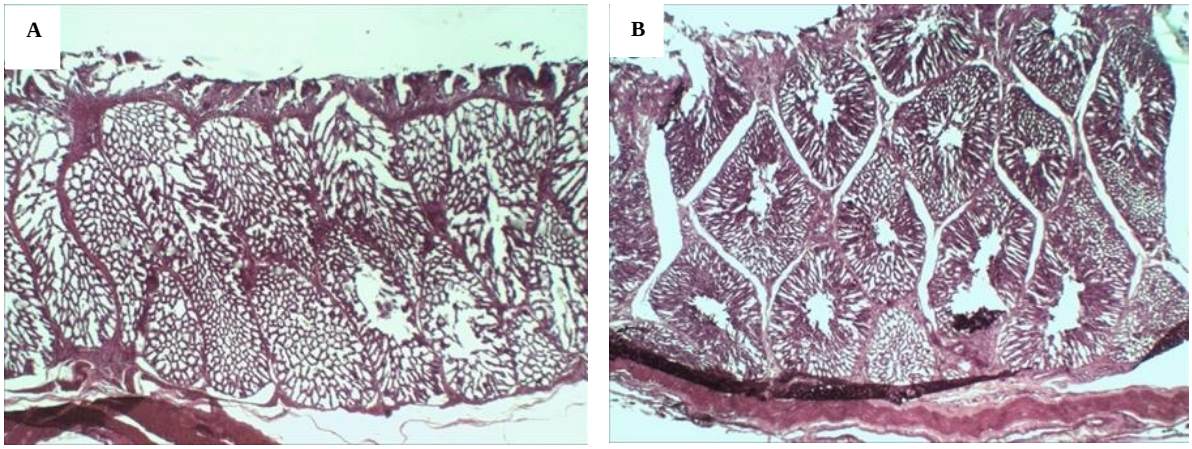

Figure 2 Thin section of (A) Changeable-hawk eagle proventriculus, and (B) Oriental honey buzzard proventriculus (40x)

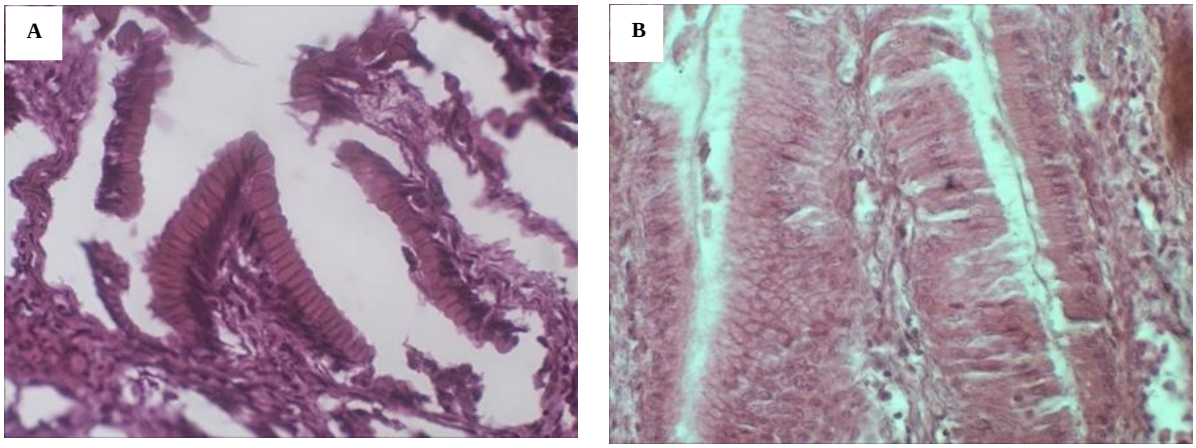

Figure 3 (A) Mucosa layer of Changeable-hawk eagle and (B) Mucosa layer of Domestic chicken (400x)

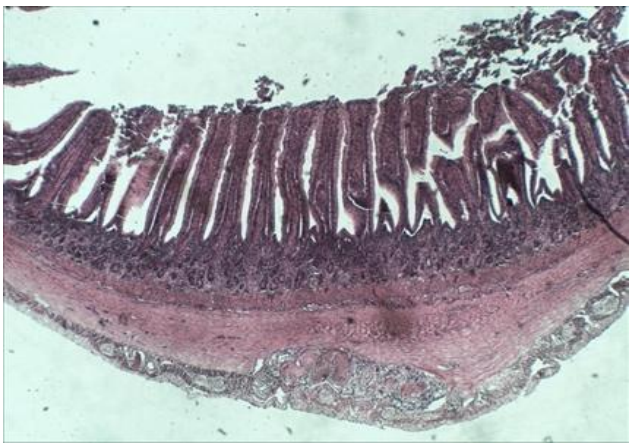

Figure 4 Duodenum of Domestic Chicken (40x)

the size of the submucosa gland is also varied in each species, and it was enormous in chickens whereas both in domestic chicken and changeable hawk-eagle, the epithelium in the mucosa layer is simple columnar. We believe that the intestine is a part of the duodenum. We find the Brunner's gland in domestic chicken and southern cassowary is denser. On the other hand, the tunica muscularis in southern cassowary is thicker than in domestic chicken (Figure 4 - 6).

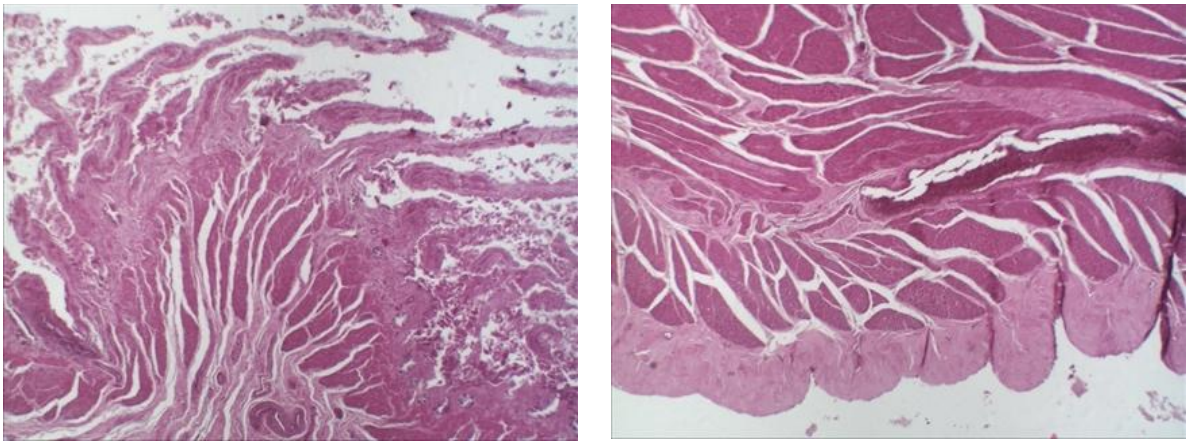

Figure 5 Duodenum of southern cassowary (40x)

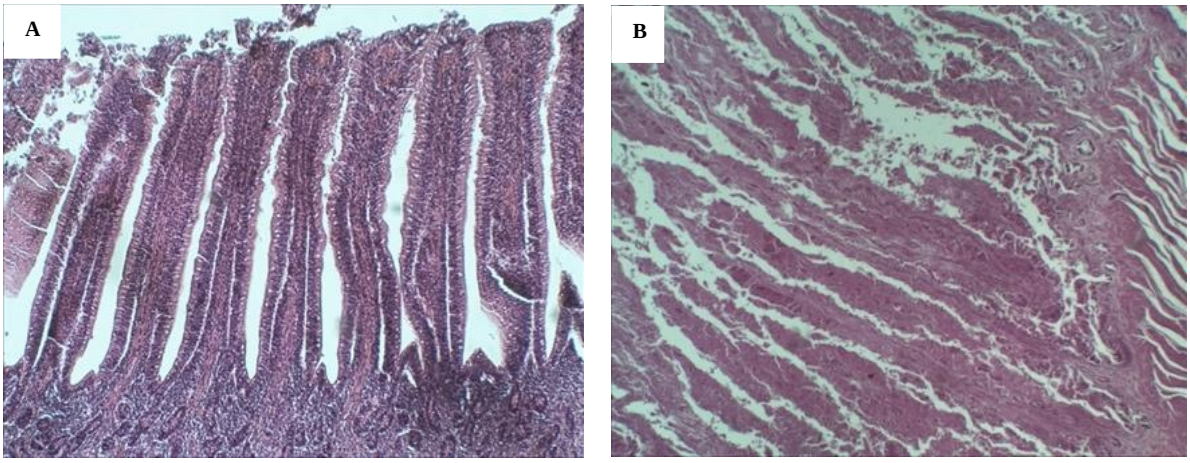

Figure 6 Duodenum Villi and Brunner's Gland of (A) Domestic Chicken and (B) Southern cassowary (100x)

4 Discussion and conclusions

Microscopic examination of proventriculus histology revealed that the chicken proventriculus layer is structured by mucosa, submucosa, muscularis, and serosa (Taher et al. 2020). The proventriculus of the chicken shows a mucosa layer that consists of simple columnar epithelium on its surface. This observation was similar to the result observed by Nasrin et al. (2012), where the author reported that the surface of the mucosa layer contains simple columnar epithelium. In the current study, it was reported that the mucosa layer of the changeable hawk-eagle is also simple columnar but lower in height than the chicken. The wall of the glandular stomach consists of submucosa glands or proventricular glands (Deka et al. 2017), which form irregular shapes in chicken, oval-like in changeable hawk-eagle, and diamond-like in oriental honey buzzard where each tubule was separated by connective tissue. The size of the glandular sub-mucosa, called adenomere, is varied, where chicken has the most significant size than others. These shape adenomere variation findings may lead to the different functions of the stomach between diet habits of birds.

The duodenum, where food molecules are absorbed (Alshamary et al. 2018), is constructed by four tissue layers i.e. tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa (Igwebuike and Eze 2010). In this study, domestic chicken villi form finger-like projections, whereas in southern cassowary these are more dynamic and longer. The Brunner's gland was denser in domestic chicken, and it can be easily recognized in the tunica submucosa. While in the southern cassowary, the Brunner's gland is slightly challenging to find. However, the tunica muscularis of the duodenum in the southern cassowary looks so thick, which consists of more muscular tissue than in the domestic chicken. Based on the results of this study, it can be concluded that there were apparent differences in some histological visually descriptive between species with different diets.

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Evaluation of DNA Isolation and Amplification from Various Organs Preserved through Frozen, Formalin-Fixed and Paraffin-Embedded Tissue Sample method

Mifta Rizqina Amalia[1], Anna Roosdiana[2], Yudit Oktanella[1], Andreas Bandang Hardian[1], Dini Agusti Paramanandi[1], Kharisma Kurnia Utami[1], Andi Tri Rakhmat Akbar[1], Made Venika Nareswari[1], Fajar Shodiq Permata[1*]

[1]Faculty of Veterinary Medicine, Brawijaya University, Puncak Dieng Eksklusif, Kalisongo, Kec. Dau, Kab. Malang 65151, Indonesia
[2]Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Kel. Ketawanggede, Kec. Lowokwaru, Kota Malang, Jawa Timur, 65145, Indonesia

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PCR

ABSTRACT

The purpose of this study was to compare the purity, concentration, and DNA band visualization of the isolated sample and PCR amplicon from three sample storage methods i.e. fresh frozen sample (-20^{o}C to -196^{o}C), preserved in formalin, and paraffin wax. For this tissue samples were collected from the sample stored at frozen temperature -20^{o}C, 10% NS formalin, and paraffin-embedded preparations, and Abs260/230 and Abs260/280 values and electrophoresis of 0.8% and 2% agarose gel visualization were analyzed. The results of the study showed a significant value of Abs260/280 for the isolated and amplified DNA purity. Among the tested three methods, frozen sample isolates and the PCR amplicon visualized a good DNA band. Meanwhile, the formalin-fixed and paraffinized tissue storage method showed a slightly lower quality DNA and no DNA band, respectively, while the PCR amplicon visualized a thin DNA band. In conclusion, all the tissue storage methods can be applied for DNA preservation and isolation, and the samples are successfully amplified on PCR examination.

* Corresponding author

E-mail: drh.fajar@ub.ac.id (Fajar Shodiq Permata)

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1 Introduction

The polymerase chain reaction is a molecular technique that allows in vitro DNA and gene amplification (Ehtisham et al. 2016). PCR can be used for diagnostic purposes to detect the presence of a specific DNA sequence of an organism such as an animal, plant, or microbial, it is also used for testing, diagnostics, or varietal selection. These days various types of PCR such as real-time PCR, quantitative PCR, Nested PCR, etc. are available for diagnostic and amplification purposes (Kadri 2019). The isolation of the DNA sample needs to be considered to obtain a good amplified result. This could be achieved by using the appropriate sample storage method which can protect the cell structures and DNA from damage.

The frozen storage method is widely used for tissue preservation at freezing temperatures (-20^{o}C to -196^{o}C) in liquid nitrogen. As a drawback, in this preservation method, there might be a chance of cell inactivation through tissue freezing, thereby inhibiting the rate of DNA damage (Wilson and Walker 2010). Cold temperatures also affect the fluidity, osmotic pressure, and tissue pH Intracellular ice formation can affect the cell's viability while the extracellular ice formation can cause an osmotic shift, leading to a concentration enhancement or reduction of extracellular fluid. This process can also damage cells' either by shrinking or swelling (Seawright et al. 2013). Therefore another alternative method is needed to avoid these reactions and simplify the storage process that does not require too many tools.

Formalin or formaldehyde is also widely used in tissue preservation for morphology, histological, and immunohistochemistry study. The diffusion process assisted formalin penetration into the cells by methylene glycol. In this method, aldehyde dehydrogenase that presents in the cell reacts to formalin and induces protein and nucleic acid cross-linking (Thavarajah et al. 2012; Groelz et al. 2013). Further, protein-DNA crosslinks binding occurs between cysteine, histidine, tryptophan, and lysine to deoxynucleosides, deoxyadenosine, deoxycytidine, and deoxyguanosine. The most common crosslinks are lysine and deoxyguanosine and cysteine and deoxyguanosine (Yu et al. 2015). In this manner, the formalin preservation method becomes potential and simple method of tissue storage for DNA isolation and PCR without using many tools as required in the freezing process.

Paraffin-embedded tissue preservation method has been also used in many clinical pathology laboratories. This method can be used for long-term storage from 1 month up to 5 years, at room temperature. According to Yi (2020), the paraffin-embedded tissue preservation method did not show any significant difference in extracted DNA and RNA purity as compared to the other method. Further, small-sized extracted DNA fragments were successfully amplified from most paraffin block samples for several years at room temperature (Nam et al. 2014). In this manner, this also becomes a potential preservation method for DNA isolation and the PCR amplification method used in medical diagnosis identification.

The pectoralis muscle is a skeletal muscle group that is composed of such muscle fibers that vary in size, shape, and arrangement of protein components. Differences in the number of DNA, RNA, and protein components will reflect cell population, size, and metabolic activity. Further, the chicken pectoralis muscle has a rapid growth and proliferation rate and is characterized by high cellular activities of satellite cells'. The higher tissue's cell proliferation indicates the high amount of DNA and high demand for cellular energy which is facilitated by mitochondria that have their own genetic material mtDNA (Mawaryana 2015).

The liver is one of the metabolic organs in the body and play important role in carbohydrate, lipid, and protein metabolism by bile juice secretion. Due to the high DNA synthesis peak of hepatocytes, the liver has a rapid and abundant regenerative capacity (Yagi et al. 2020). This indicates that the liver has a good amount of DNA to be isolated and amplified.

The chicken Growth Hormone (cGH) gene consists of 191 amino acids, located on chromosome 27 with a 4.1 kb length (Jafari et al. 2015). The cGH gene has a substantial influence on growth and metabolic function and is also expressed as Growth Hormone (GH) for body composition, egg production, reproduction, sexual maturation, and the central nervous system (CNS) functions (Khaerunnisa et al. 2017). This study aimed to compare the purity, concentration, and DNA band visualization of the isolated DNA sample and PCR amplicon from various organs preserved by three sample storage methods i.e. fresh frozen sample (-20^{o}C to -196^{o}C), preserved in formalin, and paraffin wax.

2 Materials and Methods

2.1 Paraffin-embedded preparation

The chicken pectoralis muscle and liver tissue were freshly collected and stored in 10% formalin. For this, 0.5 cm of the concerned tissue was trimmed and inserted into a tissue cassette. The tissue cassette was gradually soaked in ethanol 70%, 80%, 85%, 90%, 95%, ethanol absolute I, II, and III for 1 hour. Then it was soaked in xylol I, II, and III for 10 minutes and paraffin solution I, II, and III for 1 hour, respectively. Finally, the tissue was embedded with paraffin and stored.

2.2 Sample preparation

Broiler chicken liver and pectoralis muscle were collected from 10 Cobb Broiler chickens. In total, 30 samples were used, and these were divided into three equal groups for different storage methods. Five liver and pectoralis muscle tissue were stored at frozen

temperature -20°C, 10% NS formalin, and paraffin blocks. The frozen samples thawed to a lower temperature, and 10% NS formalin samples were rinsed with physiological saline (NaCl 0.9%) 3 times to remove the remaining formaldehyde in the tissue through the pipetting method.

The paraffin block samples were cut 100 µm using a microtome and placed into 1.5 ml PCR tubes. First, paraffin removal of the sample was carried out using 800 µl xylene, and the sample was homogenized with a vortex at a low speed for 10 minutes, then centrifuged at 14.000 rpm for 3 minutes. The supernatant was discarded using a micropipette without disrupting the pellet. This paraffin removal was repeated three times. After 2-3 times deparaffinization, the samples were gradually rehydrated with different alcohol concentrations. First, 800 µl of 100% alcohol was added to the tubes and homogenized with a vortex at a low speed for 10-15 seconds, then centrifuged at 14.000 rpm for 3 minutes. The supernatant was discarded using a micropipette without disrupting the pellet. Subsequently, this method was repeated two times with 800 µl of 70% and 50 % alcohol, and the supernatant was discarded using a micropipette without disrupting the pellet.

2.3 DNA Extraction

The DNA isolation procedure used the standard DNA isolation *Thermo Scientific Gene JET Genomic DNA Purification Kit*. The tissue sample was weighed (20 mg) and placed into a 1.5 ml Eppendorf tube, respectively. A total of 180 µl of digestion solution and 20 µl of Proteinase K were added into the tube and homogenized with a vortex for 10-15 seconds. Then the sample was incubated 2-3 hours at 56°C and homogenized using a vortex for every 30 minutes. After this, 20 µl of RNAse was added and homogenized using a vortex and incubated at room temperature for 10 minutes. This was followed by the addition of 200 µl of lysis solution and homogenization and at the end of the procedure, 500 µl of 50% ethanol was added and homogenized.

The sample was inserted into a collection tube and centrifuged at 6000 g speed for 1 minute. Afterward, the sample was taken in a new collection tube and 500 µl of wash buffer I was added and centrifuged at 8000 g for 1 minute. This was followed by the addition of 500 µl of wash buffer II and centrifuged at maximum speed for 3 minutes. In the end, 100 µl elution buffers were added to the fresh sample tube and 50 µl to the formalin and paraffin-embedded sample tube and incubated at room temperature for 2 minutes. The sample was centrifuged at 8000 g for 1 minute and isolated DNA was collected.

2.4 Polymerase Chain Reaction (PCR)

PCR tubes were prepared and added 2.5 µl of each isolated DNA sample. This was followed by the addition of a 5 µl PCR master mix solution and 2.5 µl Nuclease Free Water (NFW). Each tube was added with 1 µl cGH forward primer (5'-TCCCAGGCTGCGTTTTGTTACTC-3 ') and 1 µl cGH reverse primer (5'-ACGGGGGTGAGCCAGGACTG-3') with 429 bp targeted gene. Afterward, the PCR process was run using *miniPCR® mini8 thermal cycler bio™*.

2.5 Agarose gel electrophoresis

The agarose gel used was 0.8% for the DNA isolation sample and 2% for the PCR amplicon. The 0.8% agarose gel was made with 20 ml TBE (Tris-Borat EDTA) added with 0.16-gram agarose powder in a glass beaker, and 2% agarose gel was made with 20 ml TBE (Tris-Borat EDTA) was added with 0.4 gram agarose powder in another beaker glass. Both mixtures dissolved in a heated stirrer for 10-20 minutes. Subsequently, 2 µl peq-green (*ethidium bromide*) was added to the suspension and then inserted into the electrophoresis tray. It was set at room temperature for 10-15 minutes until the gel was dense.

An Agarose gel tray was placed on the electrophoresis machine. The DNA marker 100 bp DNA ladder, Load Ready™ was added (5 µl) to the first well. Then 10 µl of isolate DNA were added from the second well to the next well respectively, then 2 µl of loading dye (*bromophenol blue/* ethidium bromide) was added to each sample well. Afterward, the gel was run for about 25 minutes in blueGel™ Electrophoresis with a built-in transilluminator.

2.6 Statistical analysis

The purity dan concentration result was analyzed quantitatively using SPSS for windows with a one-way ANOVA statistical analysis. If there was a significant difference, proceed with the Tukey test $\alpha = 0.05$. Meanwhile, the quality was analyzed by the electrophoresis DNA band visualization.

3 Results

Most of the isolated samples have 260/280 nm and 260/230 nm values below the normal average (1.8-2 and 2.0-2.2, respectively). There is a significant value for the Abs 260/280 resulting from all the tissue storage comparisons with 0.000 values (Table 1). The paraffin-embedded pectoralis sample showed the lowest Abs260/230 nm value with 0.85±0.16. Meanwhile, the paraffin-embedded liver sample showed a better value (1.53±0.15). In addition to the Abs260/280 nm value, the paraffin-embedded pectoralis sample also has the lowest value with 0.74±0.18, followed by formalin-fixed pectoralis and liver tissue samples showed a lower value of 260/280nm (0.90 ±0.18 and 0.95 ±0.96 respectively). Paraffin-embedded pectoralis also showed the lowest concentration compared to the other samples, followed by paraffin-embedded liver samples. The Abs260/280 values then proceed to

Tukey test α = 0.05 (Table 3). PCR results showed all storage methods' showed uniform purity and concentration (Table 2). Although all of the purity levels were below the ideal absorbance average value (1.8-2 and 2.0-2.2). A significant value for the Abs260/280 proceeds to Tukey test α = 0.05 (Table 4).

The DNA bands were visualized only for frozen storage isolated samples with more than 3000 bp, while the formalin-fixed and paraffin-embedded isolated samples showed no DNA band (Figure 1A, 1B & 1C). The amplified DNA samples reached to the total DNA target at 429 bp (Figure 2A, 2B & 2C). The fresh-frozen pectoralis sample 1, 4, and 5 showed no amplified DNA band, and all formalin-fixed amplified samples visualized an amplified DNA band (Figure 2A). Fresh frozen and formalin-fixed liver tissue samples showed an amplified DNA band, with a thin band on samples 8, 9, and 10 (Figure 2B). In contrast, all the paraffin-embedded amplified samples showed very thin to no band on samples 6, 7, and 8 (Figure 2C).

Table 1 Mean of DNA isolation sample purity and concentration

DNA Isolation Sample	Abs260/230	Abs260/280	Concentration (ng/µl)
Frozen pectoralis	1.30 ± 0.61	1.07 ± 0.19	242.83 ± 270.79
Formalin-fixed pectoralis	1.08 ± 0.57	0.90 ± 0.18	223.10 ± 208.98
Paraffin-embedded pectoralis	0.85 ± 0.16	0.74 ± 0.08	7.69 ± 3.45
Frozen liver	1.33 ± 0.10	1.04 ± 0.08	114.90 ± 51.67
Formalin-fixed liver	1.05 ± 0.06	0.95 ± 0.96	150.98 ± 69.07
Paraffin-embedded liver	1.53 ± 0.15	1.18 ± 0.59	30.33 ± 10.33
Significancy value (P)	0.073	0.000	0.081

Values are average of five replicates; mean ± SE, *(P<0.05): significant

Table 2 Mean of DNA purity and concentration

PCR Sample	Abs260/230	Abs260/280	Concentration (ng/µl)
Frozen pectoralis	1.87 ± 0.12	1.62 ± 0.12	846.07 ± 169.80
Formalin-fixed pectoralis	1.79 ± 0.05	1.50 ± 0.03	899.68 ± 48.70
Paraffin-embedded pectoralis	1.85 ± 0.01	1.51 ± 0.01	908.46 ± 44.18
Frozen liver	1.83 ± 0.02	1.49 ± 0.06	891.73 ± 52.74
Formalin-fixed liver	1.80 ± 0.04	1.49 ± 0.04	969.70 ± 65.17
Paraffin-embedded liver	1.83 ± 0.02	1.48 ± 0.03	897.94 ± 26.46
Significancy value (P)	0.292	0.018	0.359

Values are average of five replicates; mean ± SE, *(P<0.05): significant

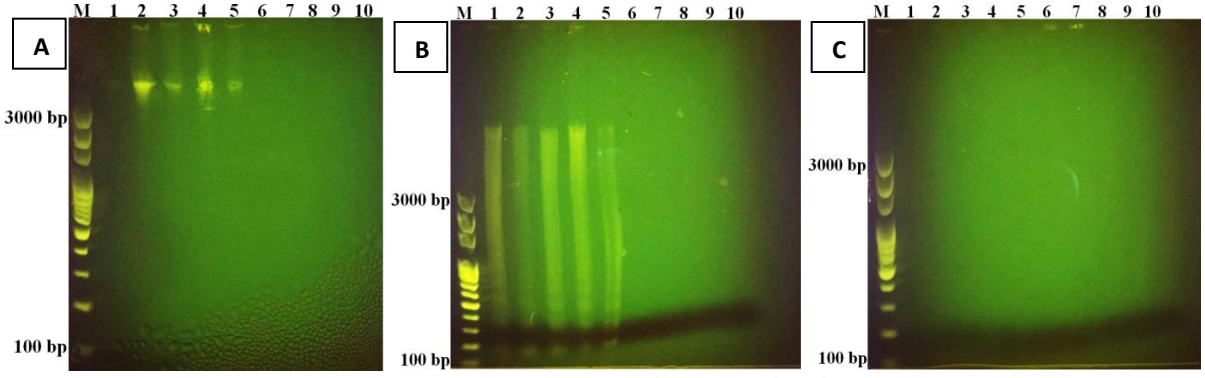

Figure 1 Agarose gel 0.8% concentration showing more than 3000 bp of isolated (A) fresh frozen pectoralis muscle sample storage DNA (1-5), (B) fresh frozen liver sample storage DNA (1-5), and (C) no DNA band of paraffin-embedded pectoralis and liver tissue (Here Lane M: DNA marker 100 bp-3000 bp; lanes 1-5: frozen pectoralis muscle DNA isolate; lanes 6-10: formalin-fixed pectoralis muscle DNA isolate).

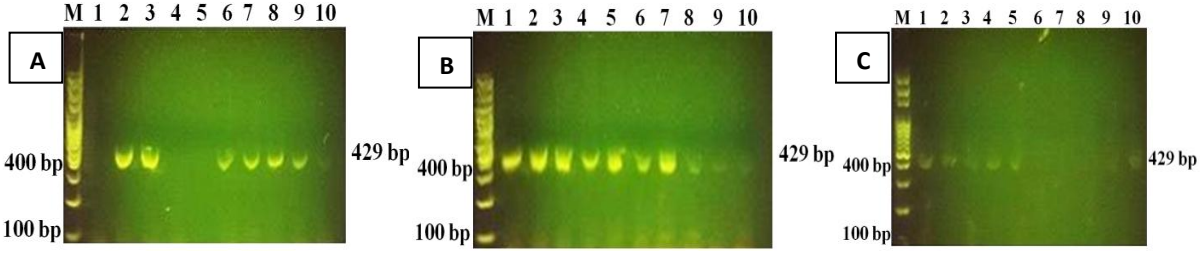

Figure 2 Agarose gel 2% concentration showing PCR amplicon band of (A) fresh frozen and formalin-fixed pectoralis muscle tissue 429 bp, (B) fresh frozen and formalin-fixed liver tissue 429 bp, and (C) paraffin-embedded liver tissue 429 bp (Lane M: DNA marker 100 bp-3000 bp; lanes 1-5: frozen pectoralis muscle PCR amplicon; lanes 6-10: formalin-foxed pectoralis muscle PCR amplicon).

Table 3 Tukey test of the Abs260/280 isolated DNA purity

(I) Storage	(J) Storage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Frozen pectoralis	Formalin-fixed pectoralis	.17200	.07990	.296	-.0750	.4190
	Paraffinated pectoralis	.32800*	.07990	.005	.0810	.5750
	Frozen liver	.02600	.07990	.999	-.2210	.2730
	Formalin-fixed liver	.12000	.07990	.666	-.1270	.3670
	Paraffinated liver	-.11000	.07990	.740	-.3570	.1370
Formalin-fixed pectoralis	Frozen pectoralis	-.17200	.07990	.296	-.4190	.0750
	Paraffinated pectoralis	.15600	.07990	.397	-.0910	.4030
	Frozen liver	-.14600	.07990	.468	-.3930	.1010
	Formalin-fixed liver	-.05200	.07990	.986	-.2990	.1950
	Paraffinated liver	-.28200*	.07990	.019	-.5290	-.0350
Paraffinated pectoralis	Frozen pectoralis	-.32800*	.07990	.005	-.5750	-.0810
	Formalin-fixed pectoralis	-.15600	.07990	.397	-.4030	.0910
	Frozen liver	-.30200*	.07990	.010	-.5490	-.0550
	Formalin-fixed liver	-.20800	.07990	.135	-.4550	.0390
	Paraffinated liver	-.43800*	.07990	.000	-.6850	-.1910
Frozen liver	Frozen pectoralis	-.02600	.07990	.999	-.2730	.2210
	Formalin-fixed pectoralis	.14600	.07990	.468	-.1010	.3930
	Paraffinated pectoralis	.30200*	.07990	.010	.0550	.5490
	Formalin-fixed liver	.09400	.07990	.843	-.1530	.3410
	Paraffinated liver	-.13600	.07990	.544	-.3830	.1110
Formalin-fixed liver	Frozen pectoralis	-.12000	.07990	.666	-.3670	.1270
	Formalin-fixed pectoralis	.05200	.07990	.986	-.1950	.2990
	Paraffinated pectoralis	.20800	.07990	.135	-.0390	.4550
	Frozen liver	-.09400	.07990	.843	-.3410	.1530
	Paraffinated liver	-.23000	.07990	.078	-.4770	.0170
Paraffinated liver	Frozen pectoralis	.11000	.07990	.740	-.1370	.3570
	Formalin-fixed pectoralis	.28200*	.07990	.019	.0350	.5290
	Paraffinated pectoralis	.43800*	.07990	.000	.1910	.6850
	Frozen liver	.13600	.07990	.544	-.1110	.3830
	Formalin-fixed liver	.23000	.07990	.078	-.0170	.4770

*. The mean difference is significant at the 0.05 level; Dependent Variable: Abs260/280; Tukey HSD

Table 4 Tukey test of the Abs260/280 amplified DNA purity

(I) Storage	(J) Storage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Frozen pectoralis	Formalin-fixed pectoralis	.11600	.03923	.067	-.0053	.2373
	Paraffinated pectoralis	.10400	.03923	.123	-.0173	.2253
	Frozen liver	.13000*	.03923	.031	.0087	.2513
	Formalin-fixed liver	.13000*	.03923	.031	.0087	.2513
	Paraffinated pectoralis	.13200*	.03923	.027	.0107	.2533
Formalin-fixed pectoralis	Frozen pectoralis	-.11600	.03923	.067	-.2373	.0053
	Paraffinated pectoralis	-.01200	.03923	1.000	-.1333	.1093
	Frozen liver	.01400	.03923	.999	-.1073	.1353
	Formalin-fixed liver	.01400	.03923	.999	-.1073	.1353
	Paraffinated pectoralis	.01600	.03923	.998	-.1053	.1373
Paraffinated pectoralis	Frozen pectoralis	-.10400	.03923	.123	-.2253	.0173
	Formalin-fixed pectoralis	.01200	.03923	1.000	-.1093	.1333
	Frozen liver	.02600	.03923	.984	-.0953	.1473
	Formalin-fixed liver	.02600	.03923	.984	-.0953	.1473
	Paraffinated pectoralis	.02800	.03923	.978	-.0933	.1493
Frozen liver	Frozen pectoralis	-.13000*	.03923	.031	-.2513	-.0087
	Formalin-fixed pectoralis	-.01400	.03923	.999	-.1353	.1073
	Paraffinated pectoralis	-.02600	.03923	.984	-.1473	.0953
	Formalin-fixed liver	.00000	.03923	1.000	-.1213	.1213
	Paraffinated pectoralis	.00200	.03923	1.000	-.1193	.1233
Formalin-fixed liver	Frozen pectoralis	-.13000*	.03923	.031	-.2513	-.0087
	Formalin-fixed pectoralis	-.01400	.03923	.999	-.1353	.1073
	Paraffinated pectoralis	-.02600	.03923	.984	-.1473	.0953
	Frozen liver	.00000	.03923	1.000	-.1213	.1213
	Paraffinated pectoralis	.00200	.03923	1.000	-.1193	.1233
Paraffinated pectoralis	Frozen pectoralis	-.13200*	.03923	.027	-.2533	-.0107
	Formalin-fixed pectoralis	-.01600	.03923	.998	-.1373	.1053
	Paraffinated pectoralis	-.02800	.03923	.978	-.1493	.0933
	Frozen liver	-.00200	.03923	1.000	-.1233	.1193
	Formalin-fixed liver	-.00200	.03923	1.000	-.1233	.1193

*. The mean difference is significant at the 0.05 level; Dependent Variable: Abs260/280 ; Tukey HSD

4 Discussion

Formalin-fixed tissue samples showed lower DNA isolation purity than the fresh-frozen tissue samples and a higher concentration than the paraffin-embedded tissue sample. A lower purity absorbance is caused by the protein-DNA crosslinks that remain in the isolated sample. The presence of a protein still attached to DNA can lower the purity result. Protein is linked with formalin in the N-terminus site of the amino acid chain, in which the formalin acts as the bridge from amino acids to DNA, where the formalin connects to the base site of DNA. These crosslinks can also induce a higher concentration because formalin protein-DNA crosslinks also induce DNA fragmentation. This fragmentation process potentially gives rise to a higher number of formalin-fixed tissue sample concentrations than the paraffin-embedded ones. This mechanism will alter the hydrogen bonds of nucleic acid base pairs and cause DNA fragmentation. Based on Kennedy-Darling

and Smith (2014), crosslink reversal can be optimally achieved with heat exposure, such as heating the sample at 120^{o}C for 25 to 40 minutes before the DNA isolation process (Mehdi and Reza 2012). Accordingly, one-time temperature exposure in the DNA isolation process is not enough to detach the formed links that affect the DNA purity and concentration.

The paraffin-embedded samples showed the most distinct DNA isolation purity and concentration compared to other storage methods. Long-term paraffin blocks the tissue-making process and potentially causes DNA damage by degradation and denaturation. The tissue soaked in different solutions from dehydration, clearing, and paraffin-embedding processes will influence the cell condition within the tissue. This is related to the cell condition that has to suffer against osmotic to pH changes. Furthermore, a lower purity value can be caused by the paraffin remains in the DNA isolate possibly preventing the aromatic ring of the nitrogenous base from absorbing UV light in the spectrophotometry. Thus, it results in a decrease in absorbance ratio value.

Although fragmentation occurs due to tissue preservation based on the DNA isolation process, the tissue PCR amplicon produced uniform purity and concentration value. Related to the crosslink reversal process, the heating temperature phases in the thermal cycler can be an influencing factor to open the protein-DNA linkage. The repeated heat exposure exponentially helps denature the residual protein-DNA crosslinks that are still attached from the DNA isolation process and become detached entirely so that the DNA is successfully amplified. These results indicate that the DNA fragments from each storage method can still serve as templates for target genes to yield a good value of DNA concentration obtained through amplification.

In electrophoresis visualization, the phosphate group of the negatively charged DNA will be ionized to migrate in the gel matrix toward the positive charge area. The ethidium bromide (EtBr) was added to the samples before electrophoresis to "stain" the DNA. It will bind between the nucleic acid bases that have a hydrophobic region. The hydrophobic environment will induce EtBr to fluorescence by the UV light and visualizing the DNA band. Consequently, the DNA damage will not provide that hydrophobic environment for EtBr to visualize.

Moreover, the DNA damage that already occurs also leads to alteration of ionized DNA structure which will decrease the DNA sensitivity to electrophoresis electric current. Hence, this could be the reason for a good DNA purity and concentration value but results in the absence of a DNA band on the gel visualization. Compared to the fresh frozen samples, the isolated formalin-fixed and paraffin-embedded tissue samples have a good purity ratio but can decrease the DNA quality. Therefore, these samples can still be amplified to the targeted number of genes.

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Incidence of Multidrug Resistance *Escherichia Coli* Bacteria in Broiler Chickens in Malang Regency

Fidi Nur Aini Eka Puji Dameanti[1*], Jeffrys Salsabila[1], HarioPuntodewo Siswanto[1], Indah Amalia Amri[1], Sruti Listra Adrenalin[1], Mira Fatmawati[1], Taty Aryanti[2]

[1]Faculty of Veterinary Medicine Universitas Brawijaya, Indonesia
[2]Center for Veterinary Research Bogor, Indonesia

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Multidrug Resistance

ABSTRACT

Chicken meat is an important source of protein but the presence of bacterial infections such as colibacillosis is a major concern for the chicken producers. Further, colibacillosis is a major cause of mortality, morbidity, and economic loss for the poultry industry. Various efforts including the use of antibiotics have been carried out to treat colibacillosis. Recently, inappropriate use of antibiotics not only induced antibiotic resistance, but sometimes it might change into multidrug resistance due to a large number of antibiotic uses. This study aimed to identify the incidence of multidrug resistance in broiler chickens on 4 farms of Malang Regency. For this, samples of 40 chicken jejunum swabs that had a history of colibacillosis with clinical symptoms of lethargy, drooping, dwarfism, hair loss, depression, thinness, diarrhea, abdominal swelling, and osteoarthritis were used in this study. Testing begins with a microscopic examination, followed by the isolation of *E. coli* on Nutrient broth (NB) and Eosin Methylene Blue Agar (EMBA) media, and finally, antibiotic sensitivity was tested against eight antibiotics namely Gentamicin, Bacitracin, Enrofloxacin, Doxycycline, Oxytetracycline, Erythromycin, Colistin, and Amoxicillin on Mueller-Hinton Agar (MHA) media. The microscopic observations showed that the chickens had a hemorrhage in the proventriculus and intestines, pericarditis, and fibrinous exudate in the air sacs and heart. Among the tested samples, 72.5% (29 samples) were found positive for *E. coli*. Further, in case of antibiotic resistance, 100% of *E. coli* positive samples were found resistant to Erythromycin, Bacitracin, and Amoxicillin, 96.6% to Enrofloxacin, 92.6% to Oxytetracycline, 37.9% to Colistin and Doxycycline, 10.3% to Gentamicin. Results of the study can be concluded that most of the E.coli positive samples have antibiotic resistance and the maximum samples are showing multidrug resistance against four or more antibiotics.

* Corresponding author
E-mail: drhfidi@ub.ac.id (Fidi Nur Aini Eka Puji Dameanti)

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1 Introduction

Chicken is a source of fulfillment of animal protein needs in Indonesia. Broiler farms of Malang regency are the second largest producer of broiler chickens with 28,929,203 chickens in East Java. These days' chicken industries are facing many challenges including the meet of growing community demand and strict supervision of the chicken's health. Further, pathogenic microorganisms such as viruses, bacteria, and various other parasites are hazardous to the health of broilers because these are consumed by humans. Among the broiler, colibacillosis also known as mushy chick disease and cellulitis is the most common one and is caused by the localized or systemic infection of *Escherichia coli* bacteria. This disease caused the highest morbidity, mortality rates, and economic loss, and it has a terrible impact on broiler production (Ibrahim et al. 2019). In this disease, *E. coli* colonized in various organs of broiler chicken such as omphalitis, perihepatitis, pericarditis, mesenteries, and others (Suryani et al. 2014). Frequent or uncontrolled use of antibiotics is the most common method used by the Indonesian chicken producer for the treatment of colibacillosis. However, as time goes on, more and more antibiotics become ineffective in treating *E. coli* infections.

Shecho et al. (2017) reported antibiotic resistance in E. coli against various antibiotics such as erythromycin, clindamycin, spectinomycin, ciprofloxacin, ampicillin, and amoxicillin, and suggested that among the tested antibiotics, E. coli had highest antibiotic resistance against the ampicillin (92.3%) while had lowest resistance against the amoxicillin (34.61%). If it continues, *E. coli* might develop a significant resistance against the various groups of antibiotics which are considered multidrug resistance. Information regarding the colibacillosis infection and multidrug resistance in Malang regency, Indonesia chicken farms are in scanty; therefore this study was carried out to identify the incidence of multidrug resistance in four broiler chicken farms of Malang Regency.

2 Materials and Methods

This research was conducted in July - August 2020 at the Laboratory of Anatomical Pathology, Faculty of Veterinary Medicine, Universitas Brawijaya, which includes the process of necropsy and swab of broiler chicken intestines. Identification and antibiotic sensitivity testing was carried out at the Laboratory of Microbiology and Immunology of the Faculty of Veterinary Medicine, Universitas Brawijaya.

2.1 Used Media and Materials

The tissue culture media and other materials used for the isolation and multiplication of *E. coli* isolates from broiler chicken intestine swabs COBB strain are phosphate-buffered saline (PBS), Nutrient Borth (NB) media (Merck® no 105443), Eosin Methylene Blue Agar (EMBA) media (Oxoid® CM0069), Triple- Sugar Iron Agar (TSIA), Simmon Citrate Agar (SCA), Sulfide Indole Motility (SIM) media, Urease media, Methyl Red (MR), Voges-Proskauer (VP) media, media catalyze, Glucose medium, Lactose medium, Sucrose medium, and Gram stain set (crystal violet, Lugol, acetone alcohol, safranin). Media Mueller-Hinton Agar (Oxoid® CM0337) supplemented with various group of antibiotics i.e. Erythromycin (Oxoid® CT0019B @ 10 µg concentration), Colistin (Oxoid® CTOO17B @ 10 µg concentration), Bacitracin (Oxoid® DD0002 @ 10 µg concentration), Enrofloxacin (Oxoid® CT0639B @ 5 µg concentration), Doxycycline (Oxoid® CT0018B @ 30 µg concentration), Oxytetracycline (Oxoid® CT0041B @ 30 µg concentration), Gentamycin (Oxoid® CT0024B @ 10 µg concentration), and Amoxicillin (Oxoid® CT0061B @ 10 µg concentration).

2.2 *E. coli* Isolation and Identification

This research used a descriptive laboratory method carried out by qualitative laboratory examination. Samples were collected from the broiler chickens aged between 14-17 days from the 4 farms of Malang Regency, East Java, Indonesia. Determination of the sample using 10% disease prevalence, sampling error rate (5%), and sampling confidence level (95%) using the Win Epi application (Thrusfield et al. 2001). The sample was selected by purposive sampling by considering the symptoms of suspected chickens suffering from colibacillosis.

The first stage of this study was to examine the chicken body as a whole to see clinical symptoms of chickens suspected of colibacillosis, followed by euthanasia by the decapitation method, and dissection of the carcass and examination of each organ to find out the pathological changes. Wahyuwardan et al. (2014) studied the results of necropsy regarding the anatomical modifications and identified *E. coli* from the jejunum swab of broiler chickens and recommended that *E. coli* can be isolated from the jejunal intestine by swab technique.

In this study, the swab sample was taken using sterile cotton and inoculated on Nutrient Borth (NB) (Merck® no 105443), and incubated at 37ºC for ± 24 hours. After incubation, it continued with primary isolation on Eosin Methylene Blue Agar (EMBA) (Oxoid® CM0069) media using the streak method and incubated at 37ºC for 24 hours. The metallic green colonies from EMBA were subject to Gram staining to see the microscopic morphology of the isolated bacteria, and biochemical tests consisting of IMViC test (Indole test, Methyl Red test, Voges Proskauer test, and Citrate test), sugar test (glucose, lactose, and sucrose), catalase test, and urease test was carried out to identify the biochemical properties of *E. coli* (Fardiaz 1992).

2.3 Antibiotic Sensitivity

The isolated *E. coli* strains were tested for antibiotic sensitivity. A total of eight types of antibiotics belonging to six groups of antibiotics were tested against the isolated *E.coli* strain. The selection of used antibiotic types was based on interviews with farmers regarding the often used antibiotics.

The disc diffusion method including the Kirby-Bauer method or filter paper disk was used for estimating the antibiotic sensitivity. The diameter of the inhibition zone was measured and analyzed for all the tested antibiotics. The standard used to determine the level of antibiotic sensitivity is based on the Clinical and Laboratory Standards Institute (CLSI) standards.

2.4 Statistical Analysis

The research results analysis was descriptive, describing the data from the isolation and identification of *E. coli* from broiler chicken intestine swabs and quantitatively analyzing the data from the diameter of the antibiotic inhibition zone obtained.

3 Results and Discussion

3.1 Necropsy

Samples that meet the criteria of inclusion are systematically operated on starting by examining the poultry body as a whole, followed by euthanasia using the decapitation method and necropsy according to the poultry necropsy protocol. Each organ of the euthanized chickens has been examined for the finding out the pathological changes (Figure 1). Examination of the carcass condition includes fluid that comes out of natural holes, nutritional status, abnormal formations, skin, wattles, cloaca, and the presence of external parasites (Poultry Industry Council of Canada 2016).

Figure 1 Chickens with Clinical Symptoms of Colibacillosis

According to Hastarinda (2016), clinical symptoms of chickens suffering from colibacillosis are generally characterized by thinness, dull fur, decreased appetite, depression, disturbed growth, diarrhea, and dirty feathers in the cloaca area. According to Mahari (2014), other clinical signs that can be found are skeletal lesions and continued systemic infection. This is following the results of the pre-euthanasian examination carried out in this study. All the collected 40 samples showed similar symptoms such as lethargy, drooping, and stunted growth. Other symptoms that appeared

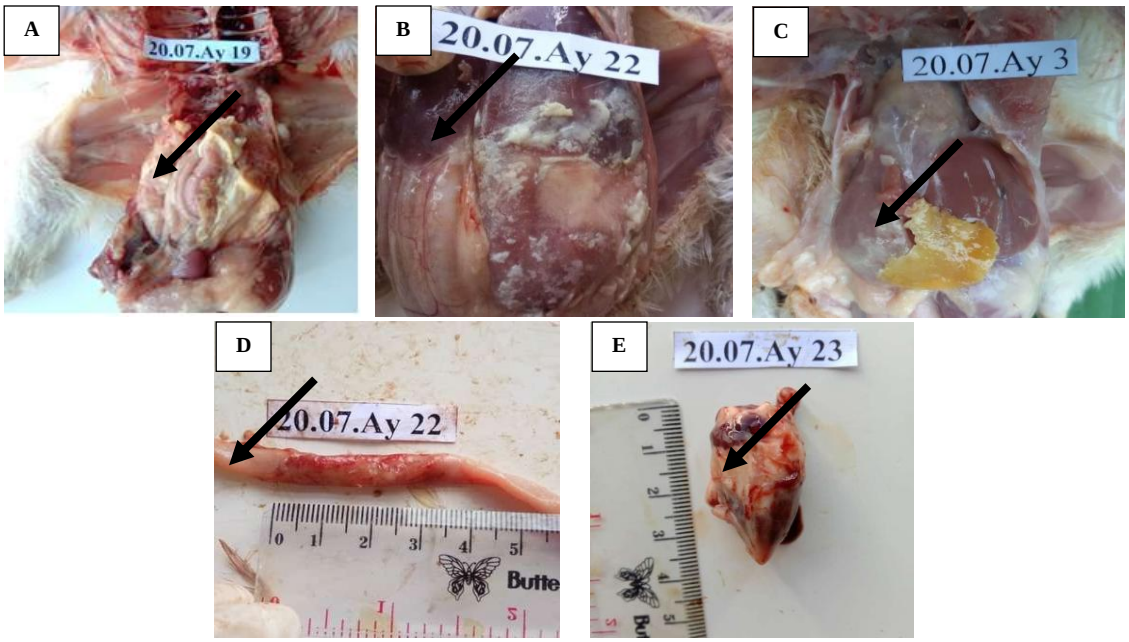

Figure 2 Appearance of macroscopic lesions resulting from the necropsy of poultry (A - fibrinous exudate on the air sac; B - Fibrinous exudate on the peritoneum; C - Chewy exudate on the liver; D - Intestinal hemorrhage; E - Pericarditis

during the study were hair loss (11 samples), depression (29 samples), thinness (28 samples), diarrhea (12 samples), and swelling in the abdominal area (7 samples).

The most common pathological changes reported from the collected broiler chicken samples after necropsy were bleeding in the proventriculus and intestines, pericarditis, and fibrinous exudate in the intestines, liver, air sacs, and heart (Figure 2). According to Mahari (2014), E. coli that infects the intestine can enter the bloodstream and infect other organs in systemic cases. *E. coli* that infects tissue will cause an inflammatory response to increase the cytokine IL-1, IL-6, and tumor necrosis factor-alpha. This inflammatory response increases vascular permeability and increases the accumulation of fluid and protein in the tissues to form a gelatinous exudate. The thick exudate becomes a visible exudate that accumulated and, in the end, caseation occurs to form a compact mass like cheese, dry and yellow.

3.2 Isolation of *E. coli*

The purpose of the necropsy is to see pathological changes and treat the swab site namely the jejunum swab. Isolation of *E.coli* was carried out on the EMBA media to obtain metallic green colonies for *E. coli* (Prawesthirini et al. 2009). The results of colony growth can be seen in Figure 3. EMBA media is a differential selective media for *E.coli* (Lindquist and John 2004), and it formed metallic green colonies on this medium by the end product of lactose fermentation by acidic *E. coli* resulting in the absorption of methylene blue color to form metachromatic, which gives the absorption of methylene blue a metallic green sheen. This medium can also inhibit the growth of Gram-positive bacteria (Lal and Cheeptham 2007). Isolation of *E. coli* from the jejunum swab of broiler chickens in the jejunum section showed that the total samples suspected to be positive for *E. coli* were 33, with a percentage of 82.5%.

3.3 Identification of *E. coli*

3.3.1 Gram stain

Metallic green colonies on EMBA media were confirmed by using Gram stain and revealed the presence of Gram-negative *E. coli* in the form of colibacillosis (Figure 4). According to Ulfah et al. (2017), *E. coli* produced metallic green colonies on EMBA, and due to the absence of a thick peptidoglycan wall; it absorbs pink-red staining in Gram staining and the shape of bacteria is rod-shaped and fimbriae. Based on the identification stage with Gram staining, it was found that all 33 samples were colibacillosis and Gram-negative bacteria.

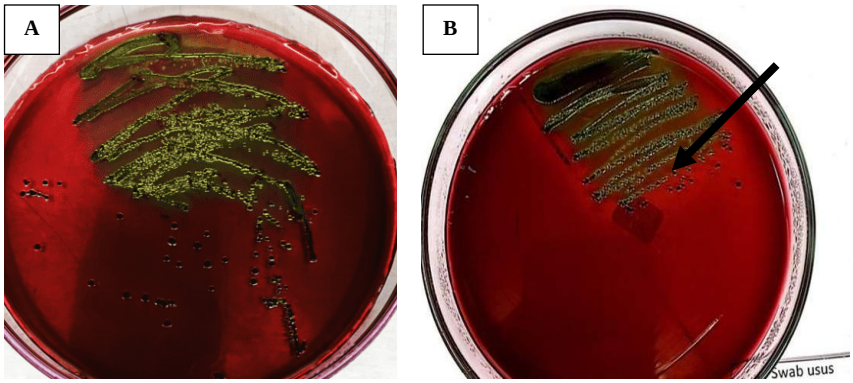

Figure 3 E. coli colony on EMBA media (A - Primary isolation; B - Secondary isolation contained separate E. coli colonies)

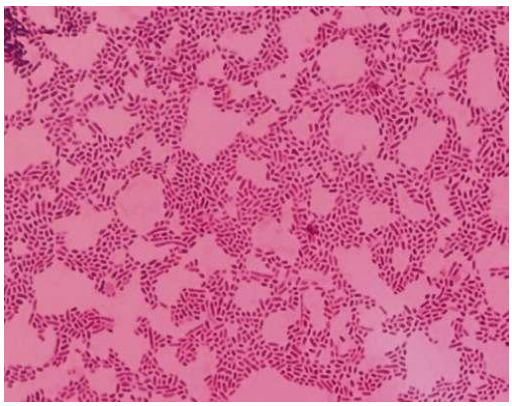

Figure 4 Gram stain results *in Escherichia coli* with 1000 x magnification

3.3.2 Biochemical Test

Samples that have been identified through Gram staining are subjected to biochemical tests consisting of the IMViC test (Indole test, Methyl Red test, Voges Proskauer test, and Citrate test), sugar test (glucose, lactose, and sucrose), catalase test, TSIA, and urease test (Figure 5- 12). Biochemical tests were carried out for differentiation based on differences in the biochemical properties of bacteria. Speciation between bacteria can be distinguished based on sugar fermentation, metabolic materials, and food ingredients based on biochemical properties. The biochemical test results showed that there were 29 samples (72.5%) were positive for *E. coli*. Elements of chemical requirements between various bacteria are also different in carbon, nitrogen, sulfur, phosphorus, metal compounds, oxygen, growth factors, and nutrient uptake (Dzen et al. 2010).

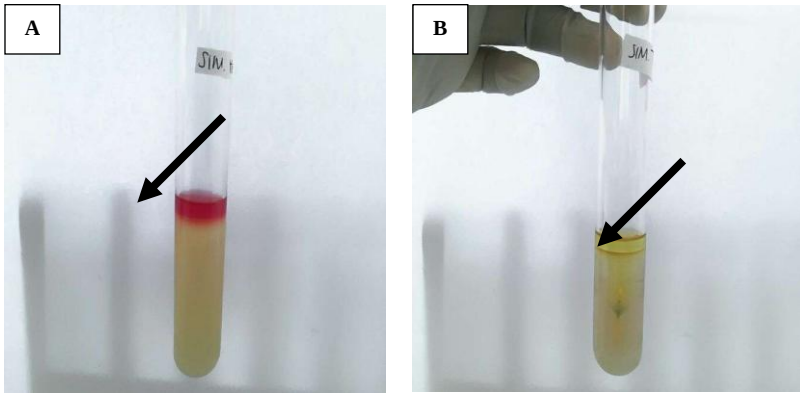

Figure 5 Results of Indole test (A - Positive indole forms a red ring; B - Negative indole does not form a red ring)

Figure 6 Results of Methyl Red - Positively marked media color change

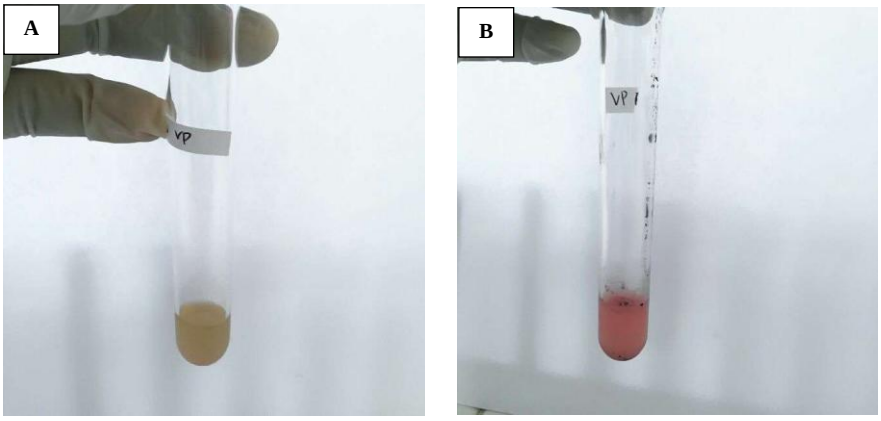

Figure 7 Results of Voges Proskauer (A - Voges Proskauer negative; B - Voges Proskauer positive)

Figure 8 Results of Citrate negative

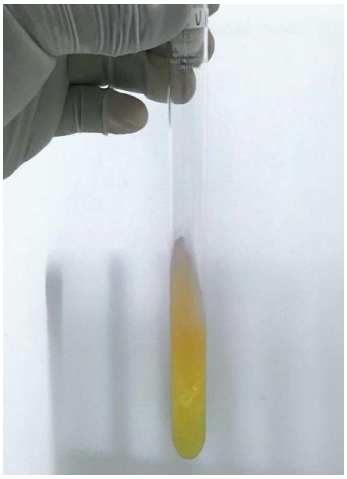

Figure 9 Results of Urease test as a negative result, media remains Yellow

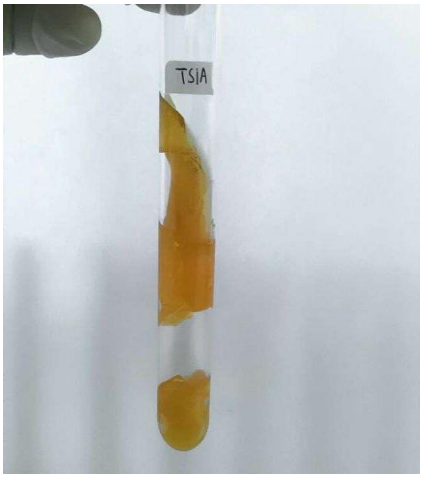

Figure 10 Results of TSIA test; Slant and Butt on the media changes color to yellow, and gas is formed

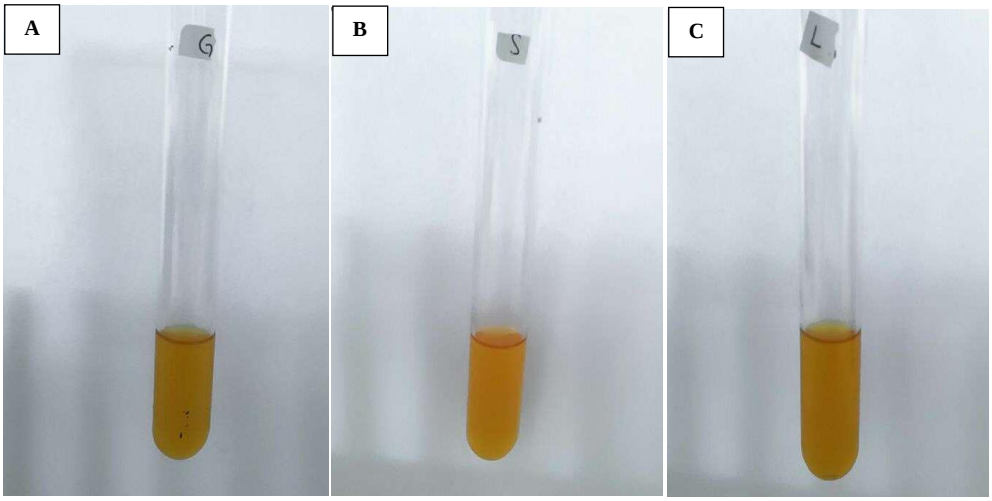

Figure 11 Results of Confectionery test (A) Glucose positive, (B) Sucrose positive, (C) Lactose positive

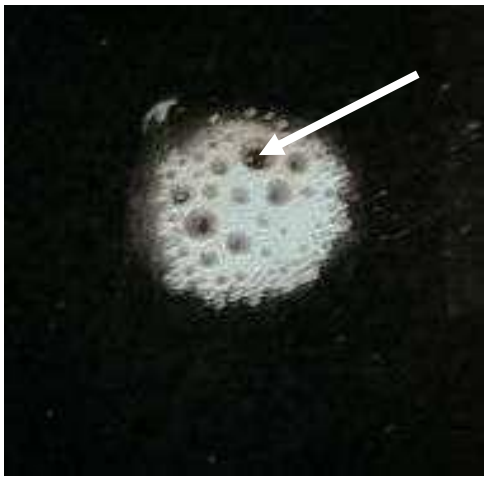

Figure 12 Results of Positive catalase showed bubble formation

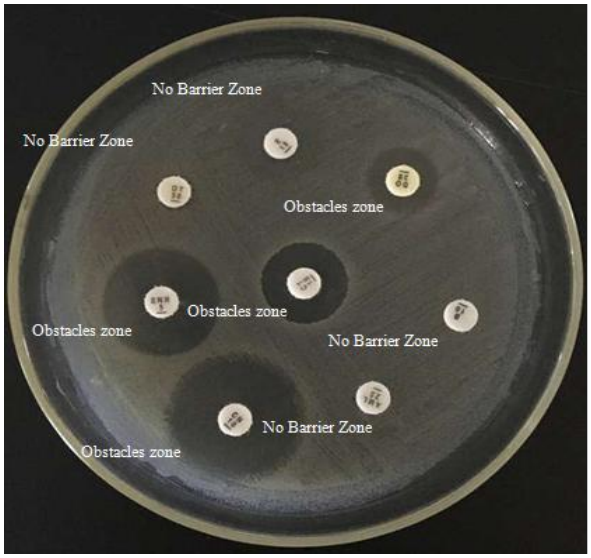

Figure 13 Observation of the diameter of the antibiotic inhibition zone

Table 1 Antibiotic sensitivity test results

No.	Sample Code		AB Susceptibility Test (mm)							
			E (15µg)	CT (10µg)	ENR (5µg)	DO (30µg)	OT (30µg)	CN (10µg)	B (10µg)	AML (25µg)
Amount and Percentage		S	0	18	1	11	2	22	0	0
			0.00	62.10	3.40	37.90	7.40	75.80	0	0
		I	0	0	0	7	0	4	0	0
			0.00	0.00	0.00	0.00	0.00	13.70	0	0
		R	29	11	28	11	27	3	29	29
			100.00	37.90	96.60	37.90	92.60	10.30	100	100

S (Susceptible), I (Intermediate), R (Resistant), E (Erythromycin), CT (Colistin), ENR (Enrofloxacin), DO (Doxycycline), OT (Oxytetracycline), CN (Gentamicin), B (Bacitracin), AML (Amoxicillin)

Table 2 Pattern of Multi-Drug Resistance in *E. coli* Isolates

Total Group	Antibiotics group	n	%
4	Macrolides + Peptides + Fluoroquinolones + Penicillins	1	3.45
	Macrolides + Peptides + Tetracyclines + Penicillins	1	3.45
5	Macrolides + Peptides + Fluoroquinolones + Aminoglycosides + Penicillins	1	3.45
	Macrolides + Peptides + Fluoroquinolones + Tetracyclines + Penicillins	24	82.76
6	Macrolides + Peptides + Fluoroquinolones + Tetracyclines + Aminoglycosides + Penicillins	2	6.90
	Amount	29	100

3.3.3 Antibiotic Sensitivity Test

Twenty-nine samples with confirmed *E. coli* were continued with the antimicrobial susceptibility test. The disc diffusion method including the Kirby-Bauer method or filter paper disk was used for estimating the antibiotic sensitivity and as a result of this study, the inhibition zone was measured which indicated the absence of bacteria growth in the area around the antibiotic disk (Figure 13). The diameter produced by this test indicates the nature of the bacteria to antibiotics based on the standards of each antibiotic, namely sensitive (S), intermediate (I), and resistance (R) (CLSI 2018). The results of the study showed that 100.00% of *E. coli* isolates were resistant to Erythromycin, Bacitracin, and Amoxicillin while 96.60% were resistant to Enrofloxacin, 92.60% to Oxytetracycline, 37.90% to Colistin and Doxycycline, and lowest resistance of 10.30% was reported for the Gentamicin (Table 1).

Table 2 shows that all samples of *E. coli* isolate experienced MDR and 6.90% of the samples were resistant to 4 and 6 classes of antibiotics while 86.21% were resistant to 5 classes of antibiotics. The results of the current study are in line with the findings of Bushen et al. (2021) those who reported a high incidence of MDR (52.5%) in healthy chickens taken from Southwest Ethiopian farms. Noor and Poeloengan (2005) suggested that inappropriate use of antibiotics due to misdiagnosis, too harsh treatment, wrong doses, and continuous use of antibiotics as growth promoters are some important causes of multi-drug resistance. According to Utami (2011), the use of antibiotics in livestock as growth promoters agents or as growth stimulators of livestock in subtherapeutic doses will increase the risk of multi-drug resistance.

Conclusion

There are 29 of 40 samples (72.50%) collected from 4 farms in Malang Regency, East Java was found positive for *E. coli*. The results of antibiotic sensitivity testing showed that 100% *E. coli* isolates were resistant to Erythromycin, Bacitracin, and Amoxicillin, while 96.6% to Enrofloxacin, 92.6% to Oxytetracycline, 37.9% to Colistin and Doxycycline and 10.3% to Gentamicin. All isolates of *E. coli* showed the occurrence of MDR against four or more classes of antibiotics.

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Effect of the combined application of Lampung Robusta Coffee Extract and *Lactobacillus acidophilus* on the Ileum and Caecum Histopathology in *Salmonella enterica* infected Balb/C Mice

Indah Amalia Amri[1*], Dhyas Meilani[1], Muhammad Zuliono D. R. P.[1], Dahliatul Qosimah[1], Dodik Prasetyo[1], Djalal Rosyidi[2], Lilik Eka Radiati[2]

[1]Faculty of Veterinary Medicine, Brawijaya University, Indonesia
[2]Faculty of Animal Science, Brawijaya University, Indonesia

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ABSTRACT

Salmonella enterica is a gram-negative bacterium that can cause Salmonellosis and gastroenteritis in humans and animals. Further, this bacterial infection is also associated with the reactive oxygen species (ROS) by lipid peroxidase that can destroy the intestinal cell's membrane. This study aimed to evaluate the preventive effect of the combined application of Lampung Robusta coffee extract and *Lactobacillus acidophilus* on the Ileum and Caecum Histopathology in *Salmonella enterica* infected Mice. In this study, male Balb-c mice aged between 8-10 weeks and weight 20-25 grams were used, these experimental animals were divided into six experimental groups namely K^- (Negative control without any infection), K^+ (Positive control with *S. enterica*), KL (Only *L. acidophilus* treated mice), P1, P2, and P3 were given a preventive extract of coffee with a concentration of 250 mg/kg BW, 500 mg/kg BW, and 750 mg/kg BW respectively and *L. acidophilus* to *S.enterica* infected mice and arrange in completely Randomized Design. Descriptive histopathological analyses were carried out after HE staining and villi's length and width for ileum's histopathology and counting goblet cells for caecum's histopathology was scored. The results of the study revealed that administration of Robusta Coffee extract @ 250 mg/ kg BW and *L. acidophilus* has a preventive effect on the ileum and caecum damage caused by salmonellosis.

* Corresponding author
E-mail: indahamaliaamri@ub.ac.id (Indah Amalia Amri)

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1 Introduction

Salmonella enterica belongs to the family Enterobacteriaceae and is associated with food-borne gastroenteritis infection in humans. *S. enterica* serovars *enteritidis* is one of the serovars associated with human salmonellosis and increases the cases of salmonellosis in humans. It infects the human body through contaminated food or drinks and causes gastroenteritis with diarrhea, stomach cramps, and bacteremia (Asminarti 2014). Furthermore, *S.enteritidis* infection also triggers inflammation in the intestine region, especially in the ileum and cecum. This inflammatory process will increase the number of free radicals (ROS) due to bacterial phagocytosis. In this manner, ROS will damage the cell components and the cell will lose its integrity and, if it occurs continuously, will cause tissue damage and oxidative stress. At higher stress, the epithelial cells also damage villi and disrupted the process of absorption of nutrients.

A wide range of antibiotics has been used for the treatment of *S. enterica* infection. However, their use is limited because, in some cases, antibiotic administration can cause local hypersensitivity to the skin, allergic reactions, and toxic reactions, as well as bacterial resistance to antibiotics that arise due to the excess and inappropriate use of antibiotics and this is one of the reasons of antibiotic treatment failure (Asminarti 2014). Natural sources of antibiotics can be used as an alternative to the synthetic antibiotic. According to Muhammad and dan Mumun (2015), coffee is a rich source of caffeine and chlorogenic acid, and among these, caffeine can damage bacterial cell amino acids and cause lysis, while chlorogenic acid of coffee can damage the polarity of the bacterial cell walls, in this manner coffee, can serve as an alternative natural antibiotic. Further, chlorogenic acid as an antioxidant can cut the chain oxidation reaction from free radicals so that free radicals will not react with other cells, and damage organs (Wulandari 2016).

Higher consumption of coffee increases the caffeine content in the body and this enhances the level of HCl production and indigestion. High HCl levels will irritate the intestinal mucosa, so excessive coffee consumption has a risk of intestinal ulcers (Rizkiani 2009). For this reason, it is difficult for coffee to be used singly so in this study, Lampung Robusta Coffee is combined with probiotics to avoid the drawbacks of excess coffee use.

Probiotics compete with pathogenic bacteria on the intestinal mucosa and have various beneficial effects on the host (Anggarini 2011). *Lactobacillus acidophilus* is one of the lactic acid bacteria that can be used as Probiotics. This bacterium has anti-microbial properties and can be inhibited the growth of pathogenic bacteria such as *Salmonella.* It can produce lactase, and vitamin K, which inhibited the adhesion or growth of bacteria (Senditya 2011). *L. acidophilus* also functions as an immunomodulator (Galdeano et al. 2019). Robusta coffee also has various oligosaccharides that serve as a source of nutrition for *L. acidophilus* bacteria (Grace 2017). Therefore, this study aimed to determine the effect of the combined application of Lampung Robusta coffee extract with *L. acidophilus* on the histopathology of the ileum and cecum of mice infected by *S. enterica*.

2 Materials and Methods

2.1 Preparation of Lampung Robusta Coffee Extracts

Lampung Green Robusta coffee extract was prepared at the Pharmacology Laboratory of the Faculty of Medicine, Universitas Brawijaya, Indonesia. For this, 100g of coffee powder was taken into the Erlenmeyer flask and mixed with 900ml of 90% ethanol. The prepared solution is shaken for 30 minutes and soaked overnight, this solution was taken into the evaporator flask and evaporated for 1.5-2 hours at 90°C in a water bath. The obtained extract was roasted and weighed to obtain a fixed extract weight (Juliantari 2018).

2.2 Animal Models

As an animal model, 24 male Balb-c mice aged between 8-10 weeks and weight 20-25 grams were used, these animals were divided into 6 cages and acclimatized for 7 days. Mice that have met BB standards are given a BR-1 feed and drink which is replaced every day.

2.3 Culturing of *Lactobacillus acidophilus* bacteria

Pure culture of *L. acidophilus* bacteria was obtained from the Faculty of Medicine, Universitas Brawijaya, Indonesia, and cultured on MRSA media with 1% $CaCO_3$. The cultured bacterial strain was confirmed by Gram staining and catalase test and bacterium that has been confirmed as *L. acidophilus* are multiplied on the Nutrient broth media. The turbidity level was confirmed using Mc Farland 0.5×10^8 to obtain bacteria with 10^8 CFU / ml concentration.

2.4 *Salmonella enteric* serovars *enteritidis infection in Balb-C mice*

Pure culture of *S. enterica* serovars *enteritidis* has been obtained from the Great Hall of Veterinary Wates, Yogyakarta, cultured on the BSA media, and culture plates were incubated at 37°C. Blackish brown colonies on the nutrient media revealed the presence of the *S. enteric*, which was further confirmed by the Gram staining. Identified *S. enterica* serovars *enteritidis* has been multiplied on the Nutrient broth. The bacteria were then tested using Mc Farland 0.5×10^8 to obtain bacteria with a concentration of 10^8 CFU/ml. Before infecting with *S. enterica* mice were kept on fasting and this was followed by giving 0.5 ml of drink with *S. enterica* culture using gastric sonde for 2 days.

2.5 Giving combinations to mice

Mice showing *S. enterica* infection symptoms were identified and used for coffee extracts and *L. acidophilus* treatment. These experimental animals were divided into six experimental groups namely K^- (Negative control without any infection), K^+ (Positive control with *S. enterica*), KL (Only *L. acidophilus* treated mice), P1, P2, and P3 were given a preventive extract of coffee with a concentration of 250 mg/kg BW, 500 mg/kg BW, and 750 mg/kg BW respectively and *L. acidophilus* and arrange in completely Randomized Design. The coffee extracts solution and *L. acidophilus* was taken in a 1 ml syringe and given with gastric sonde. This combination was given every day for 15 days. The mice's weight was calculated every 7 days, and their doses were recalculated to match the weight of the mice.

2.6 Necropsy and preparation of ileum and cecum

Before necropsy, mice were weighed first, and this was followed by cervical dislocation. These mice were taken for a necropsy by following standard procedure and ileum and cecum are taken out for histopathological observation.

2.7 Preparation of Ileum and cecum

The excised organs were cleaned with NaCl and stored in an organ pot containing 10% formalin for fixation. This was followed by the dehydration with 50-95% alcohol series, for each alcohol percent, 15 minutes were assigned. After this, organs were taken for clearing alcohol with the help of toluol fluid for 120 minutes. Then the organs were fixed in the paraffin wax and 6µm thin sections were prepared with the help of a microtome. Paraffin wax was removed in a water bath, rehydrated with 100%, 90%, 80%, and 70% alcohol for every 5 minutes, and fixed with xylol followed by Canada balsam.

2.8 Histopathology Observation

Histopathology was observed using optilab and Olympus microscopes at 100-400 magnification. Observations were made to observe the signs of inflammation such as villi and epithelial damage, inflammatory cell infiltration, and goblet cell hypoplasia.

2.9 Data Processing

Histopathology of ileum and cecum was analyzed by qualitative descriptive method with a light microscope type CX31RBSFA and photographed using optilab at 100-400x magnification. Several observations were made on the histopathology of the ileal organs, including inflammation cells, villi length, width, and the number of goblet cells. According to Harimurti and Rahayu (2009), measurements of villi length, villi width, and crypt depth are minimum of using three visual fields per slide with the help of the Imageraster application. The length of the villi is measured from the apex of the villi to the basal villi. The villi width was measured on the basal villi, and the crypt depth was measured starting from the basal villi to the basement membrane. The unit used to measure villi length and width is a micrometer (µm). In the histopathology of the caecum, the number of goblet cells is calculated by taking histopathic photographs using optilab at 400x magnification in as many as 5 fields of view/repetition, the total number obtained later in the average and scoring (Stecher et al. 2005).

3 Results and Discussion

3.1 Ileum histopathology

The Ileum histopathology examination aims to determine the degree of damage in each treatment. In this study, observations were taken using the HE staining method and 5 random fields in one preparation using a magnification of 100x and 400x. Measurement of villi length, width, and crypt depth using 100x magnification while observation of villi erosion, goblet cells, and inflammatory cells was taken at a 400x magnification.

The measurement of villi length and width are presented in Table 1. Measurement of villi length and width aims to support the scoring data of villi observations. According to Erben et al. (2014), villi scoring starts from the shortening of the light villi, which is the ratio of the length of the villi and the crypts to 2: 1 - 3:1 with a score of 1-3. In the shortening of villi, the ratio between the length of villi and crypt becomes 1: 1 to 2: 1 with a score of 2-4, and if there is villous atrophy score was recorded between 3-5 (Table 1).

Observations in mice of the negative control group (K^-) with a magnification of 400x (Figure 1) did not show any significant changes in villi with mild scoring (3). This is supported by the villi length and width measurements in Table 1. In negative controls, villi size looks uniform even though they have a shorter length when compared to the positive control group. In addition, no erosion or rupture was found in the villi, so it can be said that the villi are in good condition. Good villi conditions can improve the process of absorption of nutrients. These results are in line with the results of Putra and dan Kusdiyantini (2018), where feces of mice in negative control showed normal conditions. An indicator of inflammation is the infiltration of inflammatory cells in the intestinal villi and it can be seen with the widening of the intestinal villi.

Based on the changes in villi, the positive control group (K^+) experienced a shortening of villi size with moderate scoring (4), which means that the ratio between villi length and crypts is approximately 1: 1. Further, *Salmonella* sp. infection can cause intestinal epithelial damage. When it invading to intestinal tissue, it damages the intestinal epithelium. Based on observations, positive

Table 1 Effect of Lampung Robusta coffee extract and *L. acidophilus* applications on the various parameters of *S. enterica* infected mice intestine

Treatment Group	Villi's Length and Width		Villous blunting Score	Fecal Characteristics		Goblet's cells and predefined criteria	
	Length (µm)	Width (µm)		Fecal Characteristics	Observation	Average Cells/Field	Scoring
K^+	257.4	93.7	Moderate (4)	Oval forms; Black and Hard	Normal	29.2	0
K^-	228.1	66.2	Mild (3)	Not oval shape; Brownish; Flaccid, and little bit slimy	Diarrhea (4)	22.25	1
KL	347.4	84.3	Mild (1)	Not oval shape; Brownish, and little bit slimy	Diarrhea (3)	28.00	1
P1	305.3	73.2	Mild (2)	Oval forms; Hard and Brownish	Normal	26.73	1
P2	268	66.8	Mild (2)	Oval forms; Black, and little bit slimy	Diarrhea (3)	24.05	1
P3	249.9	74.9	Moderate (1)	Oval forms; Brownish and little bit slimy	Diarrhea (3)	24.06	1

control group mice ileum showed epithelial erosion and hypertrophy in goblet cells, infiltration of inflammatory cells consisting of PMN (Polymorphonuclear), and MN (Mononuclear). The type of PMN that can be observed is neutrophils. According to Rosales (2018), *Salmonella* sp. infection first disturbed the morphology of neutrophils. Infiltration of inflammatory cells indicated the presence of antigens that enter the body, one of which is pathogenic bacteria such as *Salmonella* sp. As the number of inflammatory cells in the tissue increases, the villi will look more expansive.

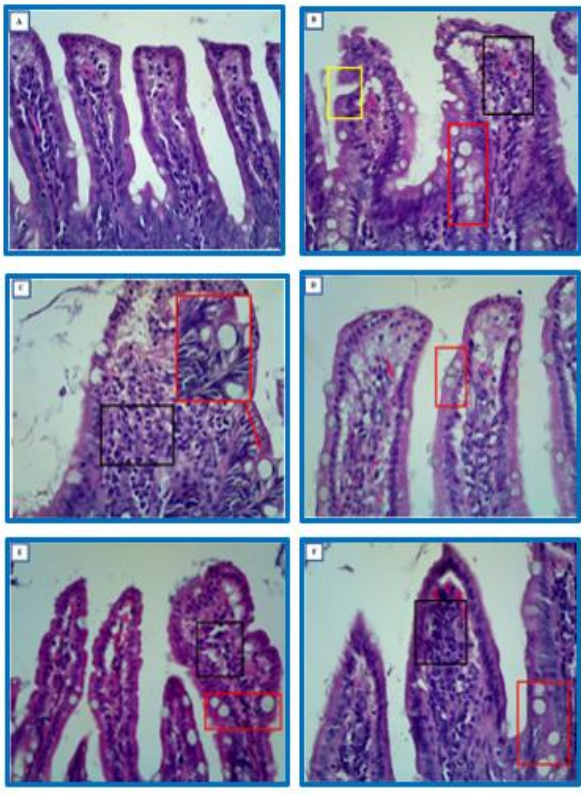

Figure 1 Microscopic observation of mice's Ileum at 400x magnification, (A) negative control, (B) positive control (C) *L. acidophilus* control, (D) Treatment P1, (E) Treatment P2, and (F) Treatment P3

Further, in the positive control, an increase in the activity of goblet cells can be seen. This is related to the body's form of compensation for the presence of antigens that can cause damage to organs. The body tries to eliminate antigens that enter the body by increasing the activity of goblet cells in mucus secretion. Increased mucus secretion aimed to protect epithelial cells from damage. Increased mucous secretion and villous damage in positive control mice can be seen in Table 1 (Balqis et al. 2015).

In the *L. acidophilus* control group (KL), goblet cell hypertrophy increases mucous production. *Salmonella* sp. can trigger cell inflammation from the blood vessels into the tissues (Figure 1C). However, *L. acidophilus* control showed no epithelial erosion compared to positive control. Lactic acid bacteria can protect the mucosal lining by colonizing the intestinal mucosal surface, making pathogenic bacteria unable to adhere. Results presented in Table 1 revealed that in *L. acidophilus* control villi length increased which might be associated with the probiotics administered. Uptake of probiotics stimulates epithelial cell proliferation and increased the production of the fatty acids which positively affect the length of villi.

In treatment groups P1, P2, and P3, several abnormalities were reported between goblet cell hypertrophy and inflammatory cell infiltration into the tissues. Entry of the *Salmonella* sp., into the body, triggers the release of various inflammatory mediators including histamine. Histamine can cause vasodilation of blood vessels. At the time of vasodilation of blood vessels, blood plasma comes out and causes edema. In the current study, the villi length of P1 (Figure 1D) has increased compared to the P2 (Figure 1E)

and P3. Further, the most severe damage has been reported in the P3 group (Figure 1F), which had the most petite villi length in all three treatment groups. This might be due to the high dose of Lampung Robusta coffee extract. According to Selviana (2015), the caffeine content of the coffee triggers excessive gastric acid secretion and damages the gastric and intestinal mucosa. Further, in mice group P2 the types of inflammatory cells were polymorphonuclear and mononuclear (Figure 1E). According to Adenin (2019), injured tissue activates the release of several cells, including leukocytes, erythrocytes, and platelets. In bacterial infections, leukocyte cells that play an important role are neutrophils. Neutrophils play a significant role in the first 24-48 hours of infection. Monocytes will assist neutrophils, later turning into macrophages after exiting the blood vessels. Among the tested three doses, the most severe level of organ damage was reported in P3 (histopathology of the ileum; Figure 1F), and this might be due to an inflammation that can elicit intestinal motility and affect the absorption of nutrients and fluids (Keraru 2017).

3.2 Histopathological Examination of the cecum

Histopathological results of a healthy negative control group without any treatment (K^-) had a normal cecum where the epithelial cells (columnar layer) were visible without any damage and normal goblet cells and did not show hypoplasia (Figure 2A). Both width and length of villi look the same without any widening due to infiltration of inflammatory cells. The histopathology of this group will be compared with other treatment groups.

The histopathology of the positive control group (K^+) showed that due to severe erosion or rupture, villi lost their original shape. Along with this, no epithelial compartment cells appeared, goblet cells were almost absent or hypoplastic, and the size of submucosal edema and villous were also reduced (Figure 2B). All these symptoms are associated with the *S. enterica* infection, which has LPS, Vi, H, and O antigens that disturbed the appearance of inflammatory cells. Further, Macrophages also produced ROS as a defense mechanism and this ROS destroyed the infection-causing bacteria. Increased ROS, an oxidant, can continuously damage the composition of the epithelial lipid membrane which damage the structure of the villi. According to Marchelletta et al. (2014), *S. enterica* infection in mice will cause submucosal edema, caecal edema, goblet cell hypoplasia, and damage to the caecal epithelium. Submucosal edema occurs due to increased permeability caused by the infiltration of inflammatory cells to the site of infection. Similarly, goblet cell hypoplasia is also caused by the infection of *S. enterica*, and in the intestine, these cells respond by removing mucus continuously and these cells became empty, shrink, and disappear in the HE coloring (Songhet et al. 2011).

The *L. acidophilus* control group (KL) showed poor results and various damage including erosion in some epithelium, villi fusion, and submucosal edema in villi. However, an increase in the number of goblet cells or hyperplasia was reported which represented better villous conditions as compared to the positive control (Figure 2C). Goblet cell hyperplasia seen in histopathy is

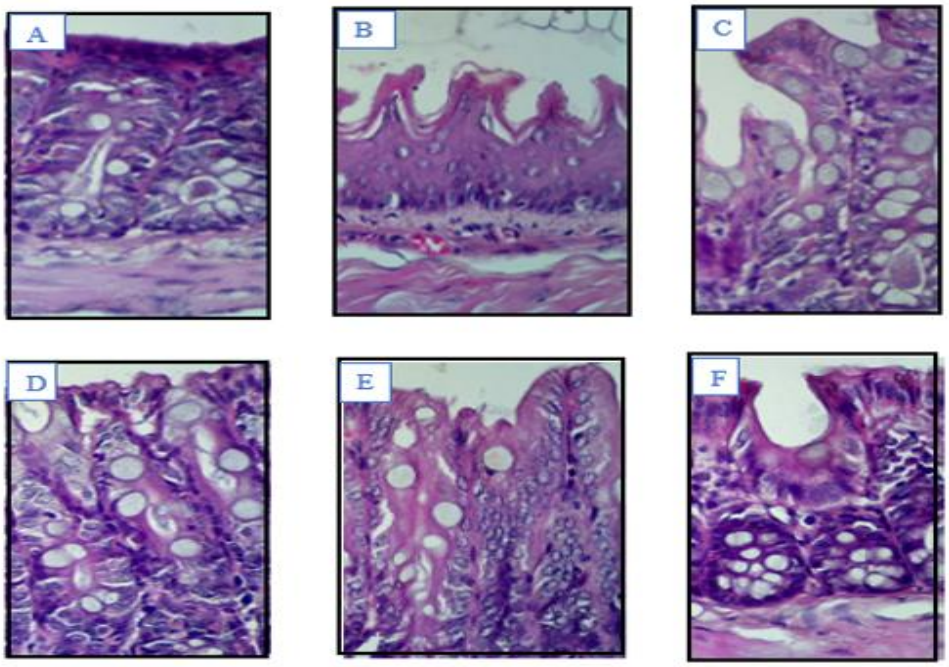

Figure 2 Microscopic observation of mice's caecum at 400x magnification; (A) negative control, (B) positive control (C) *L. acidophilus* control, (D) Treatment P1, (E) Treatment P2, and (F) Treatment P3

caused by the induction of *L. acidophilus* as a preventive function known as immunomodulators, especially IgA which increases mucous production. According to the Galdeano et al. (2019) mucus acts as the first-line defense against the antigens by binding with Ig A and forming "immune exclusion," then capturing and killing bacteria so that bacteria cannot affect adhesion to villous intestines. Arguello et al. (2017) state that administering high *Salmonella* inflammation reduced the expression of genes that encode bile acid transport. It interferes with the absorption of bile salts in the intestine and high bile salts in the intestine can reduce the amount of *L. acidophilus* in the intestine.

Experimental animal groups P1, P2, and P3 were treated with a combination of coffee extract and *L. acidophilus*. These three groups did not show any significant difference among themself and histopathological examination also did not show damage in villi as reported in the positive or *L. acidophilus* control. Here combined application of coffee extract and probiotic *L. acidophilus* play a significant role in mitigating the harmful effect of the *S. enteric* infection. Results are in agreement with the findings of Galdeano et al. (2019), those who reported the immunomodulatory role of *L. acidophilus* and competition between *L. acidophilus* and *S. enteric* which will reduce the chance of *S. enterica* infection and villi deformation.

Lampung Robusta coffee extract is a reach source of various alkaloids, including caffeine, chlorogenic acid, flavonoids, tannins, and saponins which not only have immunomodulatory properties but also antibacterial properties that work to reduce *S. enterica* infection. Chlorogenic acid also has antioxidant properties which can neutralize the ROS and prevented villous damage (Wulandari 2016). Cecum histopathology of treatment P1 is not much different from the negative control histopathology where no forms of damage such as erosion or rupture of villi, change in goblet cells were reported and the mucosa also appeared intact with the shape of a thumb (Figure 2D). Histopathology of the treatment group P2 (Figure 2E) and P3 (Figure 2F) also looked good and did not show any erosion and rupture in the epithelial cells. However, in treatment group P2, submucosal edema appeared with irregular villous form, whereas in treatment group P3, more visible submucosal edema and monocyte infiltration was reported. Further, results of the histopathological cross-section revealed that the combination of P1, namely Lampung Robusta coffee extract @ 250 mg/kg BW and L. acidophilus has a significant effect in preventing the cecum damage because its histopathic appearance approaches negative control.

Goblet cells are secretory cells found in the intestine and the number of these cells increases in the intestine crassum, especially in the distal colon and rectum. These cells are essential for mucus synthesis and secretion to the intestine lumen. This mucus lubricates the lumen and provides a defense against pathogenic bacteria (Bergstrom et al. 2008). Based on goblet cells number, Stecher et al. (2005) suggested 4 important categories i.e. 0 (the average number of goblet cells is more than 28 cells/field of view), 1 (the average number of goblet cells is 11-28 cells/field of view), 2 (the average number of goblet cells 1-10 cells/field of view), and 3 (the average number of cells is less than 1 cell/field of view).

Results of the study revealed that the average number of goblet cells in the negative control group (K^-) was 29.2 and it was included in the scoring 0 (Table 1). This result is in agreement with the findings of Stecher et al. (2005), who said that the average number of normal goblet cells in SPF mice was 28 cells/field of view. Further, the number of goblet cells in the positive control (K^+) has the lowest number, although it scored 1, this insignificant decrease in goblet cells is associated with the *Salmonella* infection, which focuses on attacking Payer patches which are dominant in the ileum. As per Santos et al. (2003) cecum does not have Payer patches, that's why in the current study less damage was recorded to the goblet cells and they scored 1. Further, Mansson et al. (2012) also suggested that *S. enterica* infection can cause epithelial damage and proliferation, continuing to deplete or reduce goblet cells. According to Songhet et al. (2011), *S. enteric* infection enhances the mucous discharge from goblet cells, and with time the goblet cells became empty and shrink and finally these cells disappear in HE staining. In this manner, the results of this study are in line with the previous findings.

The *L. acidophilus* treatment group also showed a score of 1, but the number of goblet cells was recorded 28 cells/field of view and this number was equivalent to the normal SPF mice as per the criteria of Stecher et al. (2005). This result is significantly different from the positive control and this might be due to the immunomodulatory role of *L. acidophilus* which will bind to mucus and induce goblet cell hyperplasia (Galdeano et al. 2019). Further, Liu et al. (2019) also stated that giving a mixture of *Lactobacillus* and *Bacillus* to pigs infected with *S. infantis* can increase the number of goblet cells because a probiotic can improve the integrity of the bacteria in the intestine barrier and protect the intestine from damage caused by *Salmonella* invasion, in this manner, results of the previous studies corroborate with the findings of the current study.

The treatment groups P1, P2, and P3 also had the scoring 1 with the number of goblet cells 26.73, 24.05, and 24.06 cells/field of view respectively. Here also a higher number of goblet cells was recorded as compared to the positive control treatment group. The coffee extract contains various alkaloids such as caffeine, chlorogenic acid, tannin, and antibacterial flavonoids which inhibit the entrance and multiplication of *S.enterica* bacteria. The combination of coffee extracts and *L. acidophilus* can reduce the

damage of goblet cells due to *S. enterica* invasion, and amongst the tested doses, combinations of Lampung Robusta coffee extract @ 250 mg/kg BW and *L. acidophilus* is the most effective in preventing the goblet cell hypoplasia.

Conclusions

Providing a combination of Lampung Robusta Coffee (Coffea canephora) and Lactobacillus acidophilus extracts can prevent ileum and cecum damage in mice induced by *S. enterica* with the best combination dose of Lampung Robusta Coffee extract @ 250 mg/kg and *L. acidophilus*.

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Influence of Calcium and Nonphytate Phosphorus (NPP) on Meat-Type Quail's Growth, Carcass Features, and Tibia Indices

EtabSaleh Al-Ghamdi*

Department of Food and Nutrition, College of Human Sciences and Design, KingAbdualziz University, Jeddah 21589, Saudi Arabia

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ABSTRACT

This study aimed to evaluate the role of increasing dietary calcium (Ca) and non-phytate phosphorus (NPP) supplementation on the growth, carcass, edible portions, and tibia indicators of growing quail. The current study was conducted in a 3×3 factorial design, for this, 360 1-wk-old quails were haphazardly assigned to nine groups, each group is with three gradual levels of Ca (0.60, 0.90, and 1.20 %) and NPP (0.20, 0.40 and 0.60 %). Each group was divided into five replicates with eight-quail each. Results of the study suggested that except at 2 and 6 weeks of age, dietary Ca level did not exhibit any significant ($P > 0.05$) impact on body weight. Similarly, in the case of NPP, apart from the live weight at 2 and 3 weeks of age, NPP did not have a significant impact on live body weight. Further, in comparison to the low Ca level, the moderate or high Ca levels have higher values of body weight gain. During all the experiments, dietary Ca, NPP, or their mixtures had no significant ($P > 0.05$) impact on feed consumption. Similarly, feed conversion rate and carcass metrics were also not affected by the individual or combined application of Ca or NPP supplementation Similarly, dietary intakes of Ca or P did not have any significant effect on the various tibia indicators ($P > 0.05$). Results of the study can be concluded that the effect of the Ca and NPP levels in Japanese quail diets is lowered and it does not much affect the growth rate, feed utilization, carcass yields, edible components, or tibia indices.

* Corresponding author

E-mail: asalghamdy2@kau.edu.sa (EtabSaleh Al-Ghamdi)

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1 Introduction

Among the 12 essential elements for poultry, calcium and phosphorus are the two most important elements which highly attract nutritionists, researchers, and producers who are associated with the poultry industry (Alagawany et al. 2020, 2021). These two are accounted for at least 50% of bone ash, so it can be expressed that these are the fundamental skeletal components and are also directly or indirectly participated in both quantitative and qualitative metabolic processes of poultry animals (Mc Donald et al. 2002; Rao et al. 2006; Gautier et al. 2017; Perine et al. 2022).

Calcium is also known to be the connecting material and plays an important role in connecting various blood-clotting proteins, such as protein-protein or protein-phospholipid (Brody 1994; Rousseau et al. 2016). Cytoskeleton components are another Ca-binding protein that comes from the extracellular fluid in the nervous system or cellular calcium storage organelles in muscle, and play a key role in cell communication (Brody 1994). Both nerve impulse transmission and muscle contraction also required calcium (McDonald et al. 1995, Perine et al. 2016).

Phosphorus is another primary component of bone and is also required for many metabolic processes such as protein, carbohydrate, and fat metabolism (McDonald et al. 1995, Abd El-Hack et al. 2018). The body's P content is slightly lower than its Ca content, and the bones account for 80 to 85 % of total body phosphorus. Further, in the synthesis of adenosine di- and tri-phosphates and sugar phosphates, Phosphorus is also used for energy storage and metabolism (Brody 1994; Liu et al. 2017). Moreover, phospho-proteins, phospholipids, and nucleic acids are also made up of phosphorus (Brody 1994).

Despite a long history of research on the role of calcium and phosphorus in poultry nutrition and production, there are only a few studies based on the impacts of calcium and phosphorus interaction in Japanese quail (Rao et al. 2006). Although Japanese quail have more documentation requirements than other birds because they are rare to be compared the other domestic poultry (NRC 1994). The nutrient requirements of Japanese quails are usually extrapolated from other poultry because they are not identical to other broiler chickens in terms of poultry production (Cheng 1990; Minivielle 2004). The most detailed assessment of the Japanese quail diet was reported by Shim and Vohra (1984), and Batool et al. (2021). Quail Ca and available P requirements appear to be higher than those of other birds of comparable age, according to the researchers. The available information on the influence of calcium and nonphytate phosphorus (NPP) on meat-type quail's growth and tibia indices are in scarcity. Therefore, the purpose of this study was to determine the effect of small increases of dietary calcium and nonphytate phosphorus (NPP) on the performance, carcass, edible portions, and tibia markers of the growing quails aged 1-6 weeks.

2 Materials and Methods

2.1 Diets and experimental birds

In this study, 360 growing quails weighing an average of 23.76g were fed on three levels of Ca (0.6, 0.9, and 1.2 %), and nonphytate P in a 3×3 factorial design (0.2, 0.4, and 0.6 %). Each of the nine groups was divided into five replicates, each containing eight unsexed chicks. Table 1 represented the combination of the experimental diet which was given to the one to six weeks age quails to suit their nutritional demands, according to the NRC (1994). The birds were maintained in a normal cage with free access to feed and water (50×30×50 cm^3; 1,500 cm^2 of floor area). During the experiment, the birds were kept on a 24-hour light cycle. Entire quails were raised in the same clean environment.

2.2 Data collection

At weekly intervals, each chick's body weight (BW) and body weight growth (BWG) were measured. The mortality rate was recorded daily throughout the study and it was reported zero in all experimental groups. These data were used to compute the average feed conversion ratio (FCR) and feed intake (FI) periodically and cumulatively. Daily wastage of feed was recorded, and the information was utilized to estimate FI (Alagawany et al. 2020).

At six weeks of age, six quails (one from each group) were slaughtered at random for carcass assessments and tibia characterization. Carcass weights were calculated for the main body and other edible portions. Carcass and dressed weights were compared to live body weight. The weight of each bird's right tibia was measured. Besides, the length and diameter of each tibia bone were determined using Vernier calipers (El-Faham et al. 2019; Fallah et al. 2019).

2.3 Statistical analysis

SPSS (2008) was used to conduct all of the statistical analyses. According to Snedecor and Cochran (1982), The following model was used to statistically evaluate the data as a 3×3 factorial arrangement:

$$Y_{ijk} = \mu + A_i + S_j + AS_{ij} + e_{ijk}$$

Where Y_{ijk} represents an overall mean, A_i represents the influence of the Ca level (I = 1–3), S_j represents the influence of the NPP level (j = 1–3), AS_{ji} represents the Ca and NPP interactions, and e_{ijk} represents the experimental random error. To find differences between treatments, a post-hoc Tukey's test was used. At $P \leq 0.05$, all differences and significance were measured.

Table 1 Effect of calcium and phosphorus and its interactions on live body weight of growing quails

Experimental Diets (%)									
Ca %		0.60			0.90			1.20	
P avail.%	0.20	0.40	0.60	0.20	0.40	0.60	0.20	0.40	0.60
Corn	55.44	54.31	54.15	55.20	55.42	55.90	55.75	55.88	55.72
Soybean meal 44% CP	28.55	23.00	21.65	26.62	27.26	29.28	28.50	28.70	27.76
Gluten	8.85	12.00	12.92	10.19	10.00	9.05	9.50	9.50	10.22
Bran	5.07	8.05	8.19	5.07	4.00	2.05	2.55	1.82	1.73
Mono-Ca phosphate	0.15	0.95	1.75	0.15	0.95	1.75	0.18	0.95	1.77
Limestone	1.24	0.90	0.53	2.05	1.66	1.28	2.82	2.45	2.09
Premix	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
NaCl	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Methionine	0.04	0.01	0.00	0.02	0.02	0.03	0.03	0.03	0.02
L-Lysine	0.21	0.33	0.36	0.26	0.24	0.21	0.22	0.22	0.24
Chemical analysis[1]									
CP %	24.00	24.00	24.02	24.01	24.00	24.00	24.02	24.00	24.02
ME kcal/kg	2900	2898	2900	2900	2900	2899	2900	2900	2900
Ca %	0.60	0.60	0.60	0.90	0.90	0.90	1.20	1.20	1.20
P avail.%	0.20	0.40	0.60	0.20	0.40	0.60	0.20	0.40	0.60
Lysine %	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30
M+C %	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
CF %	3.92	3.81	3.72	3.77	3.70	3.65	3.64	3.58	3.50

[1]CP: crude protein, ME: metabolizable energy, Ca: calcium, P: phosphorus, M+C: methionine pluscyctine and CF: crude fiber

3 Results And Discussion

3.1 Live body weight

The impact of dietary Ca and NPP levels on the quail's live body weight was demonstrated in Table 2. Except for 2 and 6 weeks of age, dietary Ca had no significant effect on the LBW ($P > 0.05$). As the dietary Ca level was increased, the live body weight was steadily reduced. However, in the case of dietary NPP level, no significant effect was reported on the LBW across all ages except live body weight at 2 and 3 weeks of age at which LBW significantly improved ($P = 0.001$) at the low NPP level (0.20 %) as compared to the moderate and high levels (0.40 and 0.60 %). These results are in agreement with the findings of Wang (2011) and Rao et al. (2006) those who also reported nonsignificant variations in live body weight in birds fed varying quantities of calcium (0.65 %, 0.95 %, and 1.25 %) and Nonphytate phosphorus in their diets (0.40, 0.60, and 0.80 %).

Further, the results of the study suggested that the combination between dietary calcium and phosphorus levels had a significant impact on live body weights at 2, 3, and 4 weeks of age (Table 2). The highest live body weights were found in birds given low calcium and moderate phosphorus diet (0.60 % calcium + 0.40 % phosphorus). In another study, lowering phosphorus levels in broiler chicken diets did not affect live body weight (Farhadi et al., 2017). These findings might be a result of an unbalanced calcium-phosphorus ratio, which causes the development of insoluble molecules, limiting energy and mineral uptake and utilization (Plumstead et al. 2008).

According to Wilkinson et al. (2014), the ratio of calcium to nonphytate phosphorus is more essential than individual absolute calcium and Nonphytate phosphorus dietary intakes. Our results are comparable to those of Zhu et al. (2018), who found that lowering the ratio of dietary calcium to Nonphytate phosphorus reduced live body weight considerably. During the starter and grower stages, reducing dietary calcium and Nonphytate phosphorus to 50% of the NRC (1994) had no impact on body weight (Mohamed et al. 2020). The findings of the current study are contradictory to the findings of Imari et al. (2020) those who reported that during the starting phase of broiler, reducing dietary calcium and Nonphytate phosphorus to 10% to 30% of the recommended quantity lowered body weight.

Table 2 Effect of calcium and phosphorus and its interactions on live body weight of growing quails

Treatments	Live body weight (g/d)					
	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
Calcium Percentage						
0.60	24.18	56.28[a]	94.60	114.89	137.30	205.18[b]
0.90	24.02	53.18[b]	94.94	114.76	137.06	213.58[a]
1.20	24.00	50.26[c]	94.00	108.67	132.30	213.54[a]
P value	0.69	<0.001	0.907	0.111	0.524	0.032
Phosphorus Percentage						
0.20	24.18	56.28[a]	89.60[b]	114.89	137.30	205.18
0.40	24.02	53.18[b]	99.00[a]	114.76	137.06	213.58
0.60	24.00	50.26[b]	94.94[ab]	108.67	132.30	213.54
P value	0.884	0.001	0.001	0.500	0.280	0.871
Calcium and Phosphorus Interaction						
0.60×0.20	24.22	56.33[b]	95.01[b]	119.51[a]	146.88	206.56
0.60×0.40	24.12	59.46[a]	100.22[a]	122.30[a]	136.46	204.45
0.60×0.60	24.22	53.04[b]	88.57[c]	102.88[c]	128.56	204.52
0.90×0.20	24.13	50.74[c]	86.18[c]	111.64[b]	136.49	210.01
0.90×0.40	24.00	55.44[b]	101.31[a]	114.85[b]	138.15	217.52
0.90×0.60	23.93	53.35[b]	97.32[b]	117.81[b]	136.55	213.20
1.20 ×0.20	23.99	46.78[c]	86.29[c]	108.23[b]	137.23	214.92
1.20 ×0.20	23.89	51.00[c]	95.71[b]	106.33[b]	125.40	213.27
1.20 ×0.20	24.12	53.00[b]	100.01[a]	111.44[b]	134.26	212.43
SEM	0.28	1.06	2.61	3.90	5.96	4.08
P value	0.981	0.003	0.006	0.021	0.411	0.773

Means in the same column within each classification bearing different letters are significantly differ

3.2 Body weight gain

Table 3 shows the effect of dietary Ca and NPP levels on the body weight growth of growing quails. Different levels of calcium affected body weight growth considerably (P<0.05) apart from the time from 4 to 5 weeks of age. When compared to the low level of Ca, the moderate and high levels of Ca recorded the highest values of body weight gain during the study. However, varying quantities of calcium had a substantial (P > 0.05) effect on body weight increase; however, the trend of the data is unclear due to minor changes in the results. The results of this study are in agreement with the findings of Wang (2011) those who reported that birds fed varying amounts of calcium (0.65 %, 0.95 %, and 1.25 %) and Nonphytate phosphorus (0.40 %, 0.60 %, and 0.80 %) in their meals showed no significant effect on body weight gain. According to Hamdi et al. (2015), higher calcium levels (0.9 %), have a detrimental effect on the body weight gain, whereas lower calcium levels were desirable since they fostered improved broiler performance. Our findings are similar to those of Zhu et al. (2018), who discovered that lowering the dietary calcium to Nonphytate phosphorus ratio significantly reduced body weight gain.

Wenli et al. (2005) observed that birds fed on 0.65 % calcium gained more body weight than those fed diets with higher levels of calcium (0.75 %, 0.85 %, and 0.95 %). Furthermore, varying dietary quantities of P (0.30 % vs. 0.40 %) had a substantial impact on body weight increase (Wenli et al. 2005). In another study, lowering phosphorus levels in broiler chicken diets had no impact on BWG from 1d to 7 wks-old (Farhadi et al. 2017).

Apart from the 1-2 week age, the Ca and P interactions had no significant (P > 0.05) impact on body weight gain (BWG) at any

Table 3 Effect of calcium and phosphorus and its interactions on body weight gain of growing quails.

Treatments	Body weight gain (g)					
	1-2 wk	2-3 wk	3-4 wk	4-5 wk	5-6 wk	Overall
Calcium Percentage						
0.60	32.09^{a}	38.32^{b}	20.29^{a}	22.40	67.88^{b}	180.99^{b}
0.90	29.16^{b}	41.76^{ab}	19.74^{a}	24.70	76.51^{a}	189.56^{a}
1.20	26.25^{c}	43.74^{a}	14.88^{b}	25.93	81.24^{a}	189.54^{a}
P value	<0.001	0.040	0.046	0.436	<0.001	0.027
Phosphorus Percentage						
0.20	27.17^{b}	37.87^{b}	23.96^{a}	27.07^{a}	70.29^{b}	186.38
0.40	31.30^{a}	43.78^{a}	15.63^{b}	20.14^{b}	78.41^{a}	187.75
0.60	29.04^{b}	42.16^{ab}	15.32^{b}	25.82^{a}	76.93^{a}	185.96
P value	0.001	0.021	0.001	0.045	0.013	0.854
Calcium and Phosphorus Interaction						
0.60×0.20	32.11^{ab}	38.67	24.50	27.37	59.68	182.34
0.60×0.40	35.34^{a}	40.76	22.08	14.15	67.99	180.33
0.60×0.60	28.82^{c}	35.52	14.31	25.68	75.96	180.30
0.90×0.20	26.61^{c}	35.44	25.45	24.85	73.52	185.88
0.90×0.40	31.44^{b}	45.87	13.54	23.29	79.37	193.53
0.90×0.60	29.42^{bc}	43.97	20.23	25.96	76.65	189.27
1.20 ×0.20	22.78^{d}	39.51	21.93	29.00	77.69	190.92
1.20 ×0.20	27.11^{c}	44.70	11.27	22.99	87.87	189.38
1.20 ×0.20	28.87^{c}	47.01	11.44	25.82	78.17	188.31
SEM	1.08	2.41	2.69	3.33	3.18	4.04
P value	0.004	0.120	0.136	0.445	0.074	0.765

Means in the same column within each classification bearing different letters significantly differ

ages evaluated in this study. The quails fed on a low level of calcium and a moderate dose of phosphorus (0.60 % Ca + 0.40 % P) have the largest body weight gain. However, when birds were fed on a combination of 1.20% Ca + 0.20% P, the lowest body weight gain was obtained (p=0.004). During the starter and grower stages, reducing dietary calcium and Nonphytate phosphorus up to 50% of the NRC recommendation (NRC 1994) did not affect body weight gain (Mohamed et al. 2020). Similarly, Imari et al. (2020) revealed that lowering dietary calcium and Nonphytate phosphorus level at the early stage of broiler growth has lowered the gain of the body weight as compared to the finisher or post-starter stages.

3.3 Feed intake

Results given in Table 4 show the effects of dietary Ca and P amounts, as well as their interaction effect on the feed intake of growing quails. Dietary Ca, P, or their combinations did not have any significant influence on the feed consumption at all examined ages (P > 0.05). These results are in agreement with the findings of Rao et al. (2006), and Wang (2011) those who reported the insignificant effect of Ca and P dietary supplementation on the feed intake. Wenli et al. (2005) observed that birds fed on 0.65 % calcium had better feed intake than those fed diets with higher calcium levels (0.75, 0.85, and 0.95 %). Furthermore, varying dietary quantities of P (0.30 % vs. 0.40 %) had a substantial impact on feed consumption. According to Hamdi et al. (2015), higher levels of Ca (0.9%) harmed broiler chicken feed intake. In another study, lowering phosphorus levels in broiler chicks' diets did not affect feed intake from day1 to 42 (Farhadi et al. 2017). The findings of the current study are comparable to those of Zhu et al. (2018), who found that reducing the ratio of dietary calcium to Nonphytate phosphorus reduced feed intake considerably. Further,

Table 4 Effect of calcium and phosphorus and its interactions on feed intake of growing quails

Treatments	Feed intake (g)					
	1-2wk	2-3 wk	3-4 wk	4-5 wk	5-6 wk	Overall
Calcium Percentage						
0.60	11.68	17.20	21.23	21.21	21.22	18.51
0.90	11.31	16.67	20.89	21.89	21.39	18.43
1.20	11.41	16.68	21.61	22.32	21.97	18.80
P value	0.640	0.577	0.728	0.307	0.477	0.699
Phosphorus Percentage						
0.20	10.94	16.07	22.76[a]	22.14	22.45	18.87
0.40	11.51	16.92	20.76[b]	21.24	21.00	18.29
0.60	11.95	17.56	20.21[b]	22.05	21.13	18.58
P value	0.064	0.052	0.028	0.394	0.064	0.451
Calcium and Phosphorus Interaction						
0.60×0.20	11.43	16.64	22.33	22.32	22.33	19.01
0.60×0.40	11.10	16.40	21.27	20.01	20.64	17.89
0.60×0.60	12.52	18.55	20.08	21.30	20.69	18.63
0.90×0.20	10.50	15.68	22.24	21.552	21.89	18.37
0.90×0.40	11.54	16.73	20.69	22.00	21.35	18.46
0.90×0.60	11.90	17.59	19.72	22.12	20.92	18.46
1.20 ×0.20	10.88	15.88	23.72	22.54	23.13	19.23
1.20 ×0.20	11.90	17.63	20.30	21.71	21.01	18.51
1.20 ×0.20	11.44	16.54	20.82	22.72	21.77	18.66
SEM	0.49	0.69	1.11	0.86	0.77	0.55
P value	0.358	0.253	0.847	0.623	0.862	0.839

Means in the same column within each classification bearing different letters significantly differ

the results of this study are also in agreement with the findings of Mohamed et al. (2020), and Imari et al. (2020) who reported a nonsignificant effect of dietary inclusion of calcium and Nonphytate phosphorus on feed consumption at the early stage of growth and other growth phases.

3.4 Feed conversion

The effect of gradually increasing calcium and phosphorus levels in the diet on quail chick feed conversion has been presented in Table 5. Aside from the periods of 4 to 5 and 1 to 6 weeks of age, calcium levels had a significant (P 0.05) impact on feed conversion. These results are contradictory to the findings of Wang (2011) and Rao et al. (2006) those who observed that Ca and P levels had a nonsignificant effect on feed conversion. While, Wenli et al. (2005) observed that birds fed on 0.65% calcium diets had a greater feed conversion ratio than those fed higher calcium diets (0.75, 0.85, and 0.95 %).

At all ages except 3-4 weeks, changes in dietary P had no significant influence on feed conversion and the feed conversion ratio of quail fed a 0.20 % P-based diet was reported considerably lower (P = 0.005). These results are corroborated by the findings of Wen Li et al. (2005) and Farhadi et al. (2017) who found that different dietary levels of P (0.30 % vs. 0.40 %) had no significant effect on feed conversion.

Further, Ca and P interaction also did not show any significant impacts on feed conversion ratio (Table 5), except for the 2nd week, where the higher feed conversion ratio was attained with 0.60 Ca and 0.40 P combination (P =0.011). According to Mohamed et al. (2020) reducing dietary calcium and

Table 5 Effect of calcium and phosphorus and its interactions on feed conversion of growing quails

Treatments	Feed Conversion ratio (g feed/g gain)					
	1-2 wk	2-3 wk	3-4 wk	4-5 wk	5-6 wk	Overall
Calcium Percentage						
0.60	2.58^{b}	3.20^{a}	2.37^{b}	4.88	2.24^{a}	3.58
0.90	2.73^{b}	2.83^{ab}	2.43^{b}	6.10	1.96^{ab}	3.40
1.20	3.06^{a}	2.68^{b}	3.41^{a}	6.12	1.90^{b}	3.47
P value	0.049	0.025	0.005	0.238	0.011	0.136
Phosphorus Percentage						
0.20	2.87	2.99	2.06^{b}	5.07	2.28	3.54
0.40	2.62	2.73	3.06^{a}	5.87	1.90	3.41
0.60	2.89	2.98	3.09^{a}	6.16	1.92	3.49
P value	0.089	0.245	0.005	0.238	0.110	0.136
Calcium and Phosphorus Interaction						
0.60×0.20	2.50^{b}	3.06	1.99	4.59	2.68	3.65
0.60×0.40	2.20^{b}	2.87	2.14	4.18	2.14	3.47
0.60×0.60	3.05^{a}	3.67	2.98	5.87	1.91	3.61
0.90×0.20	2.77^{b}	3.10	1.90	5.12	2.09	3.46
0.90×0.40	2.57^{b}	2.57	3.22	6.82	1.88	3.34
0.90×0.60	2.83^{b}	2.82	2.17	6.37	1.91	3.41
1.20 ×0.20	3.34^{a}	2.81	2.30	5.51	2.08	3.52
1.20 ×0.20	3.08^{a}	2.76	3.83	6.61	1.68	3.42
1.20 ×0.20	2.77^{b}	2.46	4.11	6.24	1.94	3.47
SEM	0.15	0.20	0.37	0.87	0.15	0.079
P value	0.011	0.073	0.085	0.568	0.230	0.978

Means in the same column within each classification bearing different letters significantly differ

Nonphytate phosphorus to up to 50% of the NRC (1994) recommended amounts during the starter and grower stages did not affect the feed conversion ratio. The results of this study are contradictory to the findings of Imari et al. (2020) those who reported a 10% to 30% reduction in the recommended amount of dietary calcium and Nonphytate phosphorus during the post-starter, starter, and finisher periods of broilers did not influence FCR.

3.5 Carcass characteristics

Table 6 revealed how varying levels of calcium and NPP affect the carcass characteristics of quail at different ages. Results of the study suggested that all carcass parameters were unaffected by the increasing calcium and phosphorus levels (i.e., dressing, carcass, giblets, liver, heart, and gizzard). Similarly, the combination of dietary Ca and P also did not affect the various parameters of the carcass such as giblets, liver, heart, and gizzard (Table 5). However, the interaction effect had a substantial (P <0.05) impact on the carcass (p=0.027) and dressing (P=0.046). The best results were obtained with 0.90% Ca and 0.20 % P combinations.

Reduced calcium and Nonphytate phosphorus by 10% to 30% of required levels showed no influence on internal organs and carcass attributes of broilers at 7 weeks of age, as per Imari et al. (2020), which is consistent with our findings. In agreement with this, Han et al. (2015) also observed that dietary levels of

Table 6 Effect of calcium and phosphorus and its interactions on carcass traits of growing quails

Treatments	Carcass traits (%)					
	Carcass	Dressing	Giblet	Heart	Liver	Gizzard
Calcium Percentage						
0.60	72.90	79.21	6.30	3.40	.89	2.00
0.90	72.98	79.18	6.20	3.18	.95	2.06
1.20	72.75	78.58	5.83	2.93	.91	1.98
P value	0.964	0.743	0.146	0.181	0.556	0.761
Phosphorus Percentage						
0.20	72.06	78.17	6.11	3.22	.90	1.98
0.40	73.95	80.24	6.28	3.22	.92	2.14
0.60	72.62	78.56	5.94	3.09	.92	1.92
P value	0.105	0.078	0.391	0.834	0.923	0.144
Calcium and Phosphorus Interaction						
0.60×0.20	71.74^{ab}	78.14^{ab}	6.40	3.42	.894	2.08
0.60×0.40	74.37^{a}	80.50^{a}	6.12	3.26	.881	1.97
0.60×0.60	72.60^{ab}	79.00^{a}	6.40	3.53	.915	1.95
0.90×0.20	74.45^{a}	80.58^{a}	6.13	3.29	.927	1.91
0.90×0.40	73.33^{a}	79.88^{a}	6.54	3.32	.950	2.27
0.90×0.60	71.16^{ab}	77.09^{b}	5.93	2.95	.975	2.00
1.20 ×0.20	69.99^{b}	75.80^{b}	5.80	2.94	.903	1.95
1.20 ×0.20	74.14^{a}	80.34^{a}	6.19	3.07	.939	2.18
1.20 ×0.20	74.11^{a}	79.60^{a}	5.49	2.78	.894	1.81
SEM	1.07	1.13	0.305	0.30	0.063	0.13
P value	0.027	0.046	0.502	0.841	0.966	0.430

Means in the same column within each classification bearing different letters significantly differ

Nonphytate accessible phosphorus did not affect broiler carcasses. Furthermore, Akter et al. (2016) have reported that reducing dietary calcium from 10 to 6 gm/kg and dietary Nonphytate phosphorus from 4 to 3 gm/kg did not affect the relative weights of broiler chicken internal organs. Similarly, Tizziani et al. (2019) also found that decreasing the dietary calcium level to 30% of the standard requirements did not affect carcass yield in broiler chickens. On the other hand, El-Faham et al. (2019) found that reducing the dietary calcium and Nonphytate phosphorus levels to half of the normal requirements reduced the carcass characteristics and dressing percentage age in broilers. Han et al. (2016) also found that a decreased calcium to Nonphytate phosphorus ratio resulted in reduced broiler carcass production.

3.6 Tibia indices

Table 7 showed the impacts of calcium and NPP, as well as their interactions, on the tibia indices of growing quails. All the tibia indicators were unaffected by varying dietary Ca or P intakes (P > 0.05). Regardless of the length and diameter of the tibia, the interaction between Ca and P had a significant impact on weight. Further, treatments fed with 0.60 % Ca and 0.60% P has the maximum tibia weight (0.683kg). Our findings are comparable to those of Ribeiro et al. (2016), who showed no statistical impact of calcium and NPP on bone indices (P>0.05). On the other hand, Keshavarz (2003) pointed out that ash of tibia was reduced when birds were fed diets containing 0.25, 0.20, and 0.15% of calcium.

Table 7 Effect of calcium and phosphorus and its interactions on tibia indices of growing quails

Treatments	Tibia		
	Weight (g)	Length (mm)	Diameter (mm)
Calcium Percentage			
0.60	0.59	5.46	0.28
0.90	0.62	5.47	0.28
1.20	0.61	5.53	0.28
P value	0.325	0.929	0.937
Phosphorus Percentage			
0.20	0.58	5.42	0.29
0.40	0.60	5.48	0.28
0.60	0.64	5.56	0.28
P value	0.144	0.23	0.144
Calcium and Phosphorus Interaction			
0.60×0.20	0.54^{b}	5.22	0.29
0.60×0.40	0.54^{b}	5.35	0.28
0.60×0.60	0.68^{a}	5.82	0.28
0.90×0.20	0.62^{a}	5.50	0.28
0.90×0.40	0.62^{a}	5.56	0.27
0.90×0.60	0.64^{a}	5.34	0.29
1.20 ×0.20	0.60^{ab}	5.53	0.30
1.20 ×0.20	0.65^{a}	5.54	0.29
1.20 ×0.20	0.59^{ab}	5.52	0.26
SEM	0.032	0.232	0.013
P value	0.015	0.480	0.455

Means in the same column within each classification bearing different letters significantly differ

Conclusions

Ca and NPP levels could be lowered in Japanese quail diets without affecting growth rate, feed utilization, carcass yields, edible components, or tibia indices. Results of the current study advise against raising the calcium to phosphorus ratio in the feed since this may have negative NPP on the growth performance and public health of growing quails.

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