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Exploring the Impact of Micro-plastics on Soil Health and Ecosystem Dynamics: A Comprehensive Review

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ABSTRACT

Microplastics, defined as particles measuring less than 5 mm, have emerged as widespread environmental pollutants, prompting concerns regarding their impact on soil ecosystems. This review investigates microplastics' presence, movement, and effects on soil health and ecosystem dynamics while highlighting their diverse sources, including industrial production and the breakdown of larger plastic materials. Despite their ubiquity, a significant gap exists in our understanding of the consequences of microplastics in terrestrial ecosystems, particularly within soils. The findings of this review article revealed that microplastics exert notable influences on soil properties, altering bulk density, aggregation, and water-holding capacity, which may have significant implications for soil biota and plant vitality. Furthermore, microplastics also carry toxic substances, complicating their environmental impact. The effects on soil microorganisms and soil-dwelling fauna, such as earthworms, underscore the intricate relationships within soil ecosystems. Additionally, microplastics can interact with other soil pollutants, potentially amplifying their adverse effects. The long-term impacts of microplastics on soil health remain uncertain, underscoring the imperative for sustained research endeavours. Challenges persist, including the absence of standardized methodologies for microplastic extraction and identification in soils, which hampers our ability to understand their presence and effects comprehensively. Furthermore, the lack of regulatory frameworks complicates managing and mitigating microplastic pollution. Future research should adopt a holistic approach, considering diverse microplastic types and applications. Both field and laboratory experiments

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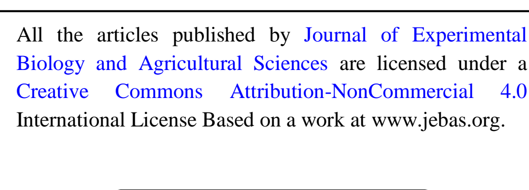

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are essential for accurately capturing the varied influences of microplastics. Efforts should concentrate on understanding the occurrence of microplastics, developing reliable detection methods, and exploring their interactions with other pollutants, especially in terrestrial ecosystems. In conclusion, mitigating microplastic pollution requires multifaceted strategies informed by ongoing research efforts and public awareness campaigns. We can effectively address the challenges posed by microplastic contamination in soil ecosystems through concerted action and comprehensive understanding.

1 Introduction

Each year, millions of tons of plastic are produced by humans, serving various purposes in our daily lives. Microplastics, a type of plastic with a thickness of less than 5 mm, are composed of a heterogeneous mixture of plastic fibers, granules, and pieces (Lusher et al. 2020). These microplastics are gaining attention as potential pollutants of concern (UNEP 2011; Sendra et al. 2021). Plastic waste in the environment can be categorized based on particle size into large plastic (Particle size higher than 5 mm), microplastic (0.1 µm to 5mm) (Barnes et al. 2009), and nano plastic (less than 0.1 µm) (Alimi et al. 2018). Owing to their greater prevalence, smaller particle size, and capacity for long-distance transmission, microplastics may be more harmful than bigger trash (Law and Thompson 2014; Gong and Xie 2020). Along with the terrestrial environment, microplastics have been reported from aquatic environments during the past few decades (Thompson et al. 2004; Thompson 2015). Previous studies suggested that microplastics may pose a hazard to various ecosystems (Andrady 2011; Peng et al. 2017).

Soil is an essential component of the land-based ecosystem. It is under significant pollution stress due to these microplastics, which affect the soil in various ways, such as the storage of carbon, the cycling of biogeochemical processes, and the support of biodiversity, which is severely affected. The term "Soil MP" describes microscopic plastic pieces that come to the soil from a variety of sources, such as processed organic waste from wastewater treatment facilities and the decomposition of used or abandoned plastic products (like plastic mulch) (Weithmann et al. 2018). The entire soil biosphere may be impacted by their ability to change the chemical and physical characteristics of soils. Li et al. (2016) suggest that microplastics may coexist with additional chemical additives during production. These additives may then leak into the environment when the plastics break down, endangering ecosystems and public health. These microplastics have been reported worldwide, including in polar zones, deep oceans, open oceans, and coastlines (Browne et al. 2011). Studies on microplastics in soil and terrestrial ecosystems are very rare, even though microplastics highly contaminate terrestrial ecosystems than aquatic ecosystems (Van Cauwenberghe et al. 2015; De Souza Machado et al. 2018a; Dissanayake et al. 2022). Because of their diminutive stature and possible hazard to aquatic and terrestrial ecosystems, these microplastics are emerging and persistent organic contaminants and have drawn attention worldwide (Huffer et al. 2019). This review delves into the broader context of soil science and how MP influences soil parameters. It is anticipated that this review will elevate the understanding of the impacts of microplastics, or MPs, on soil environments and result in more potent approaches to managing plastic pollution. It will underscore the existing gaps in our understanding, recommend areas for future investigation, and put forth effective strategies for managing and remedial measures for reducing the impact of microplastic pollution in soils.

2 Sources of Microplastic

Microplastics come from various sources and eventually find their way into soil (Figure 1). Cole et al. (2011) state that microplastics can be broadly divided into primary and secondary plastic categories. According to Castañeda et al. (2014), primary microplastics are difficult to remove from wastewater treatment systems and eventually accumulate in the environment (Liu et al. 2021). Secondary microplastics enter the soil in various ways, including soil additions from landfills, sewage sludge applied to land, irrigation using composted wastewater and organic fertilizer, tyre wear and tear, and atmospheric deposition (Liu et al. 2019).

Microplastics in these places are also made worse by sewage, sludge, mulching film, garbage disposal, etc. (Grbić et al. 2020). Sun et al. (2019) suggested that sewage treatment plants can successfully remove 99% of the microplastics from wastewater. However, these removed microplastics might accumulate in the activated sludge, and when farmers put such sludge on the farms, it can add a significant amount of microplastic pollutants to the agricultural soil (Edo et al. 2020). According to Guo et al. (2020), artificial playground turf is an indirect source causing the addition of microplastic in the soil since it can release an average of 2630 tons per year. Utilizing surface water bodies such as lakes or rivers for irrigation purposes can also introduce microplastics into soils because they are frequently contaminated by them (Wang et al., 2017). Furthermore, research indicates that airborne ultrafine plastic fibers result from home washing dryers (O'Brien et al. 2020).

In addition, through biological processes like feeding, digesting, and excretion, soil-dwelling organisms can convert plastic trash into microplastics. These microplastics substantially deteriorate the soil's quality (De Souza Machado et al. 2018b). The transportation

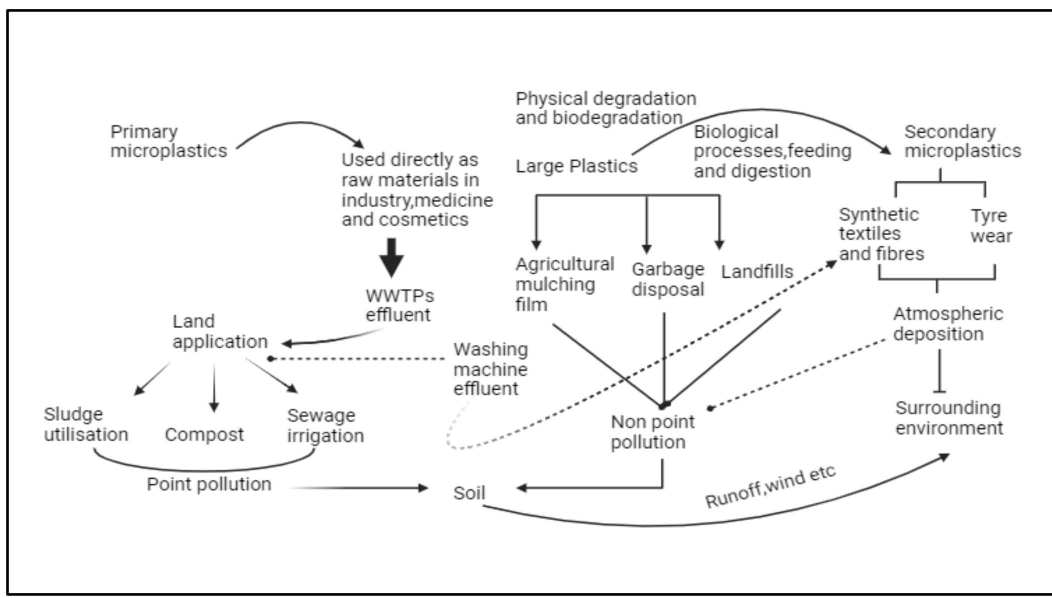

Figure 1 Varied sources of microplastics (WWTP: signifies Wastewater Treatment Plants)

Table 1 Classification of microplastics based on their density in soil

Polymer type/microplastics	Density (g/cm^3)
Poly Propylene (PP)	0.85- 0.95
PolyStyrene (PS)	1.04-1.11
Polyethylene (PE)	0.91- 0.93 (low density PE) 0.93- 0.97 (high density PE)
Poly Vinyl Chloride (PVC)	1.16-1.58
Nylon	1.08 (Nylon6) 1.31 (Nylon66)
Polyester	1.37-1.45

and transfer of microplastics seriously threaten ecosystems across food chains in severely degraded soils, particularly those irrigated with wastewater and wrapped with plastic film (Guo et al. 2020). Types of microplastic/polymer found in terrestrial and marine environments and their density are summarized in Table 1 (Andrady 2011; Hidalgo-Ruz et al. 2012; Scheurer and Bigalke 2018). Furthermore, microplastics also appear in different forms called microplastic morph types, such as fiber, fragment, foam, microbead, and granules (Tanaka and Takada 2016).

3 Impact of Microplastic On Soil

Microplastics could change the biophysical properties of soil, including its structure, porosity, moisture content, and aeration; these microplastics also have a big impact on land plants, soil animals, and soil microbes. These physical and biological impacts are also linked to the soil's chemical characteristics. These effects can be detrimental, neutral, or even beneficial (Wang et al. 2022). However, our understanding of the chain of impacts at the fundamental levels of land-based ecosystems is limited. These impacts included modifications in the non-living elements of soil and other facets of plant-soil interactions, such as soil microbial populations and traits of plants (De Souza Machado et al. 2018b).

4 Effects on Physical Properties of Soil

The physical properties of soil are a critical factor in assessing the microplastic risks posed to terrestrial ecosystems, as reported by Lehmann et al. (2019). These changes can affect the soil's bulk density, capacity to hold water, the stability of its aggregates, and its ability to repel water.

4.1 Bulk Density

The bulk density of soil, a significant determinant of estimating carbon storage in the soil, is also influenced by microplastics. This might be because plastic materials typically have a lower density than soil particles. A study by De Souza Machado et al. (2018b) revealed that the bulk density of soil was reduced more by polyester fibers than by fragments and beads. Similarly, De Souza

Machado et al. (2018b) and De Souza Machado et al. (2019) reported that PE, PS, and PP reduced the soil bulk density (Figure 2). Changes in bulk density could be partly attributed to the fact that plastics are typically less dense than numerous natural minerals prevalent in soils. Moreover, the specific type of microplastics also plays an additional role in influencing the pore space and particle interaction within the soil. Interestingly, while polyacrylic fibers and polyethylene fragments exhibit densities of approximately 86% and 71% of polyester, they did not induce as significant decreases in bulk density as polyester fibers. A reduction in the stability of soil aggregates can generally have adverse effects on the soil's biological activities and overall function, particularly on the exchange of air, water, and nutrients. This soil feature can be altered by the ongoing entry of microplastics into the soil environment.

4.2 Soil Aggregation

Various researchers have deeply investigated the effect of microplastics on soil aggregation (Boots et al. 2019; De Souza Machado et al. 2019; Lehmann et al. 2019; Zhang et al.2019) and reported that the form of the polymer affects soil aggregates when it comes from microplastics. In comparison to non-linear plastic particles, it was found that linear plastic particles altered higher soil aggregate stability. Furthermore, Zhang et al. (2019) observed that the inclusion of polyester microfibers resulted in a decrease in the number of pores smaller than 30 µm and an increase in the number of pores larger than 30 µm. This can be explained by the microfibers' capacity to enter and occupy microspores (Figure 2). The linear nature of the polyester microfibers facilitates their ability to entangle with soil particles and create clods. Consequently, increased clods from polyester microfibers might also contribute to proliferating the soil's larger pores or macropores (Jiang et al. 2019).

4.3 Water Holding Capacity

The maximum quantity of water a specific soil can restore against the force of gravity is known as the water holding capacity of a particular soil, also known as field capacity. Microplastics may alter the soil pore sizes, affecting the soil's water-holding capacity or its ability to hold onto water. A study by De Souza Machado et al. (2019) revealed that applying various polymers in different concentrations increased the WHC (water-holding capacity) of loamy soils. Moreover, research by Zhang et al. (2019) indicated that the soil's hydraulic conductivity (HC), which is its ability to transmit water, is enhanced when organic matter and microfibers are present together in the soil (Figure 2)

4.4 Soil water evaporation, evapotranspiration and desiccation

Depending on their size, microplastic particles can interact with the soil differently. Particles about the same size as soil aggregates (around 2 mm) could blend into the soil profile, creating pathways for water to move through. On the other hand, medium-sized particles (5–10 mm) can act as a soil cover, initially preventing water from evaporating. However, larger particles (10–15 mm) can cause the surface to crack, leading to increased evaporation and soil drying (Wan et al. 2022). In agriculture, plastic mulch is often used to conserve water. However, environmental factors like rain

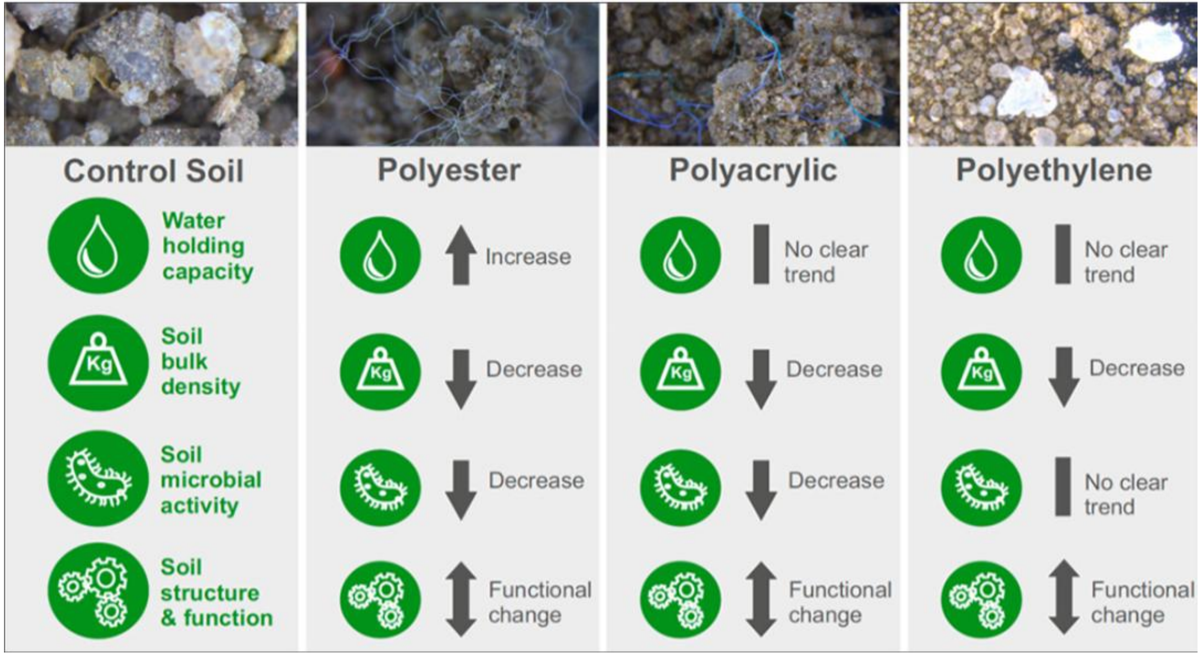

Figure 2 Impacts of Microplastics (MPs) on the Soil Biophysical Properties (Source: De Souza Machado et al. 2018b).

and sunlight can cause these materials to break down into smaller pieces. This can lead to unintended consequences such as increased evaporation, reduced soil moisture, and increased reliance on irrigation. Additionally, as the soil dries, tiny plastic particles may find their way into groundwater through fissures, which might exacerbate the issue of microplastic contamination in aquatic ecosystems (Mbachu et al. 2021). Although there isn't much research on the subject, plastic pollution in soil ecosystems can worsen water scarcity, particularly in dry regions. This emphasizes how crucial it is to carry out additional studies and create plans to control the effect of microplastics on our water and soil systems.

5 Effects on Soil Chemical Properties

Plastic materials, rich in carbon and slow to decompose, can potentially serve as a carbon source for soil microbiota as they break down over time (Rillig et al. 2019). De Souza Machado et al. (2019) claimed that certain plastic polymers, such as polyacrylonitrile and polyamide, contain nitrogen (N), and polytetrafluoroethylene contains fluorine (F) have nutritional components that could affect the biogeochemical properties of soil. However, the role of microplastics as a nutrient source in soils needs to be further confirmed because it's plausible that certain microplastics can modify the soil biogeochemical cycle by leaching. Moreover, because of their hydrophobic nature and expanded surface area, microplastics can adsorb various toxic substances, including heavy metals, hydrophobic organic compounds, and antibiotics (Sun et al. 2018). If microplastics carrying these contaminants are exposed to the environment through leaching, they could negatively impact the soil ecosystem (Kim et al. 2021).

Palansooriya et al. (2022) found variances in soil enzyme activities across all treatments. In particular, soils treated with a high temperature of 700 degrees Celsius biochar showed increased fluorescein diacetate activity during dry circumstances. Applying softwood pellet biochar heated at 700-degree Celsius increased urease activity by 146 percent in well-watered MP-contaminated soil. Biochars from OSR significantly decreased soil acid phosphatase activity in both water conditions (Palansooriya et al. 2022).

6 Effects on Biological Properties

6.1 Effects on Soil microorganisms

Microorganisms in the soil ecosystem constitute a substantial part of all land-based life forms. However, our understanding of how microplastics (MPs) affect these species is incomplete. It is conceivable that MPs could alter the soil's physical properties, such as its moisture content and porosity. These modifications may impact the soil's oxygen flow, altering the ratio of aerobic to anaerobic microbes. Furthermore, alterations brought by MPs to the pore spaces in the soil may cause the extinction of endemic microorganisms and the loss of microhabitats (Veresoglou et al. 2015). Studies have demonstrated that specific MPs, notably membrane-like polyethylene and fibrous polypropylene, can drastically shift soil microbial communities' population and alpha diversity (Figure 2). According to Yi et al. (2021) there has been a rise in soil Acidobacteria and Bacteroidetes populations, while Deinococcus-Thermus and Chloroflexi have decreased when MPs are applied to the soil. Similarly, Judy et al. (2019) suggested that the incorporation of MPs considerably changed the microbial community's structure and resulted in a considerable drop in substrate-induced respiration (SIR) rates, indicating that MPs may affect the functioning of soil microbes. Furthermore, de Souza Machado et al. (2019) suggested that MPs had variable effects on the root colonization of soil fungi *Arbuscular mycorrhiza*. In general, MPs can modify soil characteristics and impose selective pressures on soil microorganisms, which might impact the diversity and structure of communities and possible evolutionary outcomes. Further, it was reported that MPs can be more quickly biodegraded by certain bacteria like *Pseudomonas aeruginosa, Bacillus megaterium, Rhodococcus ruber, Cupriavidus necator, Pseudomonas chlororaphis* (Stawiński and Wal 2021; Kalia et al., 2021) and fungi like *Aspergillus terreus, Engyodontium album, Cochliobolus sp., A. sydowii, A. flavus, Fusarium lini, Pycnoporus cinnabarinus, Mucor rouxiiacidobacteria* that live in soil environment (Shah et al. 2008; Ali et al. 2021; Miri et al. 2022).

6.2 Effects on Soil Animal

As representative species in the soil ecosystem, Earthworms have been the focus of many studies regarding their interaction with microplastics. The influence of microplastics on earthworms is contingent upon the concentration and variety of the microplastics, which can cause a decrease in growth rate and harm to the immune system. As per the research by Cao et al. (2017), earthworms' adaptability was not significantly affected by exposure to 0.25–0.5% microplastics, and growth was only seen to drop at exposure concentrations greater than 1%. The mortality of earthworms increased by 8 and 25 percent when exposed to microplastic concentrations, which were as high as 28% and 60%, respectively (Lwanga et al. 2016). First-time negative effects of microplastic exposure on invertebrate sperm were observed in a recent study by Kwak and An (2021); these researchers suggested that these effects were independent of the size of the plastic particles. Additionally, microplastics can alter the gut microbiome of animals that live in soil, which may impact how organic materials are used and how necessary elements are cycled. While most plastics are eliminated from the body after consumption, research by Browne et al. (2011) indicated that microplastics could remain in the gut for extended periods and may have various harmful consequences. Furthermore, according to Selonen et al. (2020) microplastics can change the ecosystem which surrounds soil animals, having an indirect effect on them.

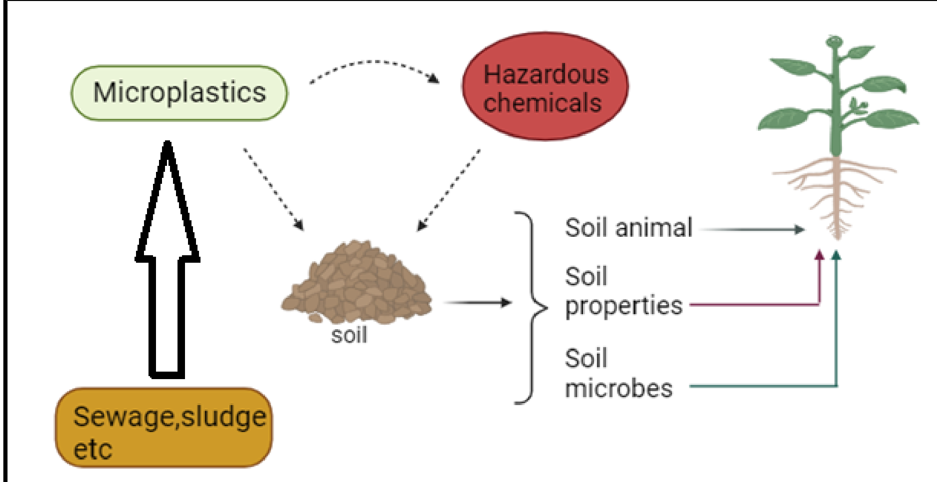

Figure 3 Picturesque representation of the impact of microplastic on various factors

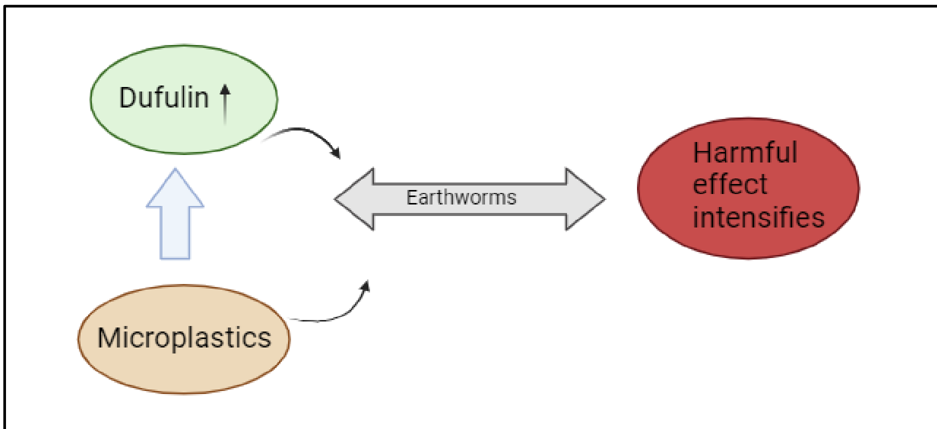

Figure 4 Impact of microplastics on earthworms

To deduce, microplastics resulting from different sources like sewage, sludges, etc., have detrimental effects on soil-dwelling organisms and other physical and chemical properties of soil. These variations in traits hamper the normal growth and development of plants if they survive under pollutant conditions; it has remnants of these compounds (dangerous substances found in microplastic), which could be lethal to living things if swallowed. Hence, microplastic sources should be considered to limit their negative impact on the health of soil, plants, microorganisms, and living beings. This pattern/ chain of microplastic to the health of the soil and the living beings is portrayed in Figure 3.

7 Microplastic and other pollutants on soil

Toxic chemicals like plasticizers, retardants, and antioxidants can leak into the soil due to microplastics. Furthermore, weathered regions of microplastics can draw other toxic pollutants from the soil, like heavy metals and organic pollutants, converting the microplastics into chemical reservoirs (Wang et al. 2020). The interaction between arsenic and polystyrene microplastics reduced the amount of bioavailable arsenic in the soil, hence mitigating the effect of arsenic on soil microorganisms in the rice rhizosphere. Microplastics may also affect plants' capacity to absorb heavy metals. Dong et al. (2021) conducted a study to evaluate the effects of polystyrene microplastics and arsenic on carrot growth and reported that 0.2 µm polystyrene microplastics may reach the leaves and roots of carrots when As (III) is present. According to Gaussian analysis, arsenic promoted cell wall deformation and elevated the negatively charged area of polystyrene microplastics, making it easier for the microplastics to enter carrots (Dong et al. 2021). It is ambiguous how other contaminants and microplastics affect soil fauna together. However, it has been noted that exposure to both microplastics and cadmium together accelerates the cadmium buildup in earthworms, increasing oxidative damage and death (Zhou et al. 2020). According to another study, microplastics greatly accelerated dufulin's bioaccumulation in earthworms and increased its harmful effects, as presented in Figure 4 (Sun et al. 2022).

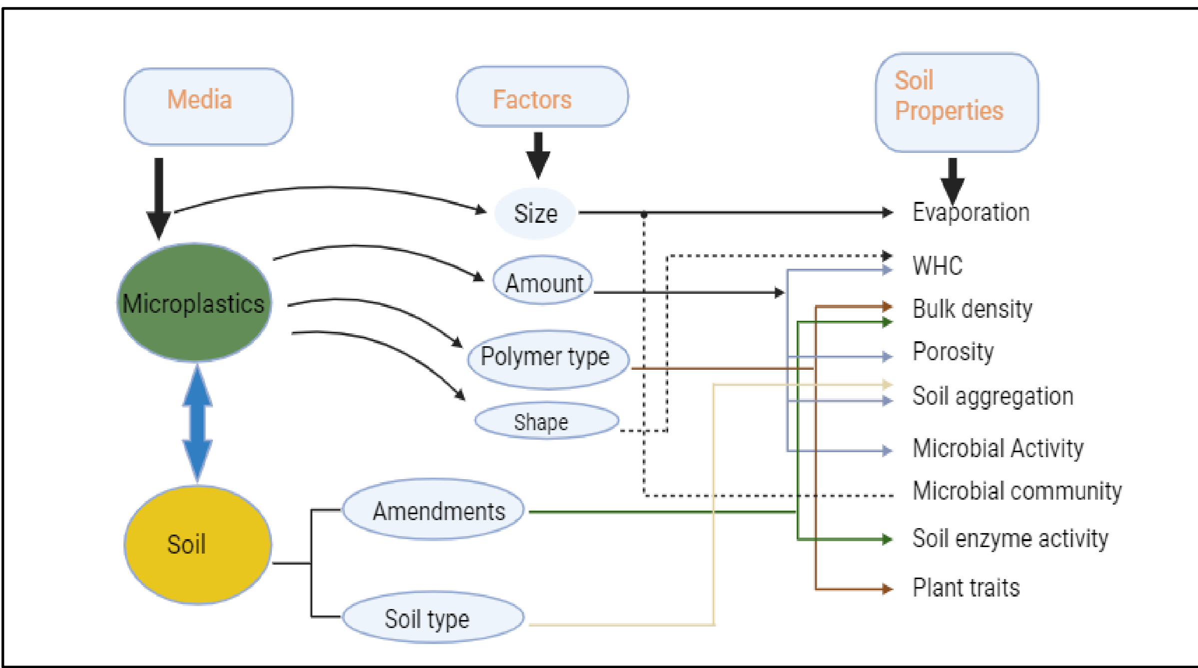

Figure 5 Demonstrating the major relationship between physical properties of soil and rhizosphere function and microplastic input components

8 Key relationship between microplastic and soil responses

This review revealed that microplastics in the soil environment can impact a wide variety of vital characteristics. This review article consolidated the relationships between input variable (such as size and shape of plastic and soil type) and their responses on soil features viz, porosity, water holding capacity (WHC), as well as microbial activity (Figure 5). These relationships demonstrate how each factor can impact a key soil parameter, providing a roadmap for future research to focus on pertinent relationships. Research has indicated that MPs' long-term build-up and concentration can modify soil's chemical, biological, and physical characteristics. The size of the microplastic and soil type can have diverse effects on the soil environment, whether positive, negative, or neutral. It's crucial to acknowledge that the abovementioned relationship is based on our present knowledge of the relationship between soil and MPs, which is still in its nascent stages. As more research is conducted and new studies are published, these relationships can be further refined, enabling ongoing research efforts to be more precisely directed.

9 Status of microplastic pollution in soil

The distribution of microplastics in marine settings has been the subject of numerous research studies (Auta et al. 2017), but minimal information is known about the status of microplastic pollution in soil/terrestrial environments. Here, this review article offers an overview of pertinent studies in Table 2 that have been published. The data highlighted below provides deeper insights into how microplastic jeopardizes the soil ecosystem, thereby influencing crop production and productivity and threatening the health of living beings.

Based on the above data, microplastics have significantly contaminated industrial regions soil, especially soils near industrial areas of Australia (Fuller and Gautam 2016), containing a range of 0.03-6.7% microplastic (mostly PVC). In Switzerland, microplastic pollution was widespread but not as severe (≤0.00555%, mostly PE) (Scheurer and Bigalke 2018). According to Gourmelon (2015), China produces, consumes, and discards a large amount of plastic annually, causing soil microplastic pollution and seeking particular attention. It is crucial to conduct extensive and ongoing microplastic surveys to prevent soil degradation, as high microplastic content has been found in certain industrial, agricultural, and even forest soils (Table 2). Information on hotspot zones, main microplastics, and related sources is essential for hazard evaluation and pollution control.

10 Prevention/Countermeasure

The influx of microplastics into soil ecosystems is expected to rise continually due to increasing production, widespread use in line with population growth, their resistance to degradation, and the significant quantities currently in existence (Eerkes-Medrano et al. 2015). Hence, there is an urgent need for potential remediation measures to reduce microplastic hazards and lessen their contamination. Implementing required policies and institutional governance initiatives could prevent microplastics and associated

Table 2 Various published sources show the proportion of microplastic pollution in soil

Source of soil	Country	Processes for quantification extraction and identification	Microplastics				References
			Size (mm)	Morphotype	Concentration (%)	Major type	
Agriculture fields	China (Northwest area)	Microplastics were treated at 130°C for 3-5 seconds, and their identification was confirmed using a microscope before and after the heat treatment.	>0.1	-	≤0.000054	PE, PP	Zhang and Liu (2018)
mix-culture ecosystem (rice-fish)	China (Shanghai)	The organic matter was identified under a microscope through density separation using a saturated NaCl solution and 30% H2O2 treatment.	<1	Mainly fibers	-	PE, PP	Lv et al. 2019
Forest buffer zone Greenhouse vegetable soils	China (Southwest area)	The process involves separating organic matter by treating organic matter with a saturated NaI solution.	<1	mainly fibers	-	-	Zhang and Liu (2018)
Vegetable fields	China (Shanghai)	separation of organic matter using a saturated NaCl solution and 30% H2O2 treatment, and its identification through a µFT-IR assay.	<1	fibers and fragments	-	PE, PP	Liu et al. 2018
Floodplain soils	Switzerland	The organic matter was treated with 27%NaCl solution and 65%HNO3 to achieve density separation, which was then identified using an FT-IR microscope.	-	-	0.03-6.7	PVC	Scheurer and Bigalke (2018)
Near industrial area	Australia	The extraction process involved pressurized fluid extraction, which was then identified using GC-MS and FT-IR spectrophotometer.	<0.5	-	≤0.0055	PE	Fuller and Gautam (2016)

Here, FT-IR denotes Fourier transform-infrared spectroscopy while GC-MS represents Gas chromatography-mass spectrometer

Potentially Toxic Elements (PTEs) from entering the human food chain. Moreover, integrating the 3R (Reduce, Reuse, Recycle) approach and circular economy principles, thereby reducing the amount of microplastics and PTEs released into the environment as well as their recontamination and spread, could aid in the mitigation process (Igalavithana et al. 2022). Research on microplastic pollution in soil must be prioritized to lessen environmental and food chain hazards. This entails investigating the origin, build-up, deterioration, movement, and possible hazards within the food chain and eco-environment (Yongming et al. 2018). Methods must be developed to eliminate microplastics. For example, reducing the quantity of microplastics entering soil ecosystems through sewage irrigation might be achieved by implementing microplastic removal techniques during wastewater treatment (Guo et al. 2020). To preserve soil quality, targeted mitigation and management plans must be created for impacted areas (Kublik et al. 2022). Some bacterial species, like *Rhodococcus ruber* and *Pseudomonas putida*, have been shown to break down plastics and may be useful for bioremediation of soil contaminated with microplastics. Nonetheless, it is important to consider the possible risk of eliminating microplastics using bioremediation (Caruso 2015).

11 Microorganisms for Plastic Management

The ability of soil microorganisms to break down natural and artificial materials in the environment is essential for the ecosystem's nutrient cycling. Therefore, they have a big natural impact on the breakdown of synthetic polymers. Utilizing an array of enzymes, these microbes may break down polymers into intermediate chemicals that can be taken up by the body and processed to meet their energy requirements. Degradation of plastic polymers by various actinomycetes, bacteria, and fungi has been reported. The rhizosphere of soil, plastic-contaminated areas, animal intestines, and landfills are the usual locations for these microbes. The inherent ability of bacteria to break down long-chain fatty acids makes them capable of degrading polymers. The breakdown of polyethylene (PE) films is largely attributed to

Arthrobacter sp. and *Streptomyces* sp., as demonstrated by the rise in the carbonyl index and CO2 evolution (Han et al. 2020).

12 Prospects and Challenges

Microplastic contamination in soil poses serious environmental issues that could jeopardize the health of the soil ecosystem and humans. Microplastics can interact with other contaminants found in soil, including organic and heavy metal pollutants. However, our understanding of these interactions is still limited, making it hard to predict the environmental hazards associated with microplastic pollution. The intricate and variable characteristic of the soil matrix makes it difficult to separate and identify microplastics. As there are no standard methods for removing, identifying, and measuring microplastics from soil samples, it is difficult to compare the outcomes of various studies, which ultimately hinders the development of a comprehensive understanding of microplastic pollution. The long-lasting impacts of microplastics on the health of soil and ecosystem functionality are largely unknown, underscoring the need for more long-lasting studies to evaluate the potential risks and effects of microplastic pollution on soil ecosystems. Currently, there are no regulatory frameworks for microplastics in soil, which complicates the management and mitigation practices of the impact of pollution generated by microplastics. Typically, the degree of microplastics in soil is lower than those used in experimental settings. Therefore, future studies should aim to use microplastics at concentrations that more closely mirror those found in the environment to reflect their ecological impacts more accurately. Additional research in this area is advised, mainly for soil types apart from light-textured soils, such as heavy-textured and carbon-rich soils, where it is unclear how adding microplastics to soil may affect its hydraulic conductivity. Research on microplastics in agricultural environments looks towards future directions and focus, offering reasonable suggestions for current issues to provide a theoretical foundation for future related research.

13 Future Research Priority Recommendation

Due to their fundamental structure and qualities, microplastics, with their different forms, shapes, sizes, and uses, have unique reactions on soil. Therefore, studies investigating the environmental impacts of microplastics must include a diverse spectrum of microplastics with varying functions and origins. Studies that consider the quantitative or qualitative features of soil microplastics might not adequately account for the impact these particles have on the soil and living things. Experiments in the field and the lab are required to determine the lowest concentration and length of exposure that result in negative consequences. Consequently, more field research is needed to confirm microplastics' effects on soil's physical properties. Using population models, Browne (2015) established a system to examine and direct the management of ecological consequences and to establish ecological links between anthropogenic garbage and the environment. This suggests that rather than focusing solely on the existence of microplastics and their sublethal consequences, further analysis of the effects on ecological relationships is needed, and to help minimize the risks that microplastics pose, research on various microplastics is required. Microplastic has an adsorption capacity that is often comparable to other environmental contaminants; nevertheless, the ability of different microplastic materials to adsorb various antibiotics under different ecological conditions varies significantly (Guo et al. 2020).

Conclusion

Soil contaminated by microplastics is an emerging environmental concern with potential repercussions for soil health and ecosystem integrity. Exploring the Impact of Microplastics on Soil Health and Ecosystem Dynamics comes with challenges, such as a lack of standardized methodologies and regulatory frameworks, emphasizing the need for concerted research efforts. Mitigating microplastic pollution in soils requires a multi-faceted approach, including developing effective removal technologies, policy interventions, and public awareness campaigns. Recognizing the potential synergies or antagonisms between microplastics and other pollutants is crucial for informed environmental management. As microplastics continue to proliferate, especially in agricultural and forestry sectors, it is imperative to prioritize research that informs sustainable practices, minimizes ecological footprints, and safeguards soil ecosystems for future generations.

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Exploring the Potential Role of *Lactobacillus plantarum* in the Reversal of Induced Cognitive Long-term Memory Impairment

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ABSTRACT

Long-term Memory (LTM) is formed by sequential changes in the different brain regions due to synaptic plasticity changes. This synaptic plasticity changes formed in the brain due to the acquittance of unexposed information and its retrieval due to learning and memory formation (LMF). In a normal condition, LMF uses RNA and protein synthesis machinery to form LTM, which lasts till the end of an organism's lifetime. Formed LTM shows sequential changes happening in the presynaptic and postsynaptic neurons. Stated sequential changes are initiated when the released neurotransmitter binds with the postsynaptic neuronal receptors and activates the brain's ERK - CREB neuronal signaling pathway. Based on the previous findings, the present study was designed to study the interrelationship between cognitive impairment and oral/gut dysbiosis with the help of a probiotic strain (*Lactobacillus plantarum*). Two phases of behavioural analysis (first and second phase) were used to identify the effect of oral microbial infusions on impaired LTM formation and its reversal using restoration of dysbiosed gut/oral microbiota. The first phase of behavioural analysis (FPBA) reported that oral microbial infusion plays a major role in developing oral/gut dysbiosis, which results in impaired cognitive functions. Further, formed oral/gut microbiota dysbiosis was reversed with the help of probiotic strain in the second phase of behavioural analysis (SPBA). Thus, a comparative two-phase behavioural analysis revealed that probiotics may play a significant role in reversing induced cognitive decline. The outcome of the present study also proved that probiotic treatment might play a major role in the reversal of dysbiosed microbiota in the oral cavity and the gut.

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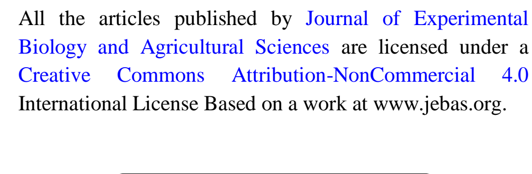

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1 Introduction

Microorganisms are tiny creatures of our nature present throughout the globe. These microorganisms have beneficial and harmful effects on all host systems, including humans. Fermented food products like cheese, yogurt, and other canned products contain some beneficial microorganisms which may promote beneficial effects in humans through the production of some primary/secondary metabolites by these microorganisms (Rezac et al. 2018; Xiang et al. 2019; Maraz and Khan 2021; Zapaśnik et al. 2022; Hakim et al. 2023; Icer et al. 2023). These microorganisms are categorized as beneficial microorganisms, and various lactobacillus species like *Lactobacillus casei*, *L. curvatum*, *L. delbrueckii*, *L. bulgaricus*, *L. plantarum*, *L. sakei* are also included in this (Lorenzo et al. 2018; Rezac et al. 2018; Hakim et al. 2023; Icer et al. 2023). These lactobacillus species play an essential role in developing oral/gut homeostasis mechanisms within the host. Disruption in this oral/gut homeostasis mechanisms results in oral/gut dysbiosis, which further results in systemic diseases like neurodegenerative disorders (Radiac and Kapila 2021; Hou et al. 2022; Deandra et al. 2023; Ma et al. 2023; Mukilan 2023).

Neurodegenerative (ND) disorders are complex progressive disorders that affect aged persons, especially those aged above sixty years. Several health parameters are associated with the development of this ND disorder; among others, improper oral hygiene plays a major role in the development of cognitive decline (CD). This CD is one of the characteristic features of ND disorders, which progress through memory loss (Gómez- Gómez and Zapico 2019; Alvarenga et al. 2021; Guo et al. 2022; Hou et al. 2022; Lamptey et al. 2022; Gu et al. 2023). Recent reports have shown that poor oral hygiene plays a significant role in the initiation of cognitive decline during mild cognitive impairment/long-term memory formation. This poor oral hygiene may be a result of pathogenic microbial colonization, which affects the normal oral microflora of the oral cavity (Chung and Chan 2023; Gu et al. 2023; Kulkarni et al. 2023; Mukilan et al. 2024; Pruntel et al. 2024). Imbalances in the oral microbiota further result in gut dysbiosis through the transmission/transport of formed pathogenic colonizes from the oral cavity to the gut. Imbalances in this gut microbiota may reduce the transmission of neurotransmitter precursor (NP) compounds from the gut to the brain through the vagus nerve (Park et al. 2021; Yu et al. 2021; Lu et al. 2023). Reduced amount of NPC compound results in reduced synthesis and release of neurotransmitters from presynaptic neurons (PrSN). Released neurotransmitter further binds with postsynaptic neuron membrane receptors and activates a reduced amount of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), and enzyme-regulated kinase – 1/2 (ERK-1/2) through reduced calcium influx happening inside the postsynaptic neuron (PoSN). Less expressive cAMP, PKA, and ERK-1/2 may reduce the phosphorylation of cAMP response element binding protein – 1 (CREB-1). Further, reduced phosphorylation of CREB - 1 down-regulates the expression of immediate early genes and postsynaptic density proteins (Ganesh et al. 2012; Olguín et al. 2016; Mukilan et al. 2015; Sivasangari and Rajan 2020; Rajan 2021; Mukilan 2023). Reduced expression of this neuronal molecule may further result in cognitive impairment (CI) initiation. Formed CI resulted from reduced long-term memory formation under dysbiosed conditions (Országhova et al. 2021; Mukilan 2022; Mukilan 2023). In the present study, we examined the unavoidable role of probiotic strain (*L. fermentum*) in reversing formed LTM impairment with the help of two-phased reward-based learning paradigms (RBLP).

2 Materials and Methods

2.1 Study animals (SA)

Commercially available healthy naïve goldfish (*Carassius auratus*) with a body length of 6.5-8 cm and 6-15 g weight were purchased from a local aquarium shop in Coimbatore, Tamil Nadu, India. Purchased animals were shifted from the aquarium to the laboratory with utmost care to prevent the formation of stress. Once shifting was over, study animals (SA) were housed in a standard rectangular glass tank with a length, breadth, and height of 42 X 30 X 21 inches for five days during the assimilation process. During assimilation, SA was maintained at standard controlled laboratory conditions with a photoperiod of 12 (light):12 (dark) hours, a controlled temperature range between 28 ± 2º C, and continuous aeration. SA fed with commercially available food resources (dry food pellets – Taiyo Pet Products Pvt Ltd, India) three times a day with a time interval of five hours in their assimilation environment (9.00, 14.00, and 19.00 h). The water quality was measured to maintain the home tank water with the needed amount of dissolved oxygen content during the experimental period (EP). The home tank was continuously replaced with fresh water on alternative days till the end of EP to maintain a dust-free environment. All experimental study designs follow the institutional animal care guidelines of the institution (Sri Ramakrishna Group of Institutions, Coimbatore, Tamil Nadu, India).

2.2 Study Design

A reward-based learning paradigm (RBLP) was used in this study design (SD) to study the effect of normal and dysbiosed microbial flora on LTM formation. SA underwent three phases of RBLP (exploration, training, and testing) in a glass experimental chamber (GEC) having a length, breadth, and height of 42 X 30 X 21 inches. This GEC contains two feeding chambers (FC) and one central chamber (CC). The designed GEC, CC, and FCs were differentiated based on the length and breadth size. CC acts as an entry space for the SA in the GEC with a size of 30 (length) X 20

(breadth) X 21 (height) inches. Compared to CC, both the FCs have a size of 6 (length) X 5 (breadth) X 21 (height) inches and serve as either positive/negative reward chambers. The commercially available dry food pellets were given as a reward for learning the cue in positive FC, not negative FC (Mukilan 2023).

2.3 Collection and Analysis of Oral Swab Sample

The oral swab samples were collected from ten healthy individuals (without dental plaques – aged between 18-21 years), six diseased individuals (without dental plaques – aged between 18-24 years) from the Department of Biotechnology, Sri Ramakrishna College of Arts & Science (SRCAS), Coimbatore, Tamil Nadu, India. After determining their concerns, oral swab samples were taken from healthy and diseased individuals. All oral samples were collected using a sterile cotton swab by swabbing the teeth and their associated surface. Collected samples were spread on nutrient agar plates under aseptic conditions in laminar airflow. Spread plates were incubated at 37º C for 24 - 48 hours. Later, incubated grown plates were used to isolate the desired microorganisms using pure culture techniques from both samples. Isolated microorganisms (M1, M2, and M3) were infused into EGs 2-5, and their impact on LTM formation was studied using RBLP behavioural scores. Behavioural scores were calculated based on the amount of time spent by the EA in the LC, CC, and RC. RC and LC act as positive and negative reward chambers. After RBLP analysis, three oral-infused cultures were identified using biochemical test results.

2.4 Biochemical Characterization

Isolated microorganisms (M1, M2, and M3) were characterized using biochemical characterization tests like indole, methyl-red, catalase, voges-proskauer, and oxidase tests. Biochemical characterization tests were performed according to the standard conditions (Alghamadi 2021).

2.5 Oral Infusion Mixture Preparation

The grown overnight cultures were used to prepare the oral infusion mixture with the help of phosphate buffer saline (PBS) for its infusions in the four experimental groups (EGs) except the control. Experimental group 1 (EG–1) did not receive any oral infusion as it was designed as a control, while EGs – 2, 3, and 4 received oral infusions of culture M1, M2, and M3 in the ratio of 50:50 (contains 50% desired microorganism and 50% PBS) as a single dose of 500 microlitres, and EG – 5 received oral infusion mixture ratio of 20:20:20:40 [contains M1, M2, and M3 cultures (each in every 20%) and PBS (40%)]. Formulated blends were orally infused into the specific EGs as a single dose. Following oral infusions, all EGs were provided 24 hours to recover from the handling stress before the first phase of behavioural analysis (FPBA). Followed by FPBA, the second phase of behavioural analysis (SPBA) was carried out with the help of a probiotic strain *L. plantarum* (MTCC No. 12921) acquired from MTCC, IMTECH, Chandigarh, Punjab, India. The acquired probiotic culture was used to prepare an oral probiotic mixture in a ratio of 50:50 (Mukilan et al. 2024).

2.6 Behavioural Analysis

Behavioural analysis was carried out in two consecutive phases, i.e., the first and second phases, to study the effect of desired cultures on LTM impairment and its reversal using a probiotic strain, *L. plantarum*. In FPBA, SAs were separated into four different experimental groups (EGs) according to the needs of the study. The EGs consist of experimental group – 1 (control), experimental group – 2 (infused with isolate M1), experimental group – 3 (infused with isolate M2), experimental group – 4 (infused with isolate M3), and experimental group – 5 (infused with M1, M2, and M3). Before infusions, experimental animal activities were confirmed in the exploratory phase. After the exploratory phase, microbial oral infusions were given to the EG – 2, 3, 4, and 5. Followed by infusions, training, and testing were performed in the FPBA. In SPBA, all four experimental groups (EGs – 2 – 4 and 5) receive probiotic oral infusions before taking the training and testing phases in RBLP. In RBLP, behavioural responses were calculated based on the time spent in the LC, CC, and RC. Other than RBLP, open field test (OFT), and predator exposure test (PET) were also performed to test the effect of microbial oral infusions on the development of anxiety-like behaviour, and fear memory formation in all EGs.

2.7 Predator Exposure Test

The predator exposure test (PET) was performed after completion of FPBA to identify the development of fear memory formation in the infused and non-infused EGs. For PET, we have used rectangular glass tanks for the creation of three different compartments [complete fear (CF), mid fear (MF), and No fear (NF) zones] within a single behavioral experimental chamber having a size of 42 X 30 X 21 inches (length, breadth, and height). Inside the NF zone, a separate chamber is created to hold the predator in an isolated manner. *Pseudotropheus demasoni* (cichlid fish) was used as a predator for this study. Behavioral responses were calculated based on the time spent in the CF, MF, and NF zones during the 900 seconds of exposure to the predator (Thangaleela et al. 2018).

2.8 Open Field Test

Following PET, all EGs were allowed to perform an open field test (OFT) in the designed GEC with the equal-sized individual box (10 x 5 cm) diagram placed below the bottom of the GEC. After completion of PET, EGs were introduced to the behavioral setup (BS) for 900 seconds, and their mobility was recorded based on the time spent in the inner compartment (TSI) and time spent in the

outer compartment (TSO). Behavioural responses of the EGs were used for identifying the presence/absence of anxiety-like behavior in the BS (Horka et al. 2024).

2.9 Data Analysis and Graphical Representation

Behavioural scores of all experimental groups were recorded in a single Microsoft Excel Sheet according to the behavioural parameters like IPBS exploration, training, and testing for IPBS and SPBS, amount of time spent in CF, MF, and NF zones for PET, and TSI, and TSO for the OFT. Recorded scores of consecutive days were used to calculate the mean value (MV), standard deviation, and standard error (SE) for the statistical analysis. MV and SE were used to create bar diagrams.

3 Results

3.1 Effect of Microbial Oral Culture Infusions on Long-Term Memory Retrieval

The present study uses FPBA to explore the role of pathogenic/non-pathogenic oral infusions on cognitive learning and memory formation during LTM retrieval with the help of RBLP in a habituated environment.

3.1.1 Identification of Induced Cognitive Memory Impairment

Initially, IPBS was used to study the role of oral microbial infusions in the induction of cognitive memory impairment. In IPBS, all five EGs (EGs 1 -5) underwent a habituation (H) process in the home tank for seven days (Days 1-7). All EGs were maintained in the home tank between days 1-7 during the H process to adapt to the laboratory setup. Following the process of H, the exploration phase (E) was carried out for all EGs in the ES between days 8-10 for 15 minutes/day. Behavioural scores of the exploration E phase showed that all EGs' animals were active, and ES did not provide any stressed environment for the habituated EGs (Figure 1). Following the E phase, four experimental groups (EGs 2-5) received oral infusions of desired microorganisms in pure (EGs 2-4) and mixed forms (EG 5). After receiving oral infusions, EGs were maintained in separate tanks for transportation and colonization in the gut for three days (Days 11-13).

Microorganisms present on the collected oral swab samples were grown on a nutrient agar medium under aseptic room conditions for 24-48 hours. After incubation, unknown microbial colonies were picked up from the mother plate, and their purity was confirmed by the quadrant streaking method (Figure 2). After purity confirmation, a quadrant individual colony was used to prepare the respective overnight cultures. Grown overnight cultures were named M1, M2, and M3 and infused orally into designated EGs in pure and mixed forms. The result of biochemical characterization tests (indole, methyl red, catalase, Voges-Proskauer, and oxidase tests) showed that isolated culture M1 was identified as *P. aeruginosa* based on the positive results of catalase and oxidase tests, culture M2 showed positive results for methyl red and catalase tests and characterized the presence of *E. coli*, and isolated culture M3 showed positive reaction for all four tests except methyl red which shows the presence of *A. hydrophila* (Figure 3 & Table 1).

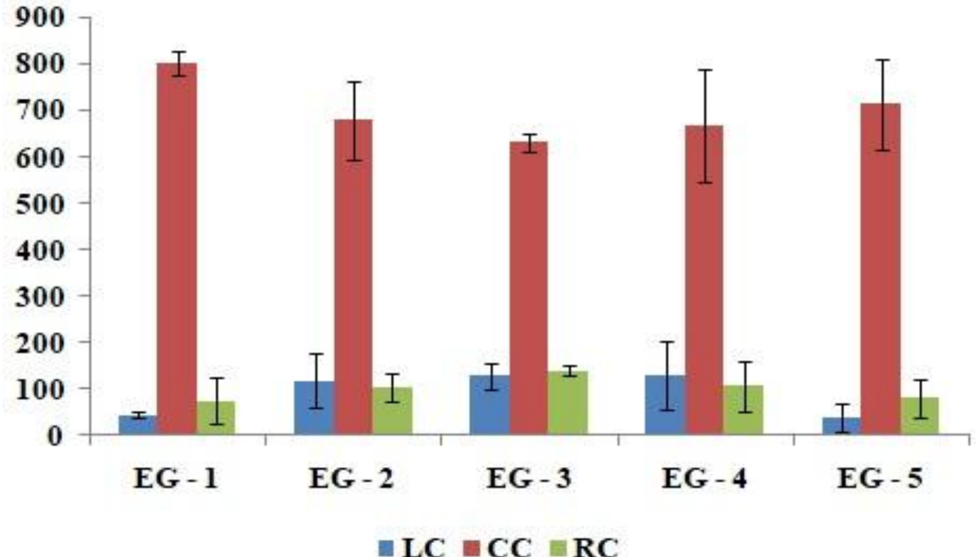

Figure 1 Exploratory phase of the first behavioral analysis phase showed that all experimental animals were active and adapted to the experimental setup. The amount of time spent in the left chamber (LC), central chamber (CC), and right chamber (RC) were used for the preparation of the bar diagram with standard error and mean (SEM) values

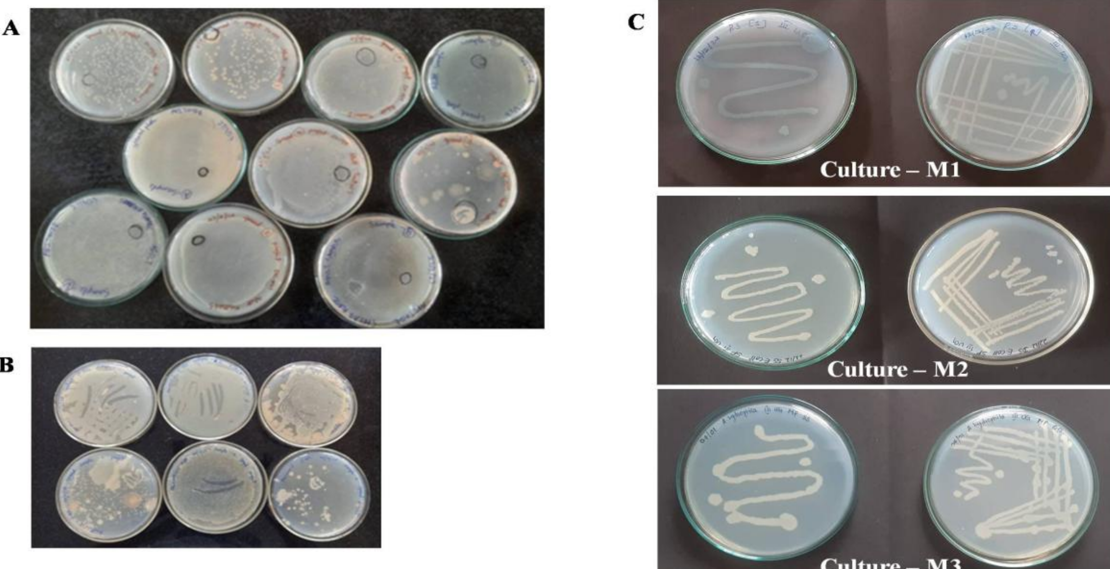

Figure 2 Representative plate pictures isolated from the collected oral swab samples (A – healthy individuals, B – diseased individuals), (C) Based on their colony morphology, three different bacterial colonies were picked up and grown on nutrient agar medium and named as culture – M1, M2, and M3

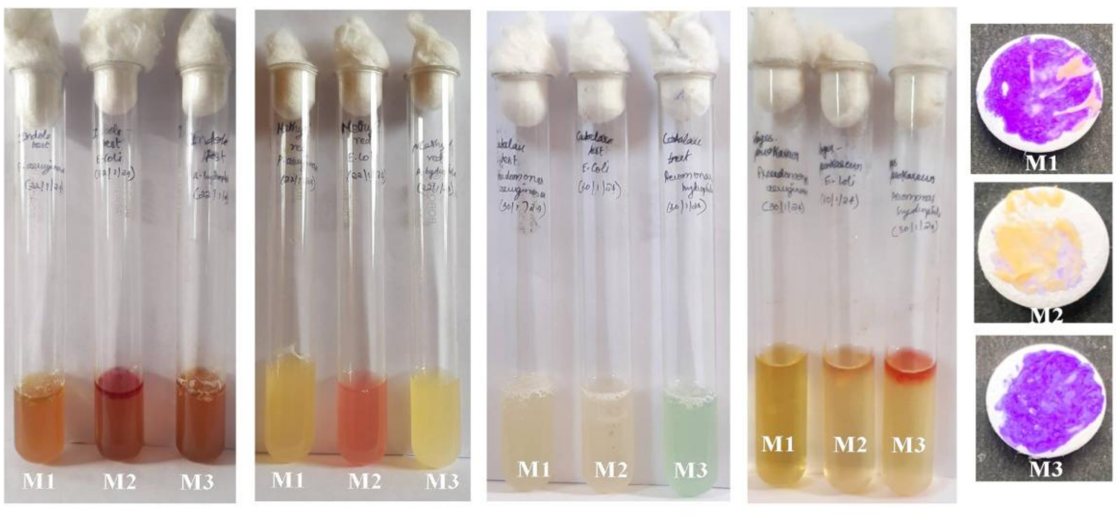

Figure 3 Biochemical characterization results showed that selected desired cultures – M1, M2, and M3 were identified as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Aeromonas hydrophila*

Table 1 Results of biochemical characterization test performed in this study

Name of the biochemical test	*Pseudomonas aeruginosa*	*Escherichia coli*	*Aeromonas hydrophila*
Indole	-	-	+
Methyl red	-	+	-
Catalase	+	+	+
Voges-proskauer	-	-	+
Oxidase	+	-	+

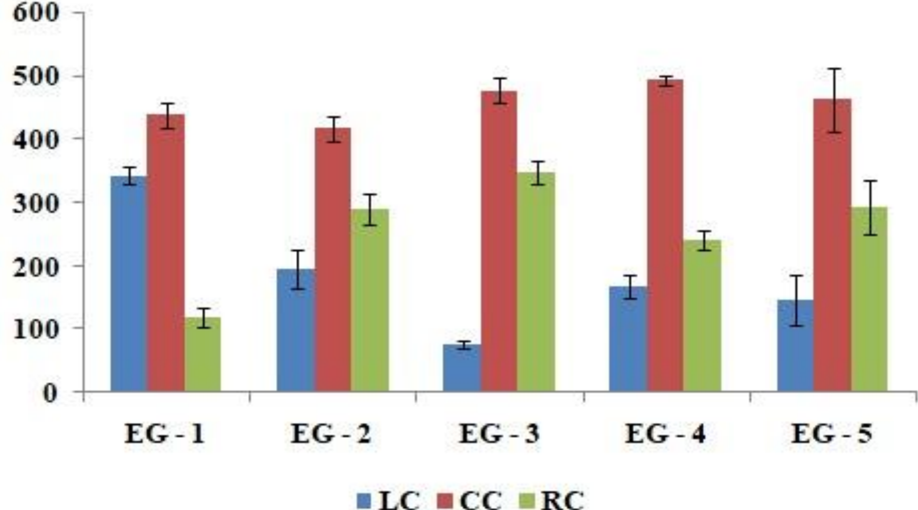

Figure 4 The first phase of behavioural training showed that all experimental animals were trained in the experimental setup with the help of positive and negative reward conditioning. The amount of time spent in the left chamber (LC), central chamber (CC), and right chamber (RC) were used for the preparation of the bar diagram with standard error and mean (SEM) values

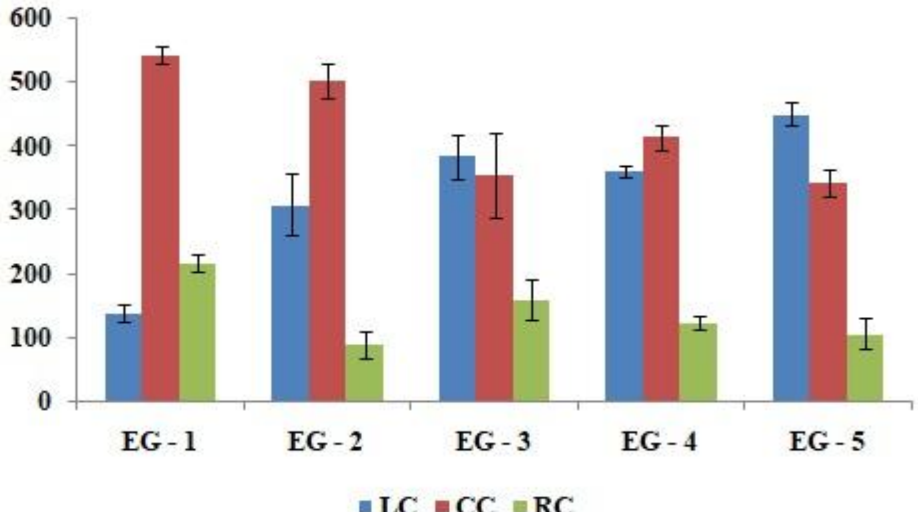

Figure 5 The first phase of behavioural testing proved that microbial oral infusions induced impaired cognitive memory retrieval. The amount of time spent in the left chamber (LC), central chamber (CC), and right chamber (RC) were used for the preparation of the bar diagram with standard error and mean (SEM) values

After infusions, the training (Tr) phase was carried out in ES for three days between days 14-17 for all EGs (EG 1-5). Behavioural responses of the Tr phase showed that microbial oral infusions did not impact EGS 2-5 compared to EG-1 (did not receive any infusion). Obtained scores also proved that microbial oral infusions showed no impairment in the Tr phase of FPBA. It also proved that the infused oral microbial mixture did not involve gut dysbiosis formation during the Tr phase (Figure 4). Further, the testing (Te) phase was carried out after a seventy-two-hour interval of the Tr phase (Days 21-23). Consolidated behavioural scores of the Te phase showed that oral microbial infusions had a varying range of memory impairment in EGs 2-5 during the memory retrieval in FPBA compared to EG-1 (Figure 5). The results showed that EG 3, 2, 4, and 5 gradually developed memory impairment compared to the non-infusive group (EG – 1). Observed results proved that pathogens like *P. aeruginosa* and *A. hydrophila* play a significant role in developing cognitive memory impairment through gut dysbiosis compared to commensal microorganisms like *E. coli*. The outcome of the FPBA showed that oral microbial infusions play a major role in the development of cognitive memory impairment compared to the impact of memory formation on non-infusive EG (Figure 6).

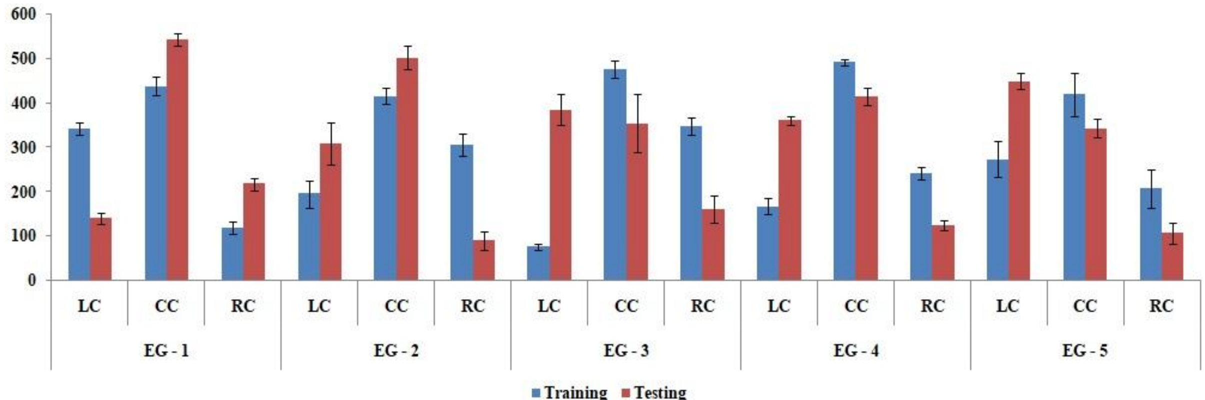

Figure 6 Comparative analysis of the first phase of behavioural training and testing proved that microbial oral infusions had a greater impairment on memory retrieval compared to the training scores [left chamber (LC), central chamber (CC), and right chamber (RC)]. SEM value was represented in the bar diagram

3.1.2 Impact of Oral Microbial Infusions on Stress Formation

Before SPBA, experimental animals were allowed to perform the predator exposure test (PET), and open field test (OFT) to identify the presence/absence of fear memory formation/stress among the infused and non-infused EGs. Initially, PET was performed to determine the amount of fear memory developed in EGs after FPBA. PET Scores showed that fear memory development was not induced in the infusive groups (in both infusions, i.e., pure and mixed form) compared to the non-infusive group. Behavioural responses of the infused EGs (EG – 2, 3, 4) showed that oral microbial infusions of *P. aeruginosa, E. coli*, and *A. hydrophila* will develop the least amount of fear memory formation compared to the non-infused EG (EG – 1). However, mixed cultures of *P. aeruginosa*, *E. coli*, and *A. hydrophila* cause higher levels of memory of fear in the EG – 5 (Figure 7). Following PET, OFT was performed to identify the effect of microbial oral infusions on motor behaviour and stress development in the experimental animals. Behavioural responses showed that microbial oral infusions do not impact the motor behaviour of the EGs. Still, it showed the development of anxiety-like behaviour in the infused group (EG - 2, 3, 4, and 5) compared to the non-infusive experimental group (EG -1). Obtained results showed that microbial oral infusions might play a major role in the development of anxiety-like behaviour due to oral/gut dysbiosis. They also proved that experimental animals were physiologically active and could explore the provided stimuli. Behavioural scores of the infusive EGs (EG – 2, 3, 4, and 5) in pure and mixed forms showed a higher amount of time spent in the outer compartment, which shows the development of anxiety-like behaviour compared to the non-infusive group (EG -1). Responses of EG -1 (non-infusive group) showed a higher amount of time spent in the inner compartment, which proves the absence of anxiety-like behaviour development. The observed pattern of the non-infusive group (EG

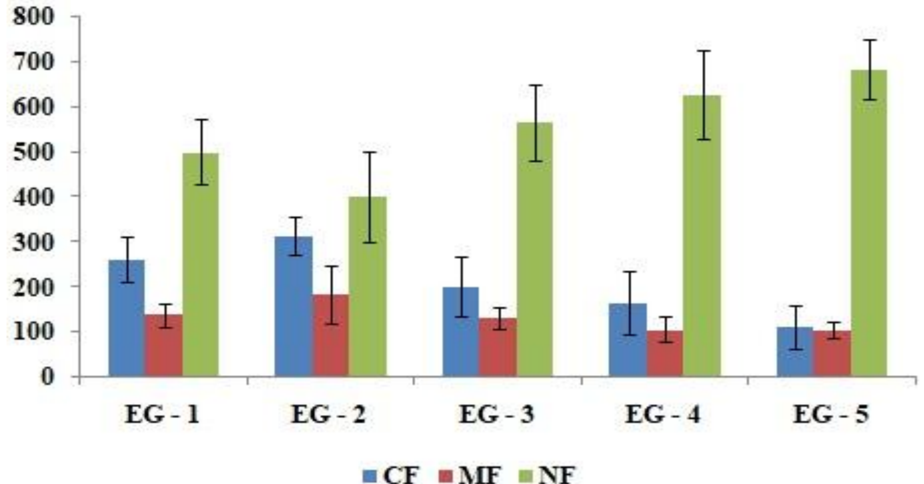

Figure 7 Behavioural scores of the predator exposure test showed that microbial oral infusions do not show any fear memory development (No fear (NF) zone) compared to mid fear (MF) zone, and complete fear (CF) zone. The represented bar diagram was created using the average mean value and standard error value presented in the form of a pin

– 1) was vice-versa in the infusive groups (EG – 2, 3, 4, and 5). The outcome results of the OFT stated that microbial oral infusions induce anxiety-like behaviour in the infused group as a result of dysbiosed oral and gut microbiota. Dysbiosed oral and gut microbiota are further involved in the production of the stress hormone cortisol in the host, which may affect cognitive memory retrieval (Figure 8).

3.2 Impact of probiotic strain *L. plantarum* on the reversal of cognitive impairment

Following FPBA, SPBA was performed to understand the impact of probiotic strain (*L. plantarum*) on the reversal of oral/gut dysbiosis-induced LTM impairment.

3.2.1 Reversal of Induced Cognitive Impairment by the Reversal of Dysbiosed Microbiota

After completion of OFT and PET, the Tr and Te phase was carried out in the SPBA to test the effect of probiotic strain on the reversal of cognitive impairment. After FPBA, probiotic oral infusions were given to all EGs (EGs 2, 3, 4, and 5) in pure and mixed form. After probiotic oral infusions, a three-day time interval (28-30 days) was given to all EGs. Following a time interval, the SPBA Tr phase was carried out between days 31-33 to identify the impact of probiotic oral infusion (POI) on information acquisition. Behavioural scores of the Tr phase showed that POI enhanced higher learning abilities in EGs – 2, 3, and 4 compared to EG -5 (Figure 9). Observed results also showed that POI may

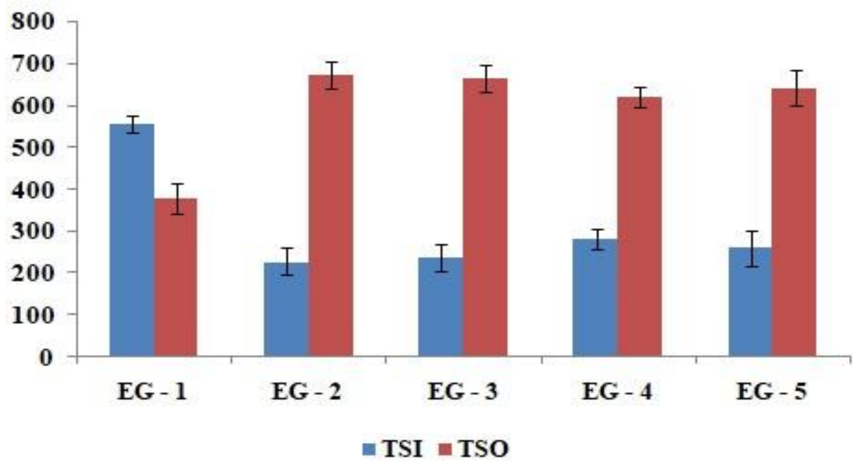

Figure 8 Behavioural responses of open field test (OFT) showed the effect of oral microbial infusions in the development of anxiety-like behaviour Represented bar diagram showing the SEM value in the form of a pin on bars (TSI – time spent in the inner compartment; TSO - time spent in the outer compartment)

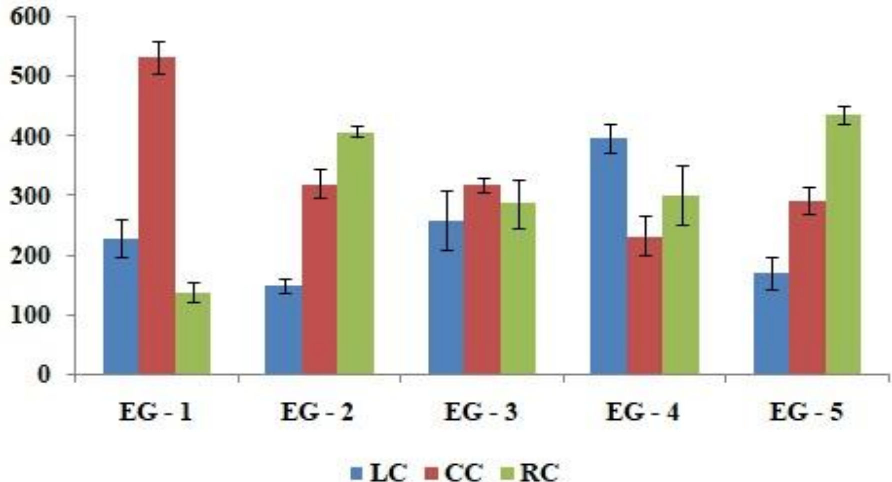

Figure 9 Behavioural responses of second phase training showed that probiotic oral infusions may take part in the reversal of oral/gut dysbiosis through the restoration of dysbiosed microbiota in experimental groups – 2, 3, 4, and 5 compared to the experimental group - 1. [left chamber (LC), central chamber (CC), and right chamber (RC)]. SEM value was represented in the bar diagram.

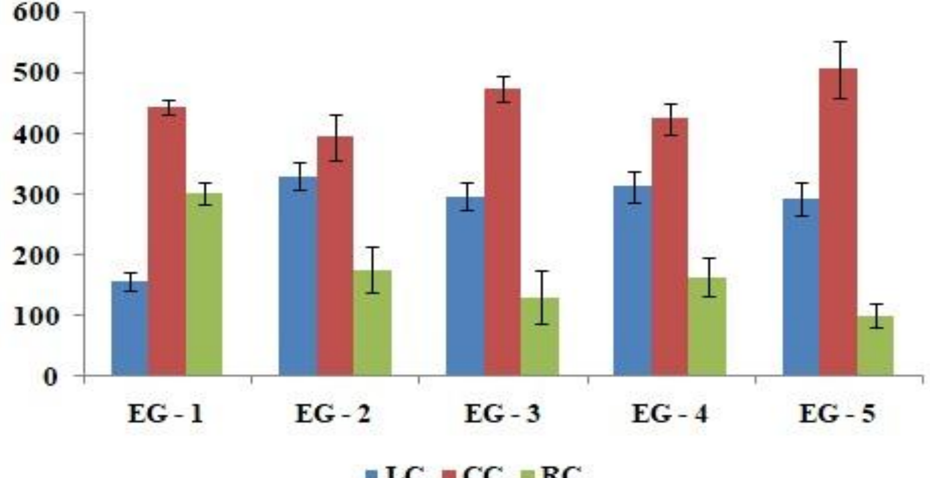

Figure 10 Behavioural responses of second phase testing proved that probiotic oral infusions completely restored dysbiosed microbiota in infused experimental groups – 2, 3, 4, and 5 compared to non-infused experimental group - 1. [left chamber (LC), central chamber (CC), and right chamber (RC)]. SEM value was represented in the bar diagram

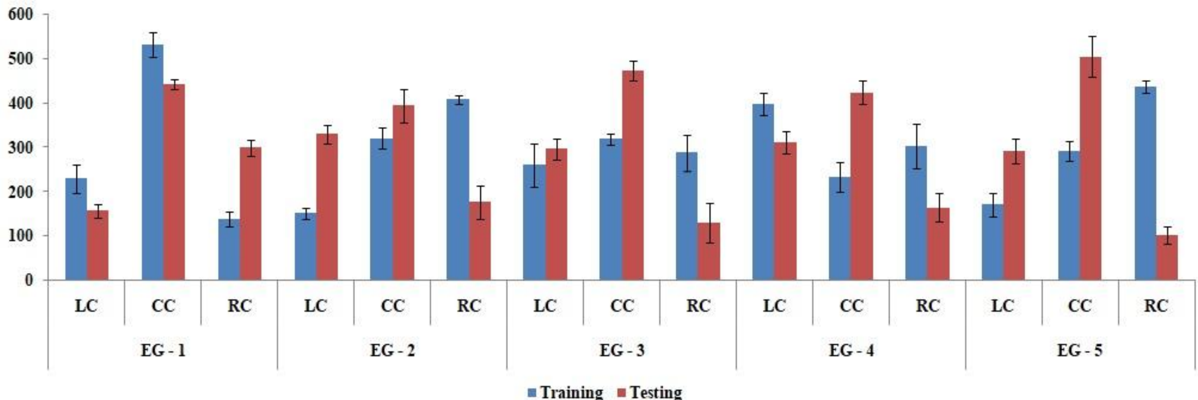

Figure 11 Comparative analysis of the second phase of behavioural training and testing showed that infused experimental groups able to retrieve learned information after receiving probiotic oral infusions. [left chamber (LC), central chamber (CC), and right chamber (RC)]

reverse the dysbiosis-induced production of neurotransmitter precursor compounds in the gut, which results in its increased transmission to the central nervous system (CNS). After three days (Days 34-36), the Te phase of SPBA was carried out between 37-39 days. Behavioural responses of the Te phase proved that POI reversed the cognitive impairment in an increased manner in EG – 2, 3, 4, and 5 compared to the control group EG – 1 (Figure 10). Comparative analysis of FPBA and SPBA showed that microbial infusion of the oral pathogen plays a major role in the formation of cognitive impairment through the aberration of beneficial microbiota in the oral cavity/gut. This dysbiosed oral/gut microbiota may play a major role in the development of cognitive impairment. The formed cognitive impairment in FPBA was reversed with the help of probiotic microorganisms used in SPBA as an oral mixture. Reversal of cognitive impairment may result from restoration of normal oral/gut microbiota or reduction of pathogenic load in the oral cavity/gut (Figure 11).

4 Discussion

The present study identified the impact of pathogenic microbial colonization in the development of impaired cognitive memory formation through the microbiome-gut-brain (MGB) axis. This MGB plays a major role in the development of cognitive health. Recent studies have shown that gut microbial dysbiosis may play a major role in the development of neurodegenerative disorders (ND) like Alzheimer's disease (AD), Parkinson's disease (PD), mild cognitive impairment (MCI), etc (Dai et al. 2022; Zhang et al. 2022; Krishaa et al. 2023; Mukilan 2023). In ND, gut dysbiosis (GD) may take part in various dysfunctions like decreased production of neurotransmitters, increased intestinal permeability, development of brain lesions/neuroinflammation, and oxidative stress (Ma et al. 2019; Chidambaram et al. 2022; Hou et al. 2022; Intili et al. 2023; Varesi et al. 2023). These conditions may play a major role in developing imbalances in the brain homeostasis

mechanism (BHM). Imbalances in BHM may result in developing brain cognitive dysfunctions associated with LTM formation. This GD was also progressed by various conditions like poor diet, poor oral hygiene, matrix metalloproteinase expressions, infections, and antibiotic treatment (Chung and Chan 2023; Gu et al. 2023; Kulkarni et al. 2023; Pitchaikani et al. 2024). Among others, poor oral hygiene plays a major in the formation of cognitive impairment during the period of childhood and adolescence. This poor oral hygiene was caused by the attachment and colonization of unwanted pathogenic microorganisms in the oral cavity (OC). Colonization of these microorganisms in OC results in the development of tooth decay, periodontal diseases, and tooth loss. Dysbiosis in oral flora further transports highly colonized pathogens to the gut through the mucosal lining (Mukilan 2023; Wei et al. 2023; Pruntel et al. 2024). As a result, disruption of gut microflora takes place in the gut, showing the least dysbiosed amount of beneficial flora. This dysbiosis initially results in intestinal inflammatory disorders (IID) development. Further, this IID results in increased intestinal permeability, which results in the least transmission of neurotransmitter precursor compounds from the gut to the brain through the blood-brain barrier (BBB) (Ma et al. 2019; Rutsch et al. 2020; Kandpal et al. 2022; Santana et al. 2022).

Besides BBB, the enteric nervous system (ENS) also plays a major role in the development of LTM due to its interconnection with the central nervous system (CNS). In a healthy state, ENS transports secreted NPC from the gut to the brain for the synthesis of neurotransmitters in presynaptic neurons, followed by its release into the synaptic cleft (SC) (Martande et al. 2020; Fleming II et al. 2020; Orr et al. 2020; Dicks 2022). Once released into SC, it binds with postsynaptic neuronal receptors and activates neuronal molecules involved in the extracellular regulated kinase (ERK) - cAMP response element binding protein (CREB) signaling pathway in the brain. This ERK-CREB signaling pathway uses adenyl cyclase (AC), protein kinase A (PKA), immediate early genes (IEGs – *Egr-1*, *C-fos*, and *C-jun*), and postsynaptic density proteins (PSDs like PSD-95) for the formation of LTM (Mukilan et al. 2018a, b; Sivasangari and Rajan 2020; Rajan 2021; Mukilan 2022; Mukilan 2023). In the present work, we tried to elucidate the impact of a diseased/dysbiosed state on LTM formation with the help of oral microbial infusions. Our results showed that oral microbial infusions have a more significant effect on the development of cognitive decline by down-regulating the expression of neuronal signaling molecules involved in the ERK-CREB signaling pathway (Mukilan 2023). Our data also showed that oral microbial infusion may have a greater impact on the development of oral/gut dysbiosis through the secretion of LPS, bacterial toxins, and antibiotics over the beneficial flora. Dysregulated oral/gut flora may result in the decreased secretion and transportation of NPC from the gut to the presynaptic neuron through the BBB and ENS. As a result, impaired LTM was formed in the brain regions. In this study, impaired LTM was also reversed with the help of the probiotic strain *L. plantarum*.

Conclusion

The outcome of the present study showed the role of *P. aeruginosa*, *E. coli*, and *A. hydrophila* microbial infusions on oral/gut dysbiosis and also results in impaired LTM formation. Further, this study also proved that microbial colonization in the oral cavity/gut may result in the aberration of normal beneficial flora in those regions. Experimental results showed that the entry of pathogens into the oral passage through poor oral hygiene may play an important role in developing imbalanced oral/gut microbiota. Further, it results in impaired LTM formation validated by FPBA. FPBA results showed a higher level of impairment in the EGs who received oral infusions of *P. aeruginosa* and *A. hydrophila* than in the EGs who received oral infusions of *E. coli*. It also proved that cognitive decline is higher in the EG who received mixed oral mixture than other EGs (received pure oral mixture). Thus, FPBA elucidated that all three isolates showed a high level of impaired memory formation in its complex form. Later on, impaired LTM was reversed with the help of probiotic strain in SPBA. SPBA results showed that cognitive reversal was high in EGs (received pure oral infusions) and low in EGs received complex mixtures. Comparative behavioural analysis proved that induced oral/gut dysbiosis may reversed with the help of probiotics in this pioneering study. Thus, the present research findings support the use of traditional fermented foods in treating memory loss or cognitive reversal in ND.

Authors Contributions

MM performed the conceptualization, research design, funding acquisition, original investigation, draft preparation, review, and manuscript editing. VE, MV, and MD performed the experiments and data collection.

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Conflict of Interest

Authors report no conflicts of interest in this work

Data Availability

Research data is available with the authors and shall be provided upon request.

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Exploring Mechanism of Actions for Eugenol and Beta-Caryophyllene to Combat Colorectal Cancer Chemotherapy Using Network Pharmacology

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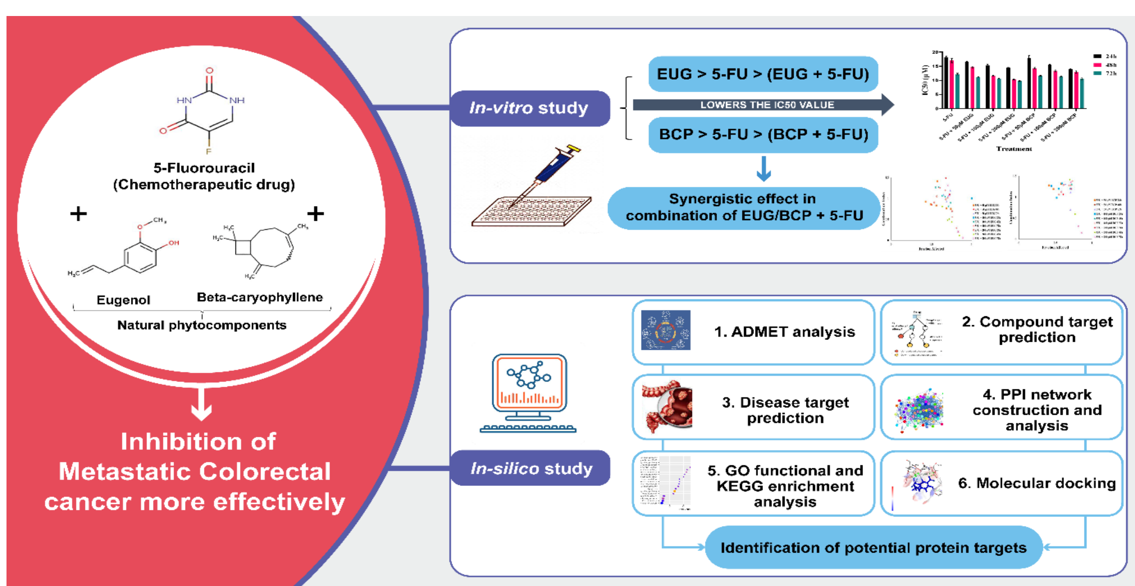
GRAPHICAL ABSTRACT



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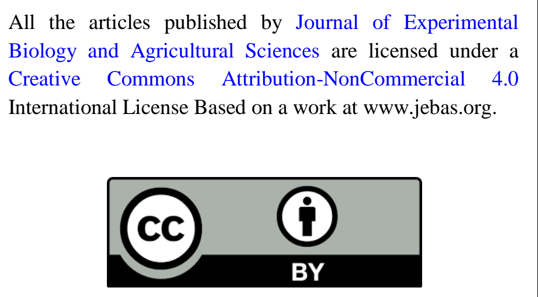


KEYWORDS

Beta-caryophyllene

Caspase-3

Colorectal cancer

Eugenol

Multi-targeted approach

Synergism

ABSTRACT

This study explores the potential of essential oils, Eugenol (EUG), and Beta-Caryophyllene (BCP) in enhancing the efficacy of the chemotherapeutic drug 5-fluorouracil (5-FU) in treating metastatic colorectal cancer (CRC). Pharmacokinetic assessment through ADMET analysis indicates that EUG and BCP adhere to the rule of five with good bioavailability, ensuring their drug-likeness properties. The study employs a multitarget strategy to reduce drug dosage and enhance effectiveness, testing the compounds on the HCT116 human colorectal cancer cell line. MTT assay revealed *in-vitro* cytotoxic effects of EUG, BCP, and 5-FU, with a noteworthy reduction in IC_{50} values observed when combining the compounds, indicating synergistic effects (CI < 1) as depicted in the Fa-CI plot. Network pharmacology-based analysis of the compound-disease-target (C-D-T) network identifies 58, 24, and 49 target proteins for EUG, BCP, and 5-FU, respectively, in metastatic CRC. Venn diagram intersection reveals 11 common target proteins, and the merged C-D-T network highlights 84 target proteins, with 16 selected based on edge count, including HSP90AA1, IGF-1R, ESR1, and CASP3. Molecular docking studies indicate that EUG, BCP, and 5-FU effectively inhibit the core target protein HSP90AA1 within the C-D-T network, suggesting their potential as modulators for CRC metastasis. These findings propose a promising approach for developing drugs targeting specific proteins to mitigate metastasis in colorectal cancer.

1 Introduction

Colorectal cancer (CRC) poses a significant global health challenge, with projections indicating a 60% increase by 2030, amounting to 2.2 million new cases and 1.1 million deaths (Toiyama et al. 2014; Arnold et al. 2017; Zhu et al. 2018; Benarba and Pandiella 2018; Huang et al. 2020). Current CRC therapies, encompassing surgery, radiotherapy, chemotherapy, and targeted therapy, face limitations, particularly in managing metastatic lesions (Fan et al. 2020). The widely used chemotherapeutic drug 5- Fluorouracil (5-FU) (Figure 1A), despite its efficacy, is hampered by side effects such as leukopenia, which can lead to life-threatening complications and therapy discontinuation (Kadoyama et al. 2011; Casale and Patel 2022).

In pursuit of alternative therapies, this study focuses on natural compounds, specifically eugenol (EUG) (Figure 1B) and beta-caryophyllene (BCP) (Figure 1C), derived from plants like *Eugenia caryophyllata* and *Ocimum sanctum.* Inspired by Hippocrates' wisdom, the research explores the medicinal properties of these compounds, known for their antioxidant, antimicrobial, anticancer, and anti-inflammatory effects (Fernández-Ruiz et al. 2013; Ulanowska and Olas 2021; Alonso Gómez et al. 2022).

Leveraging network pharmacology, the prime aim of the study is to assess the pharmacokinetic properties and interactions of EUG and BCP with core target proteins in CRC. Molecular docking validates these targets, providing insights into the mechanism of action on various cell signaling pathways. This research offers a

Figure 1 Structures of (a) 5-Fluorouracil (5-FU), (b) Eugenol (EUG), (c) Beta-caryophyllene (BCP)

promising avenue for developing safer and more effective CRC treatments based on natural compounds derived from herbal sources.

2 Materials and methods

2.1.1 Cell culture and maintenance

Human Colorectal Carcinoma cell line HCT116 was obtained from the National Centre for Cell Science (NCCS), Pune, Maharashtra, India. Cells were cultured in DMEM medium supplemented with 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin and maintained at 37 °C with 5% CO_2. Cells were sub-cultured on attaining ~ 80% confluency for further experiments using 96 or 24-well plates.

2.1.2 MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay

Cytotoxicity was measured using an MTT reagent to detect NADH-dependent dehydrogenase activity of the test compounds (Mosmann 1983). Briefly, HCT116 cells were seeded in a 96-well plate at 5×10^3 cells/well density, allowed to attach and grow for 24h in a complete DMEM medium, then treated with different concentrations of EUG (1 µM to 1000 µM), BCP (1 µM to 1000 µM), and 5-FU (3 µM to 1537.53 µM). For the combination study, three concentrations of EUG (50 µM, 100 µM, and 200 µM) and BCP (50 µM, 100 µM, and 200 µM) were combined with 5-FU (6 µM to 192.19 µM) to check their effect on IC_{50} value of 5-FU. DMSO was used as vehicle control and maintained 1% in cultures of colorectal cancer cells. After the treatment for 24h, 48h, and 72h with test compounds, the culture medium was removed, followed by PBS wash (pH 7.0), and 50 µL MTT reagent (5 mg/mL in PBS) was added into each well and incubated for 4h at 37°C in humified (5%) CO_2 incubator (Biocenter, Salvis Lab) in dark condition for formazan crystal formation. Afterward, 100 µL DMSO was added to solubilize formazan crystals and further incubated for 30 min. MTT product was quantified as absorbance using a microplate reader (Bio-Tek Epoch microplate spectrophotometer, Vermont, USA) at 570nm. Percentage Cell survival was calculated using the formula [mean A570 treated cells – mean A570 blank /mean A570 control cells – mean A570 blank]. IC_{50} value was counted using the GraphPad Prism V8.2.1.

2.1.3 Estimation of Combination Index (CI)

The CI theorem of Chou-Talalay (Chou and Talalay 1984) quantitively determines synergism or antagonism at different effect levels as indicated by Fa-CI plot in CompuSyn simulation and automated graphics which were generated for 5-FU-EUG (FE) and 5-FU-BCP (FB) combinations.

2.2 Pharmacokinetic assessment

Pharmacokinetic and pharmacodynamic studies of EUG, BCP, and 5-FU, such as absorption, distribution, metabolism, and excretion (ADME) were carried out for drug-likeness properties and toxicity. Other pharmacologically acceptable properties, such as molecular weight, solubility, hydrogen bond acceptor and donor, rotatable bonds, etc., were performed using PreADMET (https://preadmet.bmdrc.kr) and SwissADME (www.swissadme.ch). SDF (Structure Data File) and SMILES (simplified molecular input line entry system) strings were employed throughout the process. The results were analyzed and compared for each test compound and drug.

2.3 Compound targets prediction

3D structure of EUG, BCP, and 5-FU were downloaded in SDF (Structure Data File) format from PubChem (https://pubchem.ncbi.nlm.nih.gov/), which were deposited to find Potential targets for all three compounds into PharmMapper (http://www.lilab-ecust.cn/pharmmapper/), ChEMBL (https://www.ebi.ac.uk/chembl/) and Search Tool for Interacting Chemicals (STITCH) version 5.0 (http://stitch.embl.de/) database. Protein-compound interactions were analyzed at 0.900 (Highest) confidence level for *Homo sapience* species only. We combined all the protein targets to make a common list of targets for each compound.

2.4 Acquisition of disease-associated target genes

DisGeNET (https://www.disgenet.org/) and Pathcards (https://pathcards.genecards.org/) were used to identify proteins related to metastatic Colorectal cancer (Disease ID: C0009402), in which genes ≥ 0.1 score gda (Gene Disease Association) were selected for further study. Together, we clubbed both lists of genes together to make a standard list of proteins involved in metastatic CRC.

2.5 Protein-Protein interaction (PPI) network formation

PPI networks provide a valuable framework of proteins for understanding functional proteomes in much better ways. That was done using String (https://string-db.org/, ver 11.5), with organisms limited to *Homo sapience* only. PPI with the highest confidence scores (> 0.9) were reserved for this study.

2.6 Compound-Disease-Target (C-D-T) network construction and analysis

Network constructed using Cytoscape 3.9.1 software. The EUG, BCP, 5-FU PPI networks were merged with the metastatic CRC network using the "Merge" tool of Cytoscape (intersection and union options were selected) to construct the C-D-T network. We

removed duplicated edges and self-loops to present the final network. Node degree was assessed for each network using the Network Analyzer version 4.4.6 plugin (Assenov et al. 2008). The Tanimoto coefficient was calculated to compare the similarity between 5-FU and test substances using the ChemMine tool (https://chemminetools. ucr.edu/). The value of the Tanimoto coefficient falls between 0 and 1, and its higher values indicate a greater resemblance than the lower ones.

2.7 Enrichment analysis for key targets

DAVID (The Database for Annotation, Visualization, and Integration Discovery) version 8.0.0 was used for GO (Gene Ontology) and KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway enrichment analysis of EUG, BCP, and 5-FU involved in metastatic CRC to explore their functional role in the human body like Biological Processes (BP), Cellular Compounds (CC), and Molecular Functions (MF) as well as different cell signaling pathways connected to them.

2.8 Extracting core targets of the network

With the aid of Microsoft Excel, we intersected the potential target database related to compounds and the target database to get the core target associated with CRC, which was used to draw a Venn diagram online (http://www.bioinformatics.com.cn/). Protein targets from the intersected network and the top 16 proteins from a merged network of all three compounds with CRC were taken for further validation using molecular docking.

2.9 Molecular docking

Molecular docking was performed using Glide version 11.8 (Halgren et al. 2004). High-resolution protein crystalline structures were obtained by the X-ray diffraction method and presented resolutions smaller than 2.8 Å (Table 8) and were downloaded from the Research Collaboratory for Structural Bioinformatics protein databank (https://www.rcsb.org/) and prepared using protein preparation wizard tool (Schrödinger, LLC). Water and hetero molecules were removed, hydrogen atoms were added to the protein structure, optimized, and minimized by inducing potential ionization at pH 7.0 ± 2.0. Restrained minimization was defaulted at 0.30 Å coverage heavy atoms to RMSD value. The receptor grid was generated at the protein's active pocket site, the native ligand called an inhibitor or positive control. Ligand structures for EUG, BCP, 5-FU, and all positive controls were downloaded from PubChem in SDF format prepared with the Ligprep tool (Schrödinger, LLC). Molecular docking was performed at the extra precision (XP) mode for all tested compounds. The final evaluation of ligand-protein binding was performed based on glide score (Kcal/mol). The Discovery Studio program was also used for visual inspection and graphical representations of the docking results.

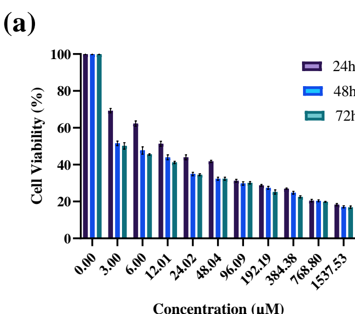
2.10 Statistical analysis

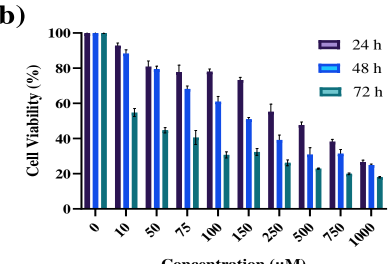
The results were expressed as Mean ± SD analyzed with two-way analysis of variance (ANOVA) for IC_{50} values using GraphPad Prism 8.2.1. Further, $P< 0.05$ was considered to be significantly different. Dunnett's multiple comparison test was performed to compare the IC_{50} value of the combination group with the 5-Fluorouracil alone group to test the reduction in inhibitory effect.

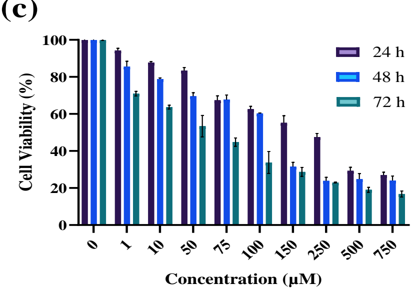
3 Results and discussion

3.1 MTT assay and CI prediction

MTT assay was used to assess the antiproliferative effect of 5-Fluorouracil (5-FU), Eugenol (EUG), and Beta-caryophyllene (BCP) against colorectal cancer (CRC) cell line HCT116 with different concentration at 24h, 48h, and 72h. The inhibitory effects are presented in figure 2 (A, B, C), which indicated dose and time-dependent decrease in the percentage cell survival of CRC cells. The inhibitory concentration (IC_{50}) values are shown in table 1. Many researchers have reported anticancer and anti-metastatic properties for all three compounds (Ahmed et al. 2022; Jubeen et al. 2022; Surducan et al. 2023). 5-FU is the most used chemotherapeutic drug in various kinds of cancer, especially colorectal cancer. Due to the narrow therapeutic window, selecting the appropriate dosage is important to avoid the problem of tumor heterogeneity and drug resistance (Kamal et al. 2020; Naren et al. 2022). We aimed to lower the IC_{50} value of 5-FU (6 to 192.19 µM) by combining it with the three concentrations of EUG and BCP (50 µM, 100 µM, and 200 µM) to reduce its side effects (Figure 2 D, E). Alone, 5-FU treatment, when compared with its co-treatment of EUG and BCP, has shown a reduction in its IC_{50} values by 1.98 for 50 µM EUG to 2.28-fold for 200 µM EUG and 1.83 for 50 µM BCP to 2.34 fold for 200 µM BCP after 24h. Similarly, 1.33 for 50 µM EUG to 1.89 fold for 200 µM EUG and 1.36 for 50 µM BCP to 1.5 fold for 200 µM BCP after 48h. Likewise, 1.58 for 50 µM EUG to 1.81 fold 200 µM EUG and 1.53 for 50 µM BCP to 1.68 fold for 200 µM BCP after 72h treatment, respectively (Table 1). A combination index of less than 1 indicated synergism for combining two drugs. Figure 2 (left and right panel) shows a Fa-CI plot for a combination index lower than 1 when fractions affected (Fa, Percent inhibition of cell survival) were found to be in range of 0.42 to 1.0 for FE and 0.41 to 0.85 for FB in HCT116. 200 µM concentrations of EUG and BCP gave best results at 48h and 72h in combinations. Ng et al. (2014) reported that *Piper betle* leaf extract which contains eugenol as major component enhances the effect of 5-FU in colorectal cancer when given in combination with 4-allylpyrocatechol and *P. betle* crude extract treatment (Ng et al. 2014). As per the previous report of Hemaiswarya and Doble (2013), the combination of eugenol and 5-FU showed synergistic effects against Human cervical cancer (HeLa) cells after 24h treatment. The recent study conducted by Bhardwaj and Dilbaghi in







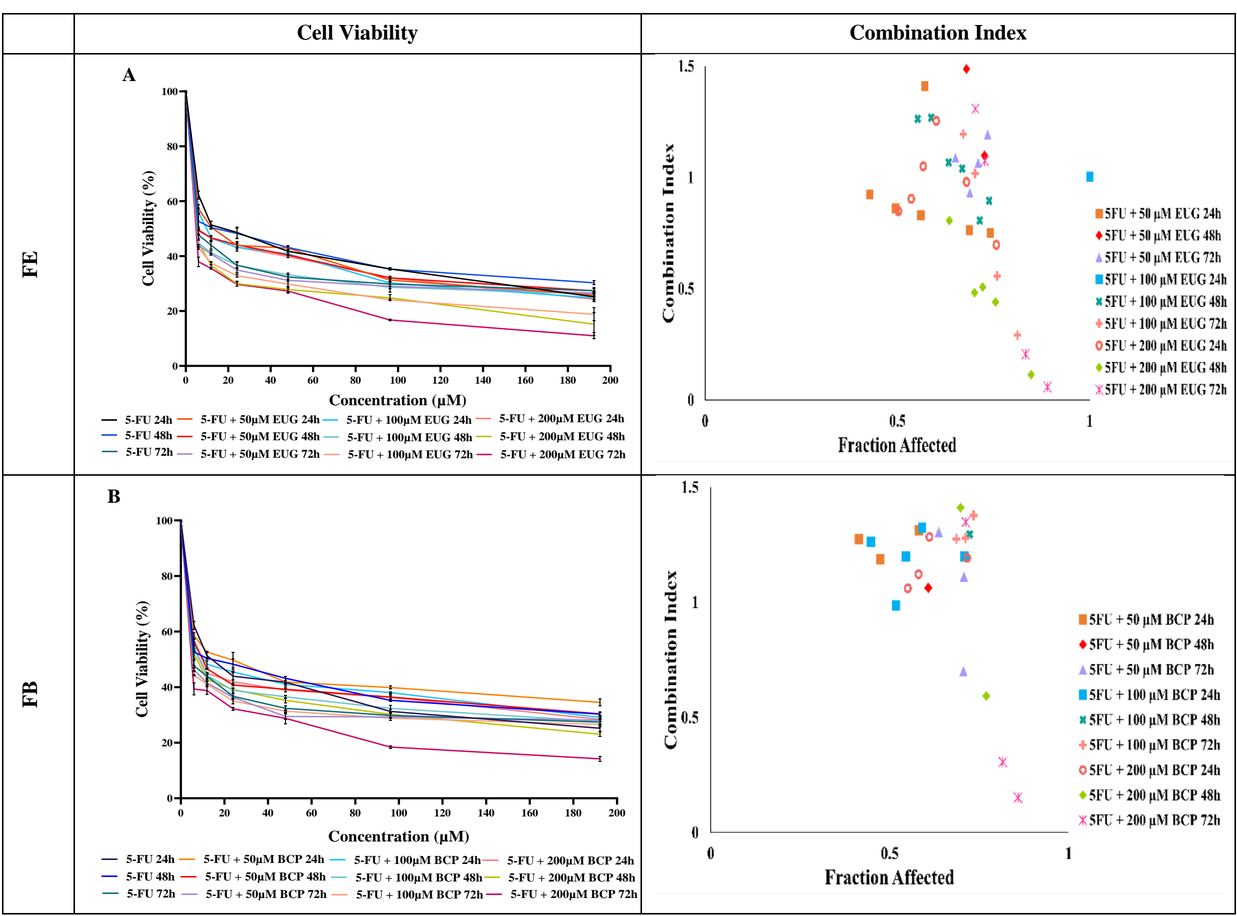


Figure 2 Effect of (A) 5-FU (B) EUG and (C) BCP; 5-Fluorouracil (5-FU) in combination with Eugenol (FE) and 5-Fluorouracil (5-FU) in combination with Beta-caryophyllene (FB) on % Survival (Left panel), Combination Index (Right panel) represented as Combination Index (CI) vs Fraction Affected (Fa) plot of HCT116 Colorectal cancer cells after treatment for 24h, 48h and 72h. Data was represented as Mean ± SD for three individual experiments.

Table 1 The inhibitory concentration 50 (IC_{50}) values of 5-Fluorouracil (5-FU), Eugenol (EUG), and Beta-Caryophyllene (BCP) alone and Combination of 5-FU and EUG (FE) as well as 5-FU and BCP (FB) for 24h, 48h and 72h in HCT116 cell line

Treatment Group	IC_{50} (µM)		
	24h	48h	72h
5-FU	32.92 ± 0.63	19.54 ± 0.77	17.74 ± 0.59
EUG	439.62 ± 2.57	152.64 ± 5.86	56.51 ± 0.75
BCP	202.98 ± 12.18	115.82 ± 3.26	53.11 ± 3.49
5FU + 50 µM EUG	16.61 ± 0.19****	14.67 ± 0.35****	11.20 ± 0.09**
5FU + 100 µM EUG	15.35 ± 0.31****	11.71 ± 0.12****	10.63 ± 0.04****
5FU + 200 µM EUG	14.40 ± 0.14****	10.29 ± 0.11****	9.77 ± 0.12****
5FU + 50 µM BCP	17.98 ± 0.79^{ns}	14.34 ± 0.21****	11.59± 0.12^{ns}
5FU + 100 µM BCP	15.51 ± 0.23****	13.37 ± 0.37****	11.31 ± 0.19*
5FU + 200 µM BCP	14.02 ± 0.19****	13.02 ± 0.43****	10.50 ± 0.39****

Results are represented as Mean ± SD; $^{****}P < 0.0001$, $^{**}P < 0.01$, $^{*}P < 0.1$, ns=nonsignificant, when 5-FU alone treated group was compared with the combination (5-FU + EUG/ BCP) groups

2022 showed the enhanced cytotoxic potential of 5-FU against the A431 skin cancer cell line by INV-FU (Beta-caryophyllene constellated 5-FU nanoinvasomes) (Bhardwaj and Dilbaghi 2022). The current experiment lowered the IC_{50} values of 5-FU by showing the synergistic effect of EUG and BCP in combination with 5-FU.

3.2 Evaluation of pharmacokinetic, ADME, and toxicity profile of EUG, BCP, and 5-FU

Evaluating compounds based on their ADME/Tox properties by computational approach has become an elemental segment in drug discovery. Orally administered drugs follow the rule of five or Lipinski's rule, according to which smaller molecules (MW < 500 g/mol) with lipophilic properties (Octanol- water partition co-efficient, AlogP98) and ≤5 Hydrogen bond donor as well as acceptor are permeable across membrane bi-layer (Lipinski et al. 1997). According to Veber et al. (2002), the number of rotatable bonds and the topological polar surface area should be within limits (Veber et al. 2002), as given in Table 2 for membrane permeability. A suitable drug-like profile has been found for all three substances according to the MDDR-like rule (Sheridan and Shpungin 2004). All three bioactive compounds fully comply with this rule and have high bioavailability.

Table 3 shows the ADME properties of EUG, BCP, and 5-FU. The drugs administered should now be able to get absorbed by intestinal cells to exert their effect by reaching target molecules. It is worth mentioning that EUG and BCP have greater HIA (Human Intestinal Absorption) (~100%), Caco, and MDCK cell permeability than 5-FU drugs. They can even cross the Blood-Brain Barrier (BBB) more effectively by binding with plasma proteins than 5-FU, justifying possible plasma transport mechanisms. Further, P-gp (p-glycoproteins) are membrane transporters that efflux the drug when taken in large amounts or quickly absorbed. P-gp inhibitors are present in BBB and other organs, making it even more difficult for the drug to efflux after absorption (Amin 2013). Cytochrome P450 enzyme family, especially CYP2D6, is important for drug metabolism due to its substrate specificity. Inhibitors of this enzyme may lead to drug elevation in the circulatory system and thus increase toxicity levels (Gibbs et al. 2006; Di 2017; Gonzalez et al. 2021). All

Table 2 Evaluation of pharmacokinetic properties of 5-FU, EUG, and BCP

Compound	Lipinski's rule[a]				Veber's rule[b]		
	MW	AlogP98	HBD	HBA	RBN	TPSA	MDDR like rule[c]
5-FU	130.08	-0.62	2	4	0	58.20	Non-druglike
EUG	164.20	2.55	1	2	3	29.46	Mid-structure
BCP	204.35	4.29	0	0	0	00.00	Mid-structure
Optimal	≤500	≤5	≤5	≤10	≤3	7-200	-

[a]MW= Molecular Weight(g/mol), AlogP98= Predicted water/octanol partition coefficient, HBD= Hydrogen Bond Donor, HBA= Hydrogen Bond Acceptor; [b]RBN= Rotatable Bond Numbers, TPSA= Topological Polar Surface Area (Å²);[c]MDDR= MDL Drug Data Report

Table 3 *In silico* ADME prediction for 5-FU, EUG, and BCP

Compound	Absorption[a]			Distribution[b]			Metabolism[c]
	HIA	QPPCaCo	QPPMDCK	PPB	Pgp binding	BBB	ADMET CYP2D6 inhibition
5-FU	76.93	16.59	0.95	8.3	No	0.28	No
EUG	96.77	46.88	342.14	100.0	No	2.25	No
BCP	100.00	23.63	45.74	100.0	Inhibitor	11.65	No
Range	70-100, well absorbed	4-70, mid permeability	4-70, mid permeability	>90, strong binding	-	-3-12, permeability to CNS	-

[a]HIA= Human Intestinal Absorption (%), QPPCaCo= in vitro Caco-2 cell permeability (nm/s), QPPMDCK= in vitro MDCK cell permeability (nm/s), [b]PPB= Plasma Protein Binding (%), Pgp binding= P-glycoprotein binding, BBB= in vivo Blood-Brain Barrier penetration (Cbrain/Cblood), [C]ADMET CYP2D6 binding= Cytochrome P450 2D6 binding

Table 4 *In silico* toxicity prediction for 5-FU, EUG, and BCP

Compound	Toxicity prediction				
	Ames_test	Carcino_mouse	hERG_inhibition	TA100	TA1535
5-FU	Mutagen	Positive	Medium risk	Positive	Positive
EUG	Mutagen	Positive	Medium risk	Positive	Positive
BCP	Mutagen	Positive	Medium risk	Positive	Positive

hERG: The human Ether-à-go-go-Related Gene, TA100 and TA1535: Salmonella typhimurium strains

three compounds under study do not show binding or inhibition of CYP2D6, hence having good absorbance and efficient metabolism.

In silico toxicity tests predicted similar toxicity results in the Ames test for the three compounds (Table 4). For TA100 and TA1535 strains of *Salmonella typhimurium* that are frequently used in the Ames test containing the same base pair replacement mutation hisG4673–75 (Prival and Zeiger 1998; das Chagas Pereira de Andrade and Mendes 2020), EUG and BCP exhibit 5-FU-like behaviour. The model predicted positive carcinogenicity results for EUG, BCP, and 5-FU in both rats and mice. All the substances here show a medium risk for hERG gene inhibition, which encodes the voltage-gated potassium channels in the heart, which is involved in the repolarization process in the heart (Garrido et al. 2020). The results indicate the insured use of EUG and BCP compared to the 5-FU analysis. The substances exhibit many toxicological similarities.

3.3 Network Pharmacology Study

Network pharmacology is a modern approach to disease mechanisms targeted by multiple synergistic compounds for drug discovery that define complex drug-disease-gene interactions (Noor et al. 2022). From 5474 genes for metastatic colorectal cancer, 1207 genes were extracted from the DisGeNet based on score-gda ≥ 0.1, which were added to the 116 genes taken from pathcards. From 1307 unique genes, 761 genes were selected for generating a disease network after eliminating duplicated genes in the Cytoscape app. Whereas 173, 91, and 139 potential genes/ proteins were selected after removing duplicated or self-loops from PharmMapper, ChEMBL, STITCH for EUG, BCP, and 5-FU, respectively, to create a network in the Cytoscape app. These identified targets were imported to Cytoscape to generate a Compound-Disease-Target (C-D-T) network. The resultant C-D-T network had 59 nodes/228 edges, 25 nodes/98 edges, and 50 nodes/190 edges against metastatic CRC for EUG, BCP, and 5-FU, respectively (Figure 3A, B, C). This indicates that EUG, BCP, and 5-FU target 58, 24, and 49 proteins of metastatic CRC, excluding the compound node from the network.

Further, by using this app's "Merge" tool, 84 potential gene nodes with 407 edges were found to be the target of all three substances for metastatic CRC. In contrast, the intersection of bioactive compounds and disease showed 11 gene nodes with 25 edges, confirmed by developing a Venn diagram (Figure 3D). The nodes with high edge count/ Node degree were taken as core targets (Table 5) because targeting one protein from the network can connectively disrupt the entire protein network, thus regulating the pathway in cancer. The first 16 common proteins were considered for further validation of the study. The first 15 proteins were present in both merged and intersected analyzed networks of CRC with all three compounds, and one protein, CHEK1, was present in the intersected network.

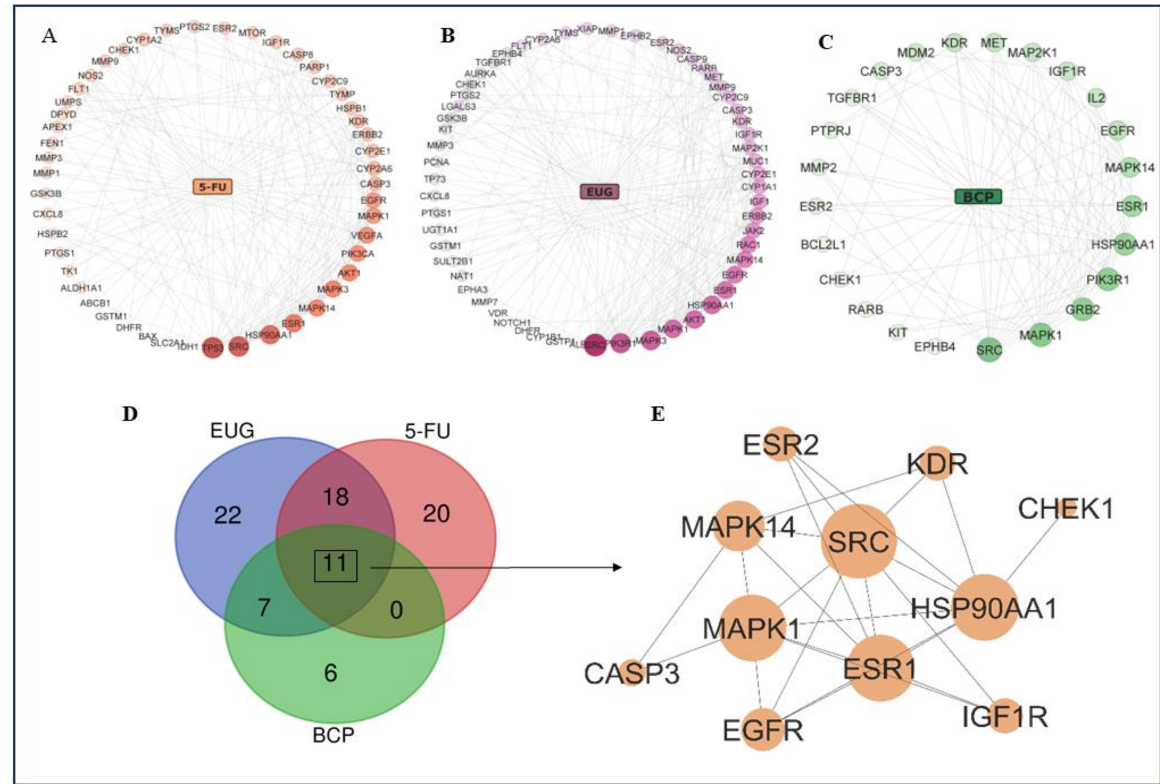


Figure 3 Compound-Disease-Target (C-D-T) network of 5-FU and Test compounds, (A) Node degree sorted C-D-T network of 5-Fluorouracil (5-FU), (B) Eugenol (EUG), (C) Beta-caryophyllene (BCP) against metastatic colorectal cancer (CRC), (D) Venn diagram of intersected hub proteins from 5-FU, EUG and BCP, (E) Protein-Protein interaction (PPI) network of 11 intersected proteins from 5-FU, EUG and BCP against metastatic colorectal cancer (CRC)

Table 5 Potential targets related to CRC with 5-FU, EUG, and BCP merged network

No.	Gene name	Protein name	UniProt ID	Edge count (Node Degree)
1	SRC	proto-oncogene tyrosine-protein kinase Src	P12931	33
2	HSP90AA1	Heat shock protein HSP 90-alpha	P07990	26
3	MAPK1	MAP kinase ERK2	P28482	23
4	ESR1	Estrogen receptor alpha	P03372	23
5	PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha	P27986	22
6	MAPK3	Mitogen-activated protein kinase 3	P27361	21
7	AKT1	RAC-alpha serine/threonine-protein kinase	P31749	21
8	TP53	Cellular tumor antigen p53	P04637	21
9	EGFR	Epidermal growth factor receptor	P00533	21
10	MAPK14	MAP kinase p38 alpha	Q16539	20
11	ERBB2	Receptor tyrosine-protein kinase erbB-2	P04626	14
12	KDR	Vascular endothelial growth factor receptor 2	P35968	14
13	CASP3	Caspase- 3	P42574	14
14	RAC1	Ras-related C3 botulinum toxin substrate 1	P63000	13
15	IGF1R	Insulin-like growth factor I receptor	P08069	13
16	CHEK1	Serine/threonine-protein kinase Chk1	O14757	9

Table 6 Gene ontology (GO) enrichment analysis of the biological process and cellular components, Molecular function for the potential metastatic colorectal cancer (CRC) targets of 5-FU, EUG, and BCP

GO term	Description	5-FU		EUG		BCP	
		GC value	*p*-value	GC value	*p*-value	GC value	*p*-value
Biological Processes (BP)							
GO:0043066	Apoptotic process	11	$4.72e^{-07}$	15	$2.30351e^{-10}$	10	$3.99736e^{-09}$
GO:0030335	Cell migration	7	$4.12e^{-05}$	14	$4.63987e^{-13}$	8	$1.43202e^{-08}$
GO:0008284	Cell proliferation	7	0.002072537	10	$2.596e^{-05}$	8	$2.09099e^{-06}$
GO:0043410	MAPK cascade	5	0.000669133	9	$2.20835e^{-08}$	5	$3.57774e^{-05}$
GO:0035556	Intracellular signal transduction	10	$1.34e^{-06}$	13	$5.70211e^{-09}$	6	0.000155802
GO:0070371	ERK1 and ERK2 cascade	3	0.004080212	5	$4.8205e^{-06}$	3	0.000944176
GO:0042060	Wound healing	4	0.002013155	5	0.000223302	3	0.006354559
Cellular Components (CC)							
GO:0005886	Plasma membrane	12	0.031705895	27	0.000461406	17	$7.31094e^{-06}$
GO:0005634	Nucleus	25	0.001722577	34	$4.96908e^{-06}$	17	$6.60645e^{-05}$
GO:0005737	Cytoplasm	34	$1.33e^{-09}$	32	$8.33057e^{-06}$	14	0.002336449
GO:0005739	Mitochondrion	17	$7.95e^{-08}$	14	0.000121741	8	0.00072934
GO:0005576	Extracellular region	10	0.059458	18	$4.892e^{-05}$	8	0.007481112
GO:0005925	Focal adhesion	6	0.003205	8	0.000179501	4	0.01181298
Molecular Function (MF)							
GO:0005515	Protein binding	41	0.008201	48	0.006371445	23	0.00112074
GO:0004712	Protein serine/threonine/tyrosine kinase activity	11	$1.08e^{-07}$	17	$1.07438e^{-13}$	9	$2.58365e^{-08}$
GO:0003677	DNA binding	-	-	8	0.091920433	5	0.067743477
GO:0004708	MAP kinase kinase activity	3	0.000955	4	$2.09929e^{-05}$	3	0.000217262

GC = Gene Count; *p*-value = Modified Fisher exact *p*-value (EASE score), the smallest, the more enriched.

Cancer is a disease that affects multiple genes, so rather than using single-target drugs, synergistic multiple medicines can act on the same or different multiple proteins to combat the disease (Li et al. 2020). For that, the tanimoto coefficient (value ranges from 0 to 1) was calculated for each bioactive compound to evaluate structural similarity with 5-FU drug, which was found to be 0.06 and 0.007 for EUG and BCP respectively, suggesting that they have the least similarity with 5-FU and can bind with same or different proteins.

3.4 Gene Ontology and Kyoto Encyclopaedia of Genes and Genomes Enrichment Analysis

The results of GO analysis (at $p< 0.05$ statistics) (Table 6) showed that all the substances under study targets affected many biological processes such as negative regulation of apoptosis, intracellular signal induction, MAPK cascade positive regulation, etc. The GO and CC annotations analysis identified various cellular components such as plasma membrane, nucleus, cytoplasm, mitochondria, etc., targeted by 5-FU, EUG, and BCP. It regulates cellular activities through protein binding, DNA binding, and protein serine/threonine/tyrosine kinase activity. KEGG pathway enrichment analysis showed that EUG, BCP, and 5-FU target many cancer cell signaling pathways such as PI3K-Akt, MAPK, VEGF, apoptosis, etc. Among all three compounds, EUG has targeted more pathways genes than the other two, suggesting its potential use in therapeutics (Table 7). An *in vitro* study of eugenol on MDA-MB-231 and SK-BR-3 breast cancer cells revealed its apoptosis-inducing effect via the PI3K-Akt pathway (Abdullah et al. 2021). An experiment on RAW264.7 showed its impact on the down-regulation of NF-kB and MAPK pathways (Deepak et al. 2015).

Table 7 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for the potential metastatic colorectal cancer (CRC) targets of 5-FU, EUG, and BCP

GO term	Description	5-FU		EUG		BCP	
		GC value	*p*-value	GC value	*p*-value	GC value	*p*-value
hsa05200	Pathways in cancer	24	$2.21e^{-15}$	30	$1.34646e^{-20}$	18	$3.73925e^{-16}$
hsa05210	Colorectal cancer	10	$1.28e^{-09}$	11	$2.0099e^{-10}$	7	$1.01772e^{-07}$
hsa04151	PI3K-Akt signaling pathway	14	$7.72e^{-08}$	18	$7.29806e^{-11}$	13	$3.33315e^{-11}$
hsa04010	MAPK signaling pathway	13	$8.54e^{-08}$	16	$5.24339e^{-10}$	11	$2.44254e^{-09}$
hsa04014	Ras signaling pathway	9	$5.43e^{-05}$	13	$3.2006e^{-08}$	10	$6.12424e^{-09}$
hsa04370	VEGF signaling pathway	10	$3.93e^{-11}$	11	$4.08898e^{-12}$	6	$5.11946e^{-07}$
hsa04150	mTOR signaling pathway	7	0.000284	8	$7.77016e^{-05}$	5	0.000864245
hsa01521	EGFR tyrosine kinase inhibitor resistance	13	$1.54e^{-14}$	14	$2.58313e^{-15}$	10	$3.49599e^{-13}$
hsa04210	Apoptosis	9	$1.06e^{-06}$	8	$3.23258e^{-05}$	5	0.000515328

GC= Gene Count; *p*-value = Modified Fisher exact *p*-value (EASE score), the smallest, the more enriched

Table 8 Docking energy score for 5-FU, EUG, and BCP for core protein targets of Compound-Disease-Target (C-D-T) network of metastatic colorectal cancer (CRC) using Glide (XP) program

Target protein			Energy Score (Kcal/mol)			
Name	PDB ID	Resolution (Å)	5-FU	EUG	BCP	Positive Control
SRC	2BDF	2.10	-5.439	-6.341	-1.898	-9.446
HSP90AA1	4BQG	1.90	-5.097	-7.515	-7.539	-7.853
MAPK1	1WZY	2.5	-3.704	-5.379	-3.594	-10.972
ESR1	5FQV	1.74	-5.028	-5.202	-7.751	-6.78
PIK3R1	3HHM	2.8	-6.015	-4.884	-5.137	-7.598
MAPK3	2ZOQ	2.3	-6.761	-6.018	-2.472	-9.638
AKT1	6CCY	2.18	-6.979	-5.504	-2.617	-8.247
TP53	5O1F	1.38	-3.869	-5.006	-	-6.975
EGFR	4WKQ	1.85	-4.821	-5.752	-3.124	-7.809
MAPK14	6SFO	1.75	-6.283	-7.628	-4.518	-13.469
ERBB2	3PPO	2.25	-5.329	-6.304	-2.194	-14.101
KDR	3WZD	1.57	-4.15	-4.871	-3.829	-10.672
CASP3	1NME	1.60	-4.492	-3.946	-1.884	-5.627
RAC1	3TH5	2.30	-5.324	-6.018	-2.472	-9.638
IGF1R	2OJ9	2.00	-5.112	-6.807	-4.953	-7.442
CHEK1	4QYE	2.05	-5.682	-5.681	-1.473	-9.868

3.5 Multitarget Molecular Docking Investigation

This method has been used to assess the interaction between various therapeutic targets of colorectal cancer with our chosen bioactive compounds, eugenol (EUG) and beta-caryophyllene (BCP), as well as standard drug 5-Fluorouracil (5-FU). Sixteen hub proteins were selected for simulation and were present in all three C-D-T networks of the compounds. The result's binding energy score (Kcal/mol) was negative for all the proteins and ligands, as shown in Table 8. The difference between the energy score of the bioactive component and the respective protein inhibitor was considered the best binding. The lowest difference between positive

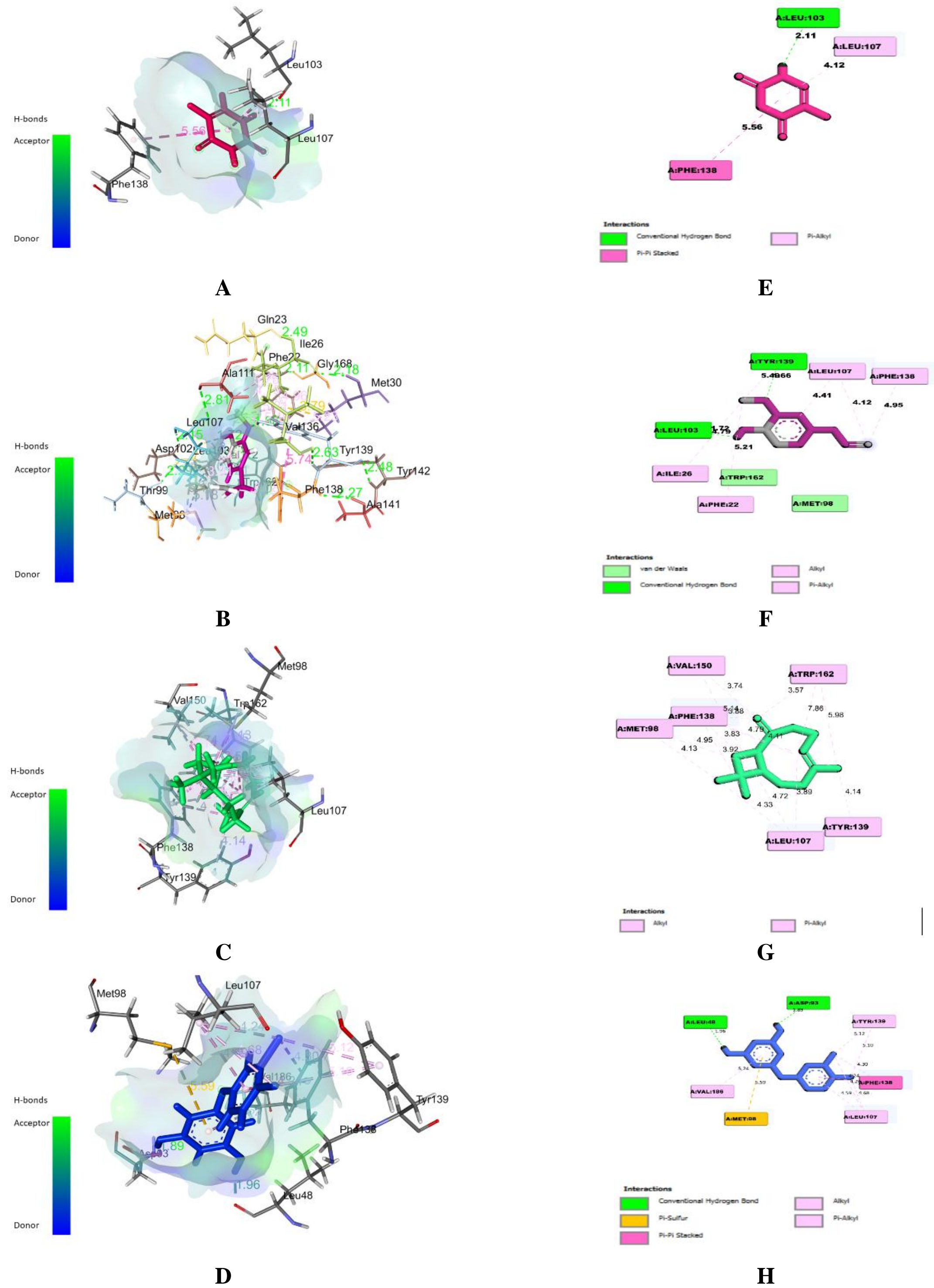


Figure 4 Protein-Ligand interaction poses of colorectal cancer target HSP90AA1 with compounds (A) A 3D docked interaction based on hydrogen bond acceptor (HBA) and donor (HBD) of 5- fluorouracil (5-FU), (B) eugenol (EUG), (C) beta-caryophyllene (BCP), (D) positive control (PC): 5-(3,4-dichloro-phenoxy)-benzene-1,3-diol inhibitor, (E) A 2D docked interaction of eugenol (EUG), (F) beta-caryophyllene (BCP), (G) 5- fluorouracil (5-FU), (H) positive control (PC): 5-(3,4-dichloro-phenoxy)-benzene-1,3-diol inhibitor at active domain of Heat shock protein 90- alpha (HSP90AA1) protein with interacting amino acids and H-bonds

control and test compounds was in four proteins, which include Heat Shock Protein- 90 alpha (HSP90AA1), Insulin-like Growth Factor 1 Receptor (IGF-1R), estrogen receptor (ESR1), and Caspase- 3 (CASP3). Figure 4 shows the docked poses of interactions in H-bond donor and acceptor form in 3D, bond length, and interacted amino acid residues in 2D of HSP90AA1 protein with test compounds. Over the last two decades, HSP90 has emerged as an intriguing target in the war on cancer. HSP90 interacts and supports numerous proteins that promote oncogenesis, thus distinguishing Hsp90 as a cancer enabler as it is regarded as essential for malignant transformation and progression (Zuehlke et al. 2015). The binding energy of 5-FU, EUG, BCP and 5-(3,4-dichloro-phenoxy)-benzene-1,3-diol inhibitor (positive control) for HSP90AA1 was -5.097,-7.515,-7.539 and -7.853 Kcal/mol respectively, which shows that eugenol and beta-caryophyllene can bind strongly with HSP90AA1 protein than the standard drug 5-Fluorouracil as shown in figure 4. A study done by Absalan and his co-workers proved that eugenol can positively interact with this small chaperone molecule of HSP90AA1 that may affect stem cell aging via telomere or telomerase-dependent pathway (Absalan et al. 2017). In addition to their findings, we found out that eugenol can bind more effectively with the hub proteins such as HSP90AA1, ESR1, CASP3, and IGF-1R than any other selected compounds. HSP90 targets multiple signaling pathways by disrupting metastatic proteins, thus inhibiting epithelial to mesenchymal transition in cancer cells (Zhang et al. 2021; Anwar et al. 2022). Type-I insulin-like growth factor receptor is a tyrosine kinase receptor that is over-expressed in many cancer processes, such as cell growth, cell proliferation, cell differentiation, apoptosis, and angiogenesis. Moreover, it is involved in PI3K-AKT and MAPK signaling pathways (Codony-Servat et al. 2017). The other key target was estrogen receptor type 1 (ESR1) is a cell cycle regulator protein that further interacts with PI3K, AKT, ERK, MAPK, and NF-KB proteins for anti-apoptosis mechanism (Williams et al. 2016; Ditonno et al. 2021). Increased Caspase 3 (CASP3) causes apoptosis, and its activation is a positive indicator of cancer treatment (Zou and Xu 2018). Eugenol exerts its apoptosis-inducing effects by cleaving caspase 3, with a significant increase in its active form showing apoptosis-inducing properties (Fathy et al. 2019), which also limits the cells' ability to metastasize and proliferate in the rise in ROS and cytochrome C's release (Abdullah et al. 2018; Anwar et al. 2022). Beta-caryophyllene also induces apoptosis in mouse blood cancer cell lines through caspase 3 induction (Amiel et al. 2012) and by downregulating PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation (Park et al. 2011; Dahham et al. 2015). All these four hub proteins (HSP90AA1, IGF-1R, ESR1, and CASP3) affect other proteins in downstream mechanisms in a way that inhibits cancer cell proliferation, induces apoptosis, and thus provides anticancer and anti-metastatic properties. Targeting any of these proteins for therapeutic use in colorectal cancer treatment by our natural compounds alone or combined with chemotherapeutic drugs can be helpful in colorectal cancer treatment.

Conclusion

In a multitarget strategy, if we combine the treatment of a drug with a phytocomponent, it can target multiple proteins to exert their effects as anticancer agents. The synergism proved effective against colorectal cancer *in-silico* and *in-vitro* when 5-FU was combined with EUG and BCP. These natural compounds effectively reduced IC_{50} value in combination with conventional chemotherapeutic drugs. They can target HSP90AA1 as a key protein to further provide anti-metastatic effect in colorectal cancer treatment through which it can promote autophagy and inhibit apoptosis through PI3K/Akt/mTOR pathway and JNK/P38 pathway, which are key pathways in epithelial to mesenchymal transition inhibition.

Abbreviations

CRC: Colorectal Cancer; EUG: Eugenol; BCP: Beta-Caryophyllene; 5-FU: 5-fluorouracil; TNBC: Triple Negative Breast Cancer; HER2 Human epidermal growth receptor 2; MMP: Matrix Metalloproteinase; TGFβ: Tumour Growth Factor beta; PI3K-Akt: The Phosphatidylinositol 3-kinase/ Protein kinase B; MAPK: Mitogen-activated protein kinase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Fa-CI: Fraction Affected-Combination Index; ADME: absorption, distribution, metabolism, and excretion; BBB: Blood-Brain Barrier; PPB: Plasma Protein Binding; HIA: Human Intestinal Absorption; P-gp: p-glycoproteins; SDF: Structure Data File; SMILES: Simplified molecular input line entry system; STITCH: Search Tool for Interacting Chemicals; PPI: Protein- Protein Interaction; C-D-T: Compound-Disease-Target; DAVID: The Database for Annotation, Visualization, and Integration Discovery; GO: Gene Ontology; KEGG: Kyoto Encyclopaedia of Genes and Genomes; FDR: False Discovery Rate; BP: Biological Processes; CC: Cellular Compounds; MF: Molecular Functions; MDDR: MDL Drug Data Report; RMSD: Root mean square deviation

Conflict of Interest

The authors have no conflicts of interest to declare relevant to this article's content.

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Author contributions (CREDIT)

D.J. (Devendrasinh Jhala) – Conceptualization, methodology, experimental studies, formal analysis, investigation, resources, funding acquisition, manuscript review and editing; K.T. (Krupali Trivedi) – Conceptualization, software, methodology, experimental studies, data curation, data analysis, writing- original draft preparation, manuscript review and editing, funding acquisition; P.R., N.P., S.P., B.G. – Conceptualization, software, validation, data analysis, manuscript review; S.C., A.P. – Manuscript review, resources, investigation, supervision. All authors have read and agreed to the publication of the manuscript.

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Influence of particle size fraction and bioactive compound contents on the biological activities of *Solanum torvum* L. leaf powder extracts

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ABSTRACT

This study investigates the bioactive compound content and biological activities of raw powder extracts and particle size fractions from *Solanum torvum* leaves. The leaves, harvested from Douala, were processed into powders and subsequently divided into different fractions. Methanolic extracts of these powders were analyzed for their content of bioactive compounds (total polyphenols, total flavonoids, and condensed tannins), antioxidant (DPPH, ABTS, FRAP assays), and antimicrobial (bacterial and fungal) activities. The particle size fraction of 250-400 µm constitutes the largest proportion (33.41%) of the raw powder. The highest contents of bioactive compounds are found in the 200-250, 400-500, and < 125 µm fractions for total polyphenols (44.62 ± 0.19 mg GEA/100 ml extract), total flavonoids (14.47 ± 0.37 mg QE/100 ml extract), and condensed tannins (22.79 ± 0.12 mg CE/100 ml extract), respectively. The antioxidant activity of extracts from 500-800µm fraction improved their DPPH and ABTS assays by

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31.50% and 40.44% compared to the raw powder. The same fraction (500-800 µm) and the raw powder extracts demonstrated bactericidal activities, while the 125-200, 200-250, and ≥ 800 µm fraction extracts exhibited moderate and significant antifungal activities against several bacterial and fungal strains, respectively. These biological activities are primarily attributed to the average flavonoid contents, which become more accessible in extracts after fractionation. This method of processing powder suggests that no plant powder should be disregarded or rejected due to its low content of bioactive compounds.

1 Introduction

The practice of incorporating powder from various plants into dishes to enhance their nutritional value, particularly micronutrients such as iron, carotenoids, and zinc, has become commonplace for many consumers, including manufacturers, patients, and researchers (Siekmans et al. 2017; Boateng et al. 2019). Many are aware that these food matrices contain not only micronutrients but also a wide variety of bioactive compounds, such as polyphenols, flavonoids, and tannins, which are essential in managing numerous diseases (Jucá et al. 2020; Borokini et al. 2022; Osei-Owusu et al. 2023). Various biological activities, including antioxidant, antimicrobial, anticancer, antidiabetic, antiobesity, and antitumoral activities, have been associated with these compounds and have been reported by various researchers (Loganayaki et al. 2010; Fombang and Saa 2016; Djoueudam et al. 2019; Senizza et al. 2021)**.** These results have led to an ever-increasing search for plant matrices rich in bioactive compounds, to the detriment of those low in them. This situation could potentially lead to a loss of biodiversity.

Several authors have identified a range of bioactive compound contents that significantly affect the variations in desired biological activities. For instance, Loganayaki et al. (2010) tested thirteen extracts. They found that the highest radical scavenging activity (DPPH) and ferric-reducing antioxidant power were reported in the fruit and leaves of *S. nigrum,* while *S. torvum* matrices corresponded to low total polyphenol contents. Similarly, Djoueudam et al. (2019) also found that the total polyphenols and flavonoids responsible for bacteriostatic and bactericidal activities vary in content. Therefore, high polyphenol contents in a plant matrix do not necessarily correlate with the most significant biological activities. Based on these observations and the health benefits consumers seek, plant matrices with low polyphenol contents should not be neglected. This is especially relevant since the literature already shows a clear distinction between plant matrices with low bioactive compound contents (fruit, stem, and leaves of *S. torvum*, *Manihot esculenta*, *Hibiscus sabdariffa* calyces, etc.) and those that are particularly rich in them (*Moringa oleifera* leaves, spirulina, etc.) (Nouman et al. 2016; Ravani et al. 2018; Tao et al. 2019).

In response to the escalating demand for plant matrices rich in bioactive compounds, many researchers have opted to increase the specific surface area of plant matrices by processing them into powders (Deli et al. 2019, 2020). *S. torvum* leaf powders have been the focus of various studies (Loganayaki et al. 2010; Ramamurthy et al. 2012; Acharyya and Khatun 2018; Djoueudam et al. 2019; Senizza et al. 2021; Osei-Owusu et al. 2023; Murugesan et al. 2024). This small shrub leafy vegetable belongs to the Solanaceae family and is widely distributed in India, Thailand, China, tropical America, and sub-Saharan Africa (Ramamurthy et al. 2012). In Cameroon, it is commonly grown near homes as wild eggplant. Recent phytochemical analyses of *S. torvum* leaf powder and their particle size fractions have revealed low contents of total polyphenols, flavonoids, and condensed tannins (Unpublished work). However, the previous researchers have not addressed the impact of these low contents on biological activities such as antioxidant, antibacterial, and antifungal activities. Each particle size fraction is characterized by the specific surface area of its particles, a major physical characteristic that influences compound extraction. Nabil et al. (2020) and Wu et al. (2022) reported that the bioactive content of plant powders increases proportionally with their specific surface area. However, they also demonstrated that an increase in the specific surface area of powders does not necessarily guarantee an increase in the content of bioactive compounds. Given that the recognized biological activities of bioactive compounds are strongly correlated not only with their presence but also with their content in a food matrix (Prithvira 2019; Gong et al. 2020), it is evident that the biological activities of a powder could be enhanced if its particle size fractions made the bioactive compounds more accessible, even if they were present in low content in the plant powder. Therefore, this study aimed to evaluate the contents of bioactive compounds in extracts of *S. torvum* leaf powder, its particle size fractions, and their effects on antioxidant, antibacterial, and antifungal activities.

2 Material and methods

2.1 Sampling and preparation of *S. torvum* leaves powders

S. torvum leaves were harvested from the field of Douala, Cameroon. These leaves were washed with clean water and dried at 25°C using an electric dryer for 5 hours. The dried leaves were ground using a Moulinex grinder (4500W, 220-240 V, France) and sieved through a 1000 µm mesh sieve to produce a powder (Assiéné et al. 2021). This powder was stored in airtight containers at 4°C until further analysis.

2.2 Determination of particle size fractions of powder

The particle size of the resulting powder was determined through sieving. A column of AFNOR sieves, decreasing in size (1000, 800, 710, 500, 400, 250, 125, 100, 50, 40 µm) down to the collector, was utilized for this purpose (Melcion 2000). A mass (M_1) of powder was placed on top of a stack of sieves and subjected to a 50 Hz vibration for 15 minutes using a Merck-type vibrator. Particles smaller than the sieve mesh passed through (Mi), while those larger were retained (Mj). Each mass was weighed to determine the particle size distribution (Rp). Depending on the sieve size, this distribution was expressed for each sample. The particle size fractions were inferred based on the mass of the sieved particles according to the formula 1.

$$R_p = \left(\frac{M_i}{M_1}\right) x\, 100 \quad (1)$$

2.3 Preparation of extracts

Extracts were prepared following the Senizza et al. (2021) method. The maceration technique was employed to obtain these extracts. Powders derived from *S. torvum* leaves were extracted with methanol at a ratio of 1:2 (mass: volume) for 48 hours at room temperature. Methanol was chosen as the extraction solvent due to its superior extraction efficiency for phytochemicals and antioxidants from *S. torvum* (Ramamurthy et al., 2012). After extraction, the samples were filtered using Whatman No 1 filter paper and dried at 40 °C using a rotary evaporator for 30 minutes. All extracts were maintained in an oven at 40 °C until a constant weight was achieved. The dried extracts were stored in amber glass bottles (to mitigate light effects) at room temperature for a maximum of 6 months. The extraction process was conducted on each fraction of particle size.

2.4 Determination of the bioactive compound contents of the extracts

2.4.1 Total polyphenol

The total polyphenol content was determined using a modified version of the method described by Makkar et al. (2007). The 100 µl of the extract was added to 200 µl of ten-fold diluted Folin Ciocalteu reagent. This mixture added 200 µl of sodium carbonate (7.5% w/v), then vortexed and incubated in the dark at room temperature for ten minutes. Subsequently, 1000 µl of distilled water was added, and the solution was vortexed again. The absorbance was measured at 760 nm using a spectrophotometer (BK-UV1600 PC visible spectrometer, China). The total polyphenol content was calculated based on a calibration curve established using gallic acid as a standard and expressed in milligrams of Gallic Acid Equivalent per 100 millilitres of extract (mg GAE/100ml Extract).

2.4.2 Total flavonoid

The total flavonoid content was determined by following a slightly modified version of the method described by Chang et al. (2002). An extract (500 µl) was combined with 500 µl of a freshly prepared aluminium chloride solution (2% w/v). This mixture was incubated in the dark at room temperature for fifteen minutes. The absorbance was then measured at 430 nm using a spectrophotometer (BK-UV1600 PC visible spectrometer, China). The total flavonoid content was calculated using a calibration curve and expressed in milligrams of quercetin equivalent per 100 milliliters of extract (mg QE/100 mL Extract).

2.4.3 Condensed tannin

The content of condensed tannins was assessed using a modified version of the method described by Ogboru et al. (2015). An extract volume of 50 µl was mixed with 3 mL of 4% (w/v) vanillin in 80% methanol and 750 µl of concentrated hydrochloric acid. Then, the mixture was vortexed and incubated at 30˚C for 20 minutes. The absorbance was measured at 550 nm using UV/visible spectrophotometry (BK-UV1600 PC visible spectrometer, China), with a hydromethanolic solvent (70/30) serving as the blank. The tannin content was calculated by comparing it with the standard curve prepared using a standard catechin solution (0.2 g/l). The results were expressed as milligrams of catechin equivalent per 100 milliliters of extract (mg CE/100 mL Extract).

2.5 Determination of antioxidant activities

2.5.1 DPPH radical-scavenging activity assay

The radical-scavenging activity was determined following the method outlined by Ramamurthy et al. (2012). For this, extracts from *S. torvum* leaves (0.3mL) were combined with 2.7 mL of 0.5 mM methanolic 1,1-diphenyl-2-picrylhydrazyl (DPPH). The mixture was incubated at 37 °C for 30 minutes, after which the absorbance was measured spectrophotometrically at 517 nm. The radical-scavenging activity (%RSA) was assessed as a percentage of DPPH discoloration using formula 2.

$$\%\,RSA = ([A_{DPPH} - A_S]/A_{DPPH})\, x\, 100 \quad (2)$$

RSA_{50} (or IC_{50}) represents the sample concentration required to reduce the total free DPPH radical by 50%. A_S denotes the absorbance of the DPPH solution with the sample extract, while A_{DPPH} refers to the absorbance of the DPPH solution without the extract. Ascorbic acid was utilized as the standard.

2.5.2 ABTS radical scavenging activity assay

The procedure was conducted following the method given by Galla et al. (2017), with some modifications. The $ABTS^+$ radical was

generated by reacting 7 mM $ABTS^+$ with 2.45 mM potassium per sulphate ($K_2S_2O_8$). After incubation at room temperature in the dark for 16 hours, the solution was diluted to achieve an absorbance of 0.70 ± 0.02 at 734 nm. The $ABTS^+$ solution (1 mL) was then added to the test sample (10 µl), thoroughly mixed, and incubated for 30 minutes. The absorbance of the mixture was measured at 734 nm. Ascorbic acid (Vitamin C) was used as the standard. The radical scavenging activity (%RSA) was calculated using the formula 3.

$$\% \text{ RSA} = ([A_{ABTS} - A_S]/A_{ABTS}) \times 100 \quad (3)$$

RSA_{50} (or IC_{50}) represents the sample concentration required to reduce the total free ABTS radical by 50%. A_S denotes the absorbance of the ABTS solution with the sample extract and A_{ABTS} refers to the absorbance of the ABTS solution without the extract.

2.5.3 Ferric reducing antioxidant power (FRAP) assay

The reducing power of iron (Fe^{3+}) extracts was assessed following the method described by Padmaja et al. (2011), with minor modifications. Approximately 25 µl of each diluted extract (2 mg/mL in methanol) was introduced into a new microplate, followed by 25 µl of 1.2 mg/mL Fe^{3+} solution. The plates were pre-incubated for 15 minutes at room temperature. Subsequently, 50 µl of 0.2% ortho-phenanthroline was added to achieve final concentrations of 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.90625, and 1.95325 µg/mL. The mixtures were further incubated for 15 minutes at room temperature, after which the absorbance was measured at 505 nm using a UV/Visible light spectrophotometer (Infinite M200 TECAN, Swiss) against a blank (comprising 25 µl methanol + 25 µl Fe^{3+} + 50 µl ortho-phenanthroline). Ascorbic acid (Vitamin C) was used as the positive control. From the obtained Optical Density, reducing percentages were calculated for each concentration and used to determine the RC_{50} from dose-response curves.

2.6 Determination of antimicrobial activities

2.6.1 Antibacterial activity

As Newton et al. (2002) described, the broth microdilution method was employed for susceptibility testing of bacterial species using 96-well microtiter sterile plates. Crude extracts were dissolved in a 5% dimethylsulfoxide (DMSO) solution and diluted with Mueller Hinton broth to achieve stock concentrations of 2000 µg/mL for the extracts, 1000 µg/mL for fractions, and 500 µg/mL for the isolated compounds. Serial two-fold dilutions of the test substances were performed with Mueller Hinton broth to yield a volume of 100 µl/well, resulting in concentration ranges of 1000-0.96 µg/mL, 500-0.96 µg/mL, and 250 to 0.96 µg/mL, respectively. One hundred microliters of each bacterial suspension obtained from the Centre Pasteur du Cameroun (CPC) (containing approximately 1.5×10^6 CFU/mL) was added to the respective wells containing the test samples and thoroughly mixed to yield final concentrations ranging from 500-0.48 µg/mL for the extract, 250-0.48µg/mL for the fraction, and 125 to 0.48µg/mL for the isolated compounds. The solvent control, 5% DMSO, did not exhibit inhibitory effects on bacterial growth. Ciprofloxacin at a concentration of 125 to 0.48µg/mL was used as the standard reference. The assay microtiter plates were incubated at 37 °C for 24 hours. Inhibitory concentrations of the extracts were detected following the addition of 50 µl of 0.2 mg/mL p-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, South Africa) and further incubation at 37 °C for 30 minutes. Viable bacteria convert the yellow dye of p-iodonitrotetrazolium chloride to a pink color. This assay is based on the ability of living cells to transform the INT dye tetrazolium ring into a pink-colored formazan structure due to the action of mitochondrial and other dehydrogenases within the cell. The lowest concentration at which no visible color changes were observed was considered the Minimum Inhibitory Concentration (MIC). Bactericidal concentrations were determined by adding 50 µl aliquots of the preparations (without INT), which showed no visible color change after subculture from MIC assays, into 150 µl of extract-free Mueller Hinton broth. These preparations were further incubated at 37 °C for 48 hours, and bacterial growth was revealed by adding INT as described above. The smallest concentration at which no color changes were observed was considered the Minimum Bactericidal Concentration (MBC). The tests were performed in duplicates. The ratio MBC/MIC was calculated to determine the bactericidal ($MBC/MIC \leq 4$) and bacteriostatic ($MBC/MIC > 4$) effects.

2.6.2 Antifungal activity

Yeast inocula were prepared from 48-hour-old cultures by selecting 2 to 3 colonies and suspending them in a sterile saline (NaCl) solution (0.9%). The absorbance was read at 530nm and adjusted with the saline solution to match a 0.5 McFarland standard solution, corresponding to approximately 106 yeast cells/mL. Conidia suspensions of dermatophyte species were prepared from 10-day-old cultures. The number of conidia was determined using a spectrophotometer and adjusted with a sterile saline (NaCl) solution (0.9%) to an absorbance of 0.600 at 450 nm, corresponding to a final concentration of about 1×10^4 spores/mL (Venugopal and Venugopal 1992).

The Minimum Inhibitory Concentration (MIC) of each extract was determined using broth microdilution techniques according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical and Laboratory Standards, NCCLS) for yeasts (M27-A2). Stock solutions of the test extracts were prepared in a 5% aqueous dimethylsulfoxide (DMSO) solution and diluted with Sabouraud Dextrose Broth

(SDB) to achieve a 1 mg/mL concentration. This was serially diluted two-fold to obtain a concentration range of 500 to 0.24 µg/mL for extracts and 125 to 0.24 µg/mL for compounds. The final concentration of DMSO in the well was less than 1% (preliminary analysis with 1% DMSO did not inhibit the growth of the test organisms).

The negative control well consisted of 195 µl of SDB and 5 µl of the inoculum (containing about 10^6 cells/mL for yeast and 1 x 10^4 spores/mL for filamentous fungi). The plates were covered with a sterile lid and incubated on a shaker at 37 °C for 48 hours (for yeasts) and at 28 °C for 7 days (for dermatophytes). MICs were assessed visually after the corresponding incubation period and were defined as the lowest product concentration at which no growth or virtually no growth was observed. The assay was repeated three times.

For the Minimal Fungicidal Concentration (MFC) determination, 5 µl aliquots from each well that showed no growth of microorganisms were replicated into 195 µl of SDB and incubated at 37 °C for 48 hours (yeasts) and at 28 °C for 7 days (dermatophytes). The lowest concentration that yielded no growth after subculturing was taken as the MFC. Nystatin (for yeasts) and griseofulvin (for dermatophytes) were positive controls.

2.7 Statistical analysis

Analyses were conducted in triplicates. Microsoft Excel 2016 software was utilized for the calculation of means and standard deviations, as well as for plotting the curves. The software Statgraphic Centurion 15.2 (StatPoint Technologies, Inc, Warrenton, Virginia, USA) was employed to analyze variance and for separating means using the Duncan multiple range test at $P < 0.05$. XLStat 2016 was used for principal components analysis, which established relationships between particle size fractions of *S. torvum* leaf powders, the mass of particles, bioactive compounds, and antioxidant activities.

3 Results and discussion

3.1 Particle size fractions and their mass distribution in *Solanumtorvum* leaf powder

The particle size distribution of *S. torvum* leaf powders (Figure 1) allowed for identifying seven distinct particle size fractions: < 125, 125-200, 200-250, 250-400, 400-500, 500-800, and ≥ 800 µm. Significant differences were observed between the masses of particles across these seven fractions, and among the tested fractions, the 250-400 µm fraction had the highest mass (33.41%). These differences can be attributed to the particle size that characterizes each fraction comprising the powder. The mass of each fraction can either negatively or positively influence the content of bioactive compounds. The accessibility of bioactive compounds significantly depends on the specific surface area of each particle during extraction (Rodriguez et al. 2019). Similar results regarding the particle size distribution of various plant powders have been reported by many authors (Bahar and Khalili 2015; Wu et al. 2022).

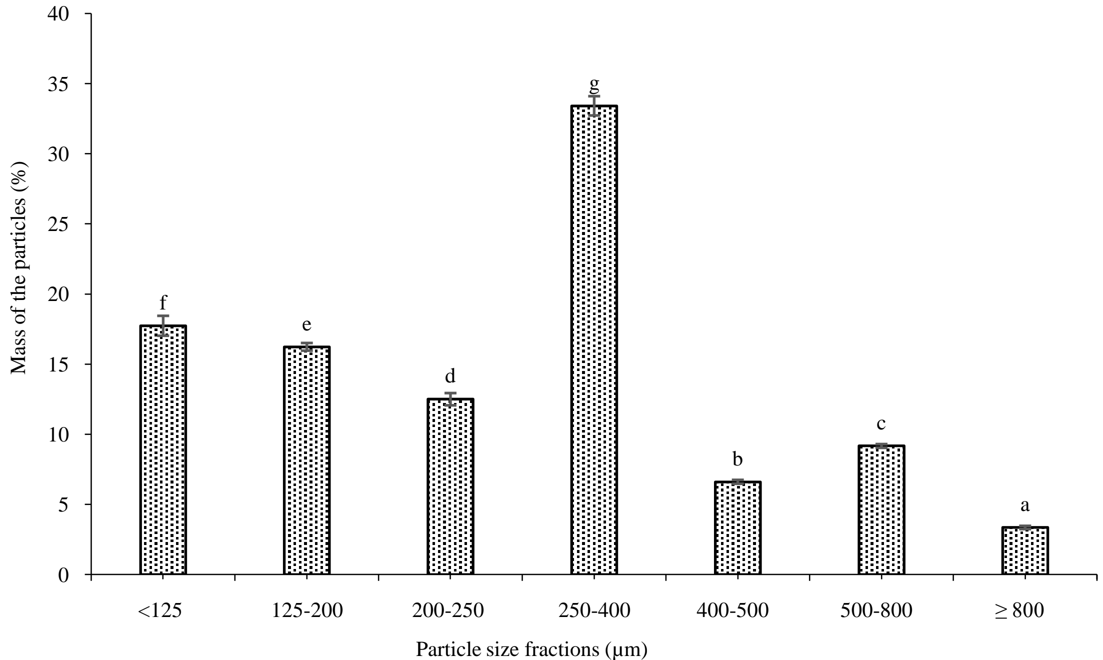

Figure 1 Mass distribution of particle size fractions in *S. torvum* leaf powder; Data are mean of five replicates; Columns without common letters differ significantly at LSD P<0.05.

Table 1 Contents of bioactive compounds in extracts of *S. torvum* leaf powder and its particle size fractions

S. torvum leaves powders	Total polyphenols mg GAE/100mL Extract	Total flavonoids mg QE/100 mL Extract	Condensed tannins mg CE/100 mL Extract
Powder (Ø < 1000 µm)	43.63 ± 0.15[cd]	10.97 ± 0.75[bc]	17.73 ± 0.27[e]
Particle size fraction (µm)			
≥ 800	40.43 ± 0.32[a]	9.62 ± 0.18[a]	13.01 ± 0.59[a]
500 – 800	43.60 ± 0.27[cd]	11.79 ± 0.36[cd]	13.51 ± 0.51[ab]
400 – 500	43.88 ± 0.24[de]	14.47 ± 0.37[f]	12.93 ± 0.85[a]
250 – 400	44.14 ± 0.20[e]	12.62 ± 0.87[de]	14.21 ± 0.61[bc]
200 – 250	44.62 ± 0.19[f]	10.37 ± 0.33[ab]	16.56 ± 0.60[d]
125 – 200	41.12 ± 0.19[b]	11.62 ± 0.20[c]	14.77 ± 0.22[c]
< 125	43.37 ± 0.15[c]	12.77 ± 0.60[e]	22.79 ± 0.12[f]

Mn ± SD - Means ± Standard Deviation; Means in the same column for each compound, with different superscripts, are significantly different from each other respectively ($p < 0.05$). GAE : Gallic Acid Equivalent; QE: Quercetin Equivalent; CE : Catechin Equivalent

3.2 Bioactive compound contents in *S.torvum* leaf powder extracts and its particle size fractions

3.2.1 Total polyphenol content

The total polyphenol content of extracts from the powder and its particle size fractions is 43.63 ± 0.15 mg GAE/100mL for the powder (Table 1). This is significantly lower (296.4 mg GAE/100mL extract) than the total polyphenol content reported by Djoueudam et al. (2019) in *S. torvum* leaf powder extracts. The polyphenol content varies significantly across various fractions, ranging from 40.43 ± 0.32 to 44.62 ± 0.19 mg GAE/100mL extract. As the particle size decreases, the accessibility of total polyphenol content increases. Among the tested fractions, 200-250 µm has shown the highest polyphenol content (44.62 ± 0.19 mg GAE/100 mL extract).

These results can be attributed to the grinding process, during which the dry leaves of *S. torvum* are reduced to small particles of varying sizes, facilitating the dissemination of polyphenols. This mechanical effect increases the specific surface area of the leaves, enhancing the exposure of polyphenols to the extraction solvent (methanol) and thereby facilitating their extraction in greater quantities. Similar observations were made by Nabil et al. (2020) on particle size fractions of a plant powder (*Opuntia ficus-indica*), further corroborating the results obtained in this study.

However, these findings contrast with those reported by Wu et al. (2022), who demonstrated that a smaller powder particle size (increases specific surface area) does not always improve the accessibility of polyphenols (catechin). This can be explained by the fact that certain compounds, such as fibers, can limit the grinding of leaves by forming agglomerates, thereby preventing the dissemination of compounds in the smaller particle-size fractions. This results in a concentration of these compounds in the larger particle size fractions, as evidenced by the work of Deli et al. (2019) on grinding and sieving for the fractionation of plant powders.

3.2.2 Total flavonoid content

The total flavonoid content of powder extracts of *S. torvum* leaves is 10.97 ± 0.75 mg QE/100 mL extract, which is lower than the 16.70 mg QE/100mL reported by Djoueudam et al. (2019). Flavonoid content significantly varies across particle size fractions, ranging from 9.62 ± 0.18 to 14.47 ± 0.37 mg QE/100mL extract (Table 1). As the particle size decreases, a significant increase in flavonoid content is exhibited (from 9.62 ± 0.18 to 12.77 ± 0.60 mL QE/100 mL extract). However, an optimum is observed in the 400-500 µm fraction (14.47 ± 0.37 to QE/100 mL extract).

As previously demonstrated with total polyphenols, the presence of polysaccharides (fibers) or heterosides (glycosides) linked to flavonoids in the leaves, and these are similar to the results reported by Prithvira (2019) those who suggested that the dissemination of flavonoids across different particle size fractions may associate their accessibility to the solvent during extraction. These results align with those reported by Wu et al. (2022) on the flavonoid (catechin) content of particle size fractions of a plant powder (*Rhizoma dioscoreas oppositae*).

Flavonoids, an essential group of natural polyphenolic compounds characterized by their flavan core, are primarily found in vegetables, fruits, and plant-based beverages. Due to their multiple biological activities, including antimicrobial, antioxidant, antidiabetic, and anticancer properties (Maizuwo 2017; Dongmo et al. 2023; Murugesan et al. 2024), these are an indispensable component in the field of health.

3.2.3 Condensed tannin content

As demonstrated in Table 1, the accessibility of condensed tannins to the solvent during extraction significantly increases as the particle size decreases (corresponding to an increase in specific leaf surface area). The values for the powders are 17.73 ± 0.27 mg CE/100 mL extract, and they range from 12.93 ± 0.85 to 22.79 ± 0.12 mg CE/100 mL extract for the particle size fractions. The fraction < 125 µm has the highest content, at 22.79 ± 0.12 mg CE/100 mL extract.

The previously mentioned factors in polyphenols can explain these results and align with findings Deli et al. (2019) reported on *Dichrostachys glomerata* and *Hibiscus sabdariffa* powders. Moreover, complexation-free polyphenols in the leaves may also promote their dissemination across various particle size fractions, particularly the finest ones (Guerriero et al. 2018), further explaining this result. Several authors have reported similar results with superfine particle sizes (Meng et al. 2019). Tannins, a group of polyphenolic compounds, are known for their biological activities (such as antioxidant and antimicrobial properties) and their role in managing several chronic diseases (Nouman et al. 2016; Rodriguez et al. 2019; Nabil et al. 2020).

3.3 Antioxidant activities of S. torvum leaf powder extract

3.3.1 DPPH radical scavenging activity

Antioxidant activity encompasses the prevention of free radicals, the neutralization of existing free radicals, and the repair of damaged biomolecules (Makkar et al. 2007). These activities are specific to pure molecules or plant extracts with this health-promoting capacity. The antioxidant capacity of *S. torvum* leaf powder extracts and their particle size fractions were evaluated and presented in Table 2. It is expressed as the concentration in µg/mL of extract required to scavenge 50% free radicals (RSa_{50}). Therefore, the lowest RSa_{50} value is considered to have the greatest antioxidant capacity for *S. torvum* powder extracts.

Results of the study revealed that the powder extracts and their particle size fractions exhibit antiradical activity, with concentrations varying significantly from one fraction to another. The most potent concentration among particle size fractions was found in the 500-800 µm fraction, at 11.40 ± 2.40 µg/mL extract, while the concentration of the powder is 36.20 ± 2.16 µg/mL extract. This represents a 31.50% improvement in the extract's antiradical activity due to the powder's fractionation. However, compared with ascorbic acid (vitamin C) (7.80 ± 1.40 µg/mL), extracts from the 500-800 µm fraction and the powder are 1.46 and 4.64 times less active against DPPH radicals, respectively. This result suggests that the extracts' bioactive compounds contribute to the studied powder's antiradical activity. Total polyphenols, flavonoids, and condensed tannins, known for their antioxidant activity (Ramamurthy et al. 2012; Ravani et al. 2018; Nabil et al. 2020), show no significant correlation (Table 3) with the DPPH antiradical activity of the powder extracts studied. This indicates that the accessibility of bioactive compounds in the extracts is insufficient to ensure the highest scavenging activity. Therefore, the contents of these bioactive compounds in the extracts are a key factor that needs to be taken into account to ensure the greatest antiradical activity. This observation is supported by the fact that the closest antiradical activity to vitamin C is not ensured by the particle size fraction with the highest contents of bioactive compounds. Deli et al. (2019) report that extracts' DPPH antiradical/antioxidant capacity becomes increasingly important as bioactive compound contents become more accessible, corresponding to smaller particle sizes of plant powder. This result

Table 2 Antioxidant activities of extracts from *S. torvum* leaf powder and its particle size fractions (µg/mL)

S. torvum leaves powders	DPPH (RSa_{50})	ABTS(RSa_{50})	FRAP (RC_{50})
Powder(Ø < 1000 µm)	36.20 ± 2.16[d]	45.00 ± 2.39[ef]	24.00 ± 1.52[b]
Particle size fraction (µm)			
≥ 800	28.60 ± 2.66[c]	48.80 ± 3.55[f]	30.00 ± 3.42[de]
500-800	11.40 ± 2.40[a]	18.20 ± 2.74[b]	33.00 ± 2.96[ef]
400-500	78.60 ± 3.09[g]	32.20 ± 2.85[c]	37.00 ± 2.44[g]
250-400	47.40 ± 2.65[e]	55.00 ± 2.18[g]	28.20 ± 1.65[cd]
200-250	57.80 ± 3.28[f]	55.40 ± 3.38[g]	26.20 ± 1.14[bc]
125-200	17.40 ± 1.50[b]	42.00 ± 2.67[de]	23.00 ± 1.04[b]
< 125	35.00 ± 3.01[d]	37.60 ± 2.15[d]	34.60 ± 1.43[fg]
*Vitamin C	7.80 ± 1.40[a]	11.20 ± 1.68[a]	18.00 ± 1.00[a]

Mn ± SD - Means ± Standard Deviation; Means in the same column with different superscripts are significantly different from each other ($P < 0.05$), RSa_{50} - 50% Scavenging activity required to scavenge 50% of free radicals; RC_{50} - Reduction Concentration required to reduce 50% of Fe^{3+}* Reference

Table 3 Pearson correlation coefficients between particle mass, bioactive compound contents, and antioxidant activities

	DPPH	FRAP	ABTS	Mass of the particles	Total Flavonoids	Total polyphenol	Condensed tannins
DPPH	1						
FRAP	0.331 (0.422)	1					
ABTS	0.298 (0.473)	-0.573 (0.137)	1				
Mass of the particles	-0.045 (0.914)	-0.503 (0.203)	0.220 (0.600)	1			
Total Flavonoids	0.475 (0.233)	0.614 (0.105)	-0.422 (0.297)	-0.132 (0.755)	1		
Total polyphenol	0.534 (0.172)	0.211 (0.615)	-0.026 (0.950)	0.242 (0.563)	0.423 (0.295)	1	
Condensed tannins	-0.061 (0.884)	0.003 (0.994)	0.096 (0.820)	0.309 (0.455)	0.039 (0.926)	0.243 (0.560)	1

r (p): Correlation coefficient (probability threshold)

contrasts with those obtained in this study. Certain phenolic compounds, free or not, in varying contents in the extracts could explain these differences.

3.3.2 ABTS radical scavenging activity

The ABTS radical scavenging activity (RSa_{50}) of *S. torvum* leaf powder extracts varies significantly across particle size fractions (Table 2). The most potent concentration in µg/mL of extract is closest to that of ascorbic acid, the reference antioxidant used in this study. Among the extracts of the particle size fractions, the most potent concentration is found in the 500-800 µm fraction at 18.20 ± 2.74 µg/mL extract. The concentration for the powder is 45.00 ± 2.39 µg/mL extract, representing a 40.44% improvement in the antioxidant activity of the extracts. However, these concentrations (18.20 ± 2.74 µg/mL and 45.00 ± 2.39 µg/mL) are respectively 1.62 and 4.01 times less active against the ABTS radical than vitamin C (11.20 ± 1.68 µg/mL).

The antioxidant activity of the extracts studied is attributed to the bioactive compounds accessible in the powder (total polyphenols, flavonoids, and condensed tannins (Ramamurthy et al., 2012; Ozcan et al., 2014). These compounds show no significant correlation with the concentrations (RSa_{50}) obtained (Table 3). This may be explained by the reasons previously mentioned for DPPH radical scavenging activity. The antioxidant activity of *S. torvum* leaf powder extracts, evaluated via the DPPH and ABTS assays, depends not only on the accessibility of the bioactive compounds but also on their content in the extracts. However, in this study, the highest antioxidant activity is not ensured by the extracts with the highest bioactive compound contents.

3.3.3 Ferric-reducing antioxidant power (FRAP)

The FRAP assay assesses the antioxidant potential of foods by measuring their ion-reducing capacity (Djoueudam et al. 2019). This capacity is attributed to the bioactive compounds present in the food (Ramamurthy et al. 2012). In this context, the concentrations of *S. torvum* leaf powder extract needed to reduce iron by 50% (Table 2). The data revealed that all extracts possess a reducing capacity for Fe^{3+} ions, which significantly varies across different particle size fractions. This observation corroborates the antioxidant potency of the bioactive compounds in the powder (Kumar and Pandey 2013). However, the fraction of 125-200 µm and the powder do not exhibit a significant difference in their $Fe^{3\pm}$ reducing concentration, and this fraction possesses 1.27 times less antioxidant power (23.00 ± 1.04 µg/mL extract) compared to vitamin C (18.00 ± 1.00 µg/mL extract). Several studies have identified flavonoids as the primary bioactive compounds responsible for Fe^{3+} reduction (Makkar et al. 2007; Kumar and Pandey 2013; Prithvira 2019). The average flavonoid content in the extracts of the 125-200 µm fraction (11.62 ± 0.20 mg EQ/100 mL extract) and powders (10.97 ± 0.75 mg EQ/100 mL extract) does not show a significant difference (Table 1). This further confirms that the antioxidant capacity of the studied extracts depends not only on the accessibility of the bioactive compounds but also on their concentration in the extract, even if it is low.

3.4 Relationship between bioactive compound content, their antioxidant activity, and grouping of *S.torvum* leaf powder extracts

Data analyzed from *S. torvum* leaf powder extracts revealed a weak correlation between the content of bioactive compounds and antioxidant activity. However, a multivariate analysis will facilitate a comprehensive understanding of the interactions among these variables, thereby enabling a more accurate characterization of the powder extracts. A principal component analysis was conducted (Figure 2). The first principal component (F1) accounted for 60.90% of the observed variations among variables, while the second principal component (F2) accounted for 15.28%.

The correlation circle (Figure 2a) identifies two major groups of variables characterized by positive correlations. The first group comprises total polyphenols, total flavonoids, DPPH, and FRAP, while the second group includes condensed tannins, ABTS, and

particle mass. These two primary groups indicate that the antiradical/antioxidant activity (DPPH), more than the Fe^{3+} reducing power (FRAP), is primarily attributed to flavonoids among the analyzed polyphenols. Condensed tannins contribute minimally to the antioxidant activity (ABTS). However, the mass of the particles containing them should be considered.

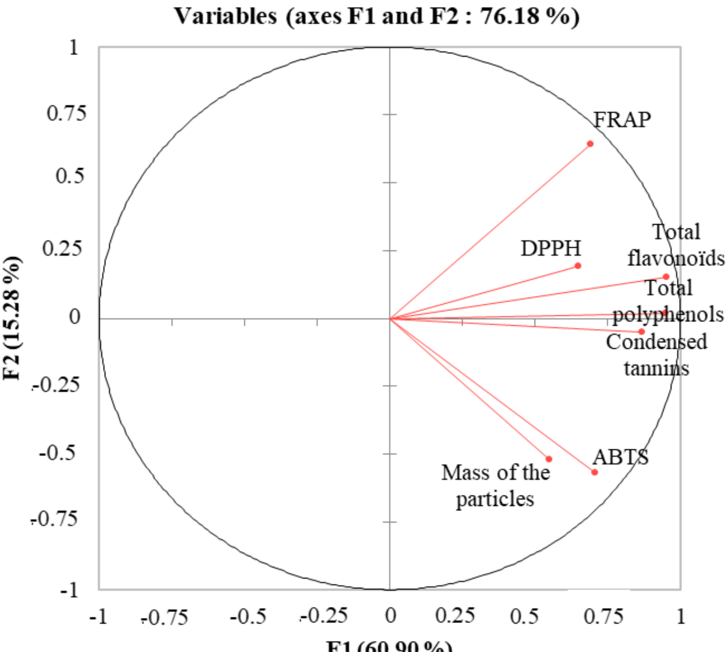

Figure 2a Correlations between the mass of the particles, bioactive compounds contents and antioxidant activities

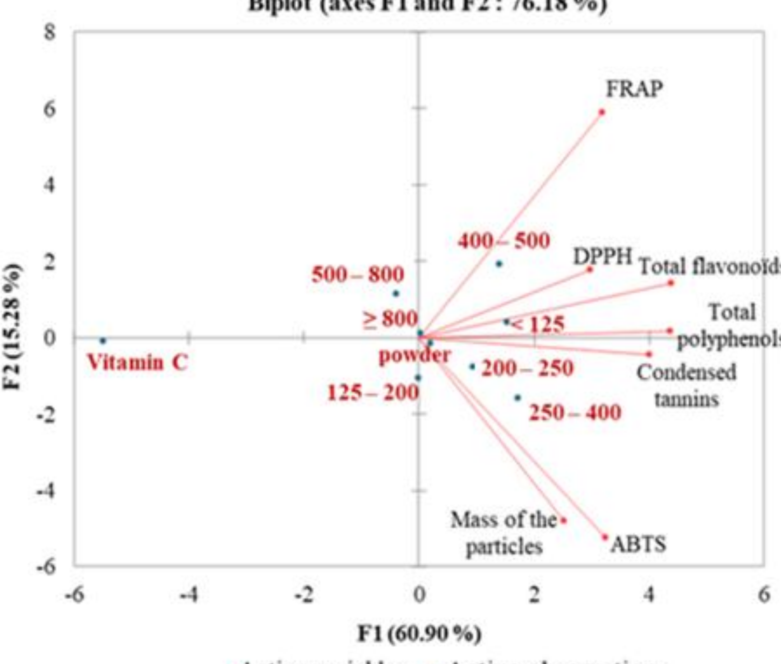

Figure 2b Regrouping *S. torvum* leaves powder extracts according to the mass of particle size fractions, bioactive compounds contents, and their antioxidant activities

Table 4 Antibacterial activities of extracts from *S. torvum* leaf powder and its particle size fractions

S. torvum leaves powders	Parameters	Bacteria								
		PA	SA	EC	KP	SF	SD	Stm	ST	SE
Powder (Ø < 1000 µm)	MIC (µg/mL)	>500	>500	>500	>500	>500	>500	250	250	>500
	MBC (µg/mL)	ND	ND	ND	ND	ND	ND	500	500	ND
	MBC/MIC	ND	ND	ND	ND	ND	ND	2	2	ND
Particle size fraction (µm)										
≥ 800	MIC (µg/mL)	>500	250	>500	>500	>500	>500	>500	>500	>500
	MBC (µg/mL)	ND	500	ND	ND	ND	ND	ND	ND	ND
	MBC/MIC	ND	2	ND	ND	ND	ND	ND	ND	ND
500-800	MIC (µg/mL)	250	>500	>500	250	>500	250	>500	>500	>500
	MBC (µg/mL)	500	ND	ND	500	ND	500	ND	ND	ND
	MBC/MIC	2	ND	ND	2	ND	2	ND	ND	ND
400-500	MIC (µg/mL)	500	500	500	500	500	250	500	500	>500
	MBC (µg/mL)	>500	>500	>500	>500	>500	500	>500	>500	ND
	MBC/MIC	ND	ND	ND	ND	ND	2	ND	ND	ND
250-400	MIC (µg/mL)	500	500	250	500	500	>500	500	500	>500
	MBC (µg/mL)	>500	>500	500	>500	>500	ND	>500	>500	ND
	MBC/MIC	ND	ND	2	ND	ND	ND	ND	ND	ND
200-250	MIC (µg/mL)	>500	>500	>500	>500	>500	>500	>500	>500	>500
	MBC (µg/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MBC/MIC	ND	ND	ND	ND	ND	ND	ND	ND	ND
125-200	MIC (µg/mL)	>500	>500	>500	>500	>500	>500	>500	>500	>500
	MBC (µg/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MBC/MIC	ND	ND	ND	ND	ND	ND	ND	ND	ND
< 125	MIC (µg/mL)	>500	>500	>500	>500	>500	>500	>500	>500	>500
	MBC (µg/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MBC/MIC	ND	ND	ND	ND	ND	ND	ND	ND	ND
*Amoxicilline	MIC (µg/mL)		0.5							
	MBC (µg/mL)	/	1	/	/	/	/	/	/	/
	MBC/MIC		2							
*Ciprofloxacine	MIC (µg/mL)	1		0.25	0.5	0.25	0.5	0.5	0.5	0.5
	MBC (µg/mL)	4	/	1	2	2	1	2	1	2
	MBC/MIC	4		4	4	0.125	2	4	2	4

PA: *Pseudomonas aeruginosa* (ATCC10145), SA: *Staphylococcus aureus* (ATCC25922), EC: *Escherishia coli* (ATCC10536), KP: *Klebsiella pneumonae* (ATCC13883), SF: *Shigella flexineri* (NR518), SD : *Shigella dysenteria* (CPC), Stm : *Salmonella typhimurium* (CPC), ST: *Salomonella typhi* (ATCC6539), SE: *Salmonella enteritidis* (ATCC13076), ND: Not Determined, MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, MBC/MIC: Determines the bactericidal (MBC/MIC ≤ 4) or Bacteriostatic (MBC/MIC > 4) effects of the extracts, 0 ≤ MIC< 10µg/mL: Very good activity, 10 ≤ MIC< 125µg/mL: Good activity, 125 ≤ MIC< 250µg/mL: Significant activity, 250≤ MIC<500µg/mL: Moderate activity, MIC> 500µg/mL: Low activity (Kuete et al. 2010), *Standard

Table 5 Antifungal activities of extracts from *S. torvum* leaf powder and its particle size fractions

Solanumtorvum leaves powders extract	Parameters	Yeast				Dermatophyte		
		CA	CK	CP	CN	TM	MA	EF
Powder(Ø < 1000 µm)	MIC (µg/mL)	>500	>500	>500	>500	>500	>500	>500
	MFC (µg/mL)	ND	ND	ND	ND	ND	ND	ND
	MFC/MIC	ND	ND	ND	ND	ND	ND	ND
Particle size fractions (µm)								
≥ 800	MIC (µg/mL)	500	500	250	250	250	>500	>500
	MFC (µg/mL)	>500	>500	500	500	500	ND	ND
	MFC/MIC	ND	ND	2	2	2	ND	ND
500-800	MIC (µg/mL)	500	>500	>500	500	250	>500	500
	MFC (µg/mL)	>500	ND	ND	>500	500	ND	>500
	MFC/MIC	ND	ND	ND	ND	2	ND	ND
400-500	MIC (µg/mL)	>500	>500	>500	>500	>500	>500	>500
	MFC (µg/mL)	ND	ND	ND	ND	ND	ND	ND
	MFC/MIC	ND	ND	ND	ND	ND	ND	ND
250-400	MIC (µg/mL)	>500	>500	>500	>500	>500	>500	>500
	MFC (µg/mL)	ND	ND	ND	ND	ND	ND	ND
	MFC/MIC	ND	ND	ND	ND	ND	ND	ND
200-250	MIC (µg/mL)	>500	>500	>500	>500	125	>500	>500
	MFC (µg/mL)	ND	ND	ND	ND	250	ND	ND
	MFC/MIC	ND	ND	ND	ND	2	ND	ND
125-200	MIC (µg/mL)	>500	>500	250	250	>500	>500	>500
	MFC (µg/mL)	ND	ND	500	500	ND	ND	ND
	MFC/MIC	ND	ND	2	2	ND	ND	ND
< 125	MIC (µg/mL)	>500	>500	>500	>500	>500	>500	>500
	MFC (µg/mL)	ND	ND	ND	ND	ND	ND	ND
	MFC/MIC	ND	ND	ND	ND	ND	ND	ND
*Nystatine	MIC (µg/mL)	0.5	0.25	1	0.25	/	/	/
	MFC (µg/mL)	2	1	2	1			
	MFC/MIC	4	0.25	2	0.25			
*Griseofulvine	MIC (µg/mL)	/	/	/	/	0.25	1	0.5
	MFC (µg/mL)					1	2	2
	MFC/MIC					0.25	2	4

CA: *Candida albicans* (CPC), CK: *Candida krusei* (CPC), CP: *Candida parasilosis* (CPC), CN: *Cryptococcus neoformans* (CPC), TM: *Trichophyton mentagrophytes* (CPC), MA: *Microsporium audouinii* (CPC), EF: *Epidermophyton flocosum* (CPC), ND: Not Determined, MIC = Minimum Inhibitory Concentration, MFC : Minimum Fungicidal Concentration, MFC/MIC: Determines the bactericidal (MBC/MIC ≤ 4) or Bacteriostatic (MFC/MIC > 4) effects of the extracts, 0 ≤ MIC< 10µg/mL: Very good activity, 10 ≤ MIC< 125µg/mL: Good activity, 125 ≤ MIC< 250µg/mL: Significant activity, 250≤ MIC<500µg/mL: Moderate activity, MIC> 500µg/mL: Low activity (Kuete et al. 2010), *Standard

When these two major groups are plotted on the F1xF2 factorial plane, these accounts for 76.18% (Figure 2b) of the interactions between these variables (bioactive compounds and antioxidant activities) and the factors (powder extracts), four groups of powder extracts can be distinguished: 400-500 and < 125, 200-250 and 250-400, 125-200, and 500-800 µm. All these groups are characterized by antioxidant activities due to bioactive compounds in all extracts, which become accessible after extraction. Among these four, only the 125-200 µm and 500-800 µm fractions exhibit the highest antioxidant activities, comparable to that of vitamin C, the reference antioxidant. The antiradical/antioxidant (DPPH) and ion-reducing (FRAP) activities of the 500-800 µm fraction are attributed to their average flavonoid content. The antioxidant activity (ABTS) of the 125-200 µm fraction is weak due to condensed tannins. The fraction ≥ 800 µm and powder are underrepresented on the F1xF2 factorial plane. This suggests that when the content of bioactive compounds is low in a plant powder, it would be beneficial to fractionate it into finer particles. This would enhance the accessibility and content of bioactive compounds, thereby improving biological activities.

3.5 Antimicrobial activities and influence of bioactive compound contents of powder and its particle size fractions of *S. torvum* leaf extracts

3.5.1 Antibacterial activities

The antibacterial properties of *S. torvum* leaf powder extracts were assessed against gram-positive bacterial strains and clinical bacterial isolates obtained from the Centre Pasteur du Cameroun (CPC). These include *Pseudomonas aeruginosa* (PA: ATCC10145), *Staphylococcus aureus* (SA: ATCC25922), *Escherichia coli* (EC: ATCC10536), *Klebsiella pneumoniae* (KP: ATCC13883), *Shigella flexneri* (SF: NR518), *Shigella dysenteriae* (SD: CPC), *Salmonella typhimurium* (Stm: CPC), *Salmonella typhi* (ST: ATCC6539), and *Salmonella enteritidis* (SE: ATCC13076). The microdilution broth susceptibility assay was employed for this purpose, as noted by Newton et al.(2002).

The results indicate that all powder extracts exhibit antibacterial activity, ranging from moderate (250 ≤ MIC < 500µg/mL) to low (MIC > 500µg/mL) (Table 4) (Kuete et al. 2010). The powder and the 500-800 µm fraction demonstrated bactericidal activity against two (Stm, ST) and three (PA, KP, SD) bacteria strains, respectively. This antibacterial activity is attributed to phenolic compounds in the *S. torvum* leaf powder extracts (Acharyya and Khatun 2018; Senizza et al. 2021), which are made more accessible by particle size distribution. It is observed that even a low content of phenolic compounds is sufficient to exhibit antibacterial activity, as these activities are observed at micro concentrations of the extracts. Consequently, the obtained polyphenol contents are adequate. However, the observed weak antibacterial activities can be attributed to the lipophilic nature of all the isolates, which hinders their entry into the hydrophilic periplasmic space of bacterial strains of isolates (Dongmo et al. 2023). These results affirm that *S. torvum* leaves are a valuable source of bioactive compounds used in traditional medicine for treating infectious diseases (Loganayaki et al. 2010; Senizza et al. 2021; Rajapaksha and Premathilake 2020; Jan et al. 2024).

3.5.2 Antifungal activities

The antifungal properties of *S. torvum* leaf powder extracts are presented in Table 5. These extracts were evaluated against clinical fungal isolates obtained from the Centre Pasteur du Cameroun (CPC), including *Candida albicans* (CA: CPC), *C. krusei* (CK: CPC), *C. parasilosis* (CP: CPC), *Cryptococcus neoformans* (CN: CPC), *Trichophyton mentagrophytes* (TM: CPC), *Microsporium audouinii* (MA: CPC), and *Epidermophyton flocosum* (EF: CPC).

All powder extracts showed antifungal activities ranging from significant minimum inhibitory concentrations (125 ≤ MIC < 250µg/mL) to low minimum inhibitory concentrations (MIC > 500µg/mL). The powder extracts demonstrated low activity (MIC > 500 µg/mL) against all studied fungal strains. The fraction 125-200 µm showed moderate activities (MIC = 250 µg/mL) against two yeast strains (CP and CN), while the fraction 200-250 µm fraction exhibited significant activity (MIC = 125 µg/mL) against the dermatophyte, and the ≥ 800 µm fraction displayed moderate activities against the two yeasts (CP and CN) and the dermatophyte. The activities of these fractions are fungicidal. Bioactive compounds (total polyphenols, flavonoids, and condensed tannins), made more accessible in the extracts studied after particle size distribution, may explain these results (Kumar and Pandey 2013; Senizza et al. 2021). Interestingly, it was not the extracts from the particle size fractions with the highest contents of bioactive compounds that produced the most significant results. However, the weak solubility of isolates in polar solvents can also account for the observed low activities. These results justify using these leaves as a medicinal plant for treating certain skin conditions and gastric diseases.

Conclusion

Evaluating the bioactive compound contents in leaf powder extracts, their particle size fractions, and their impact on biological activities leads to the following conclusions. When the content of bioactive compounds is low in a plant powder, such as the *S. torvum* leaf powder, which is considered of little value in terms of its biological activity, one effective method to enhance these activities is to proceed with the fractionation of the powders. This process improves the accessibility of bioactive compounds (polyphenols, flavonoids, condensed tannins) and increases their content in the powder extracts, particularly in the extracts of the

finest particle-size fractions. This enhancement has specific positive effects on their antioxidant (DPPH, ABTS, FRAP), antibacterial (*S. typhimurium, S. typhi, P. aeruginosa, K. pneumoniae, S. dysenteriae*), and antifungal (*C. parapsilosis, C. neoformans, T. mentagrophytes*) activities. Flavonoids appear to be the primary bioactive compounds responsible for these activities. The mass of the particle size fraction must be considered. This method of processing powder suggests that no plant powder should be disregarded or rejected due to its low content of bioactive compounds.

Author's contribution

ASSIENE AGAMOU Julien Armel: Conceptualization, Investigation, Methodology, Data Analysis, Writing-Original draft, Writing-review and editing; DJEUKEU ASONGNI William: Methodology; Data analysis, Review original draft; ASSIENE OYONG Damase Serge: Methodology, Data Analysis, Review original draft TIZE Zra: Methodology, Data analysis; OBONO NDONG Tatiana Léa, MBANGO EKE Pauline, MBOUBE NGONGANG Oriane, KEUBING FEUDJIO Suzanne Rose: Methodology; FONGNZOSSIE FEDOUNG Evariste: Supervision, Methodology, Review original draft

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Declaration of interest

The authors declare that they have no conflict of interest.

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Optimization of Formic Acid-Formalin-Based Decalcification Protocol for Rat Calvarial Bone Histology

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ABSTRACT

Decalcification is crucial in histological processing, particularly for studying mineralized tissues like bone. The choice of decalcification method can significantly impact the quality of histological sections and the preservation of tissue morphology. This study aims to establish a standardized protocol for decalcifying rat calvarial bone using a formic acid-formalin-based decalcification solution. The protocol was systematically optimized and evaluated based on various parameters, including decalcification time, formic acid concentration, and tissue integrity preservation. The decalcification process was evaluated through comprehensive assessments, including gross physical examination, chemical analysis, and radiographic imaging techniques. Our result demonstrated that the 10% formic acid concentration proved most effective for decalcifying rat calvarial bone samples within eight days, excelling in mineral

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Histopathology

Bone regeneration

content removal while preserving specimen structural integrity. In contrast, the 5% concentration failed to complete decalcification within ten days, and the 15% compromised sample quality within eight days. Histological analyses confirmed the efficacy of the 10% formic acid concentration in maintaining tissue integrity and achieving optimal staining quality. The standardized protocol presented in this study provides an effective and reliable approach for achieving consistent and high-quality histological sections of rat calvarial bone. An ideal decalcification agent should effectively remove calcium salts, preserve structural integrity and molecular components, facilitate rapid yet minimally damaging decalcification, and ensure ease of handling for laboratory personnel. Further exploration of its applicability to different bone types or species is recommended to broaden its research utility.

1 Introduction

Teeth and bones fall into the group of the toughest tissues, being denser and less reactive chemically than other body tissues. The microscopic analysis is challenging due to the high calcium and phosphorus levels in the biological apatite that forms these tissues (AbouNeel et al. 2016). It is crucial to extract inorganic calcium from the organic collagen matrix, calcified cartilage, and nearby tissues to obtain well-defined bone sections. This process, referred as decalcification, is essential for successful analysis (Khangura et al. 2021). Decalcification removes calcium salts from mineralized tissues for histologic sectioning. But with balanced acidity, any acid can affect tissue stability. The impact depends on solution acidity and decalcification duration. Factors like solution strength, temperature, agitation, and tissue suspension affect decalcification speed (Callis and Sterchi 1998; Bancroft and Gamble 2008).

The samples should always be properly fixed before decalcification (González-Chávez et al. 2013). Various agents, such as weak and strong acids and chelating agents, are used for decalcification. Choosing the appropriate decalcification agents is crucial in handling mineralized tissues as it can impact the tissues' integrity and immunohistochemical properties (Savi et al. 2017). An ideal decalcification agent possesses several key characteristics such as it should effectively remove calcium salts from mineralized tissues without compromising the structural integrity of tissue or cellular details (Prasad and Donoghue 2013). Furthermore, it must preserve the antigenicity and molecular components of tissue, allowing for accurate histological and immunohistochemical analysis (Kim et al. 2016). An ideal agent should also facilitate a reasonably rapid decalcification process while minimizing tissue damage or distortion. It should also be easy to handle, ensuring safety for laboratory personnel and compatibility with subsequent staining techniques (Sanjai et al. 2012).

Methods include acid/manual, microwave, ion exchange resins, electrolytic, and ultrasonic decalcification (Skinner 2003). Precisely determining the endpoint is vital due to acids' harmful effects on tissues. The endpoint determination is achieved through physical, chemical, and radiographical techniques (Savi et al. 2017). Critical steps in the decalcification include meticulously assessing the specimen beforehand, ensuring thorough fixation, preparing slices of optimal thickness for fixation and processing, selecting an adequate volume of a suitable decalcifier changed at regular intervals, precisely determining the endpoint, and executing comprehensive processing according to a suitable schedule (Skinner 2003).

Since there is no ideal decalcification agent, the choice and standardization of the decalcification protocol depend on the specific bone type and the intended examination. This study aims to standardize a formic acid-formalin-based decalcification protocol for the histological evaluation of rat calvarial bone.

2 Materials and Methods

2.1 Specimen Preparation and Fixation

All experimental protocols adhered strictly to the guidelines established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA Guidelines 2003). The study protocols were approved by the Institutional Animal Ethics Committee (IAEC) of the ICAR-Indian Veterinary Research Institute, ensuring compliance with ethical standards for animal research. Specimens used in this study were obtained from adult rats previously sacrificed in other approved experimental studies. The present study consisted of three groups, each containing six samples. A total of 18 rat calvarial bone samples were collected and underwent fixation in neutral buffered formalin for three days.

2.2 Decalcification Procedure

A formic acid-formalin-based solution was utilized for decalcification. Formic acid concentrations of 5%, 10%, and 15% were tested to optimize the protocol. The samples were randomly assigned into three groups – groups A (5% formic acid concentration), B (10% formic acid concentration), and C (15% formic acid concentration). The concentration of 40% formaldehyde was kept at 5% level. A decalcification solution was employed in a 100-fold volume relative to the specimen to ensure

adequate coverage. The decalcification solution was replaced daily, and the specimens were washed before replenishment with freshly prepared solutions to maintain optimal decalcification conditions. The endpoint of decalcification was monitored daily using chemical analyses and radiographic imaging techniques to assess the completion of the process (Skinner 2003).

2.3 Evaluation of Decalcification

Evaluation of the appearance, texture, and integrity of the specimens after each decalcification cycle was done. Daily chemical analyses were performed to track mineral content changes and verify decalcification progress. The calcium oxalate test was employed to determine the endpoint of decalcification. A 5 ml spent decalcification solution was mixed with 5 ml of concentrated ammonium hydroxide and 5 ml of saturated aqueous ammonium oxalate. Any white precipitate suggested calcium presence and incomplete decalcification (Shahid et al. 2023).

Daily radiographic imaging was employed to visualize the extent of mineral removal and determine the completeness of the process. The specimens were exposed at 25 KVp and 3 mAs for 10s. The tissue sections were processed, paraffin-embedded, and subjected to standard staining procedures for histological analysis. Routine staining using H&E and special staining using RGB Trichrome were employed to evaluate the tissue integrity (Mamachan et al. 2023).

After the staining was completed, a final grading system was employed, utilizing a scale from 1 to 4, and here,1 for poor, 2 for fair, 3 for good, and 4 for excellent, allowing for a detailed classification based on predefined criteria. Following this grading, the results were tabulated and analyzed using appropriate statistical methods to determine significant differences and optimal conditions for decalcification.

The statistical analysis employed in this study involved a One-Way ANOVA to assess the significance of the duration of decalcification across different groups. The Kruskal-Wallis test was also utilized to evaluate the significance of staining scores. A significance level of $p < 0.05$ indicated statistical significance, underscoring the rigor applied in determining the impact of varying decalcification durations and staining outcomes.

3 Results

The results highlighted that among the tested formic acid concentrations (5%, 10%, and 15%), the 10% formic acid concentration proved most effective for decalcifying rat calvarial bone samples. The mineral content was removed within 8 days while maintaining specimen structural integrity and texture. In contrast, the 5% formic acid concentration failed to complete decalcification within 10 days. Alternatively, the 20% formic acid concentration achieved decalcification in 5 days but compromised sample quality. By day 8, evident changes in appearance, texture, and integrity were noticed and the time for decalcification varied notably among the groups. Specifically, group B showed considerable variation compared to Groups C and A. Group A required significantly more days to achieve decalcification. In contrast, Group C achieved it significantly faster than Group B (Figure 1A).

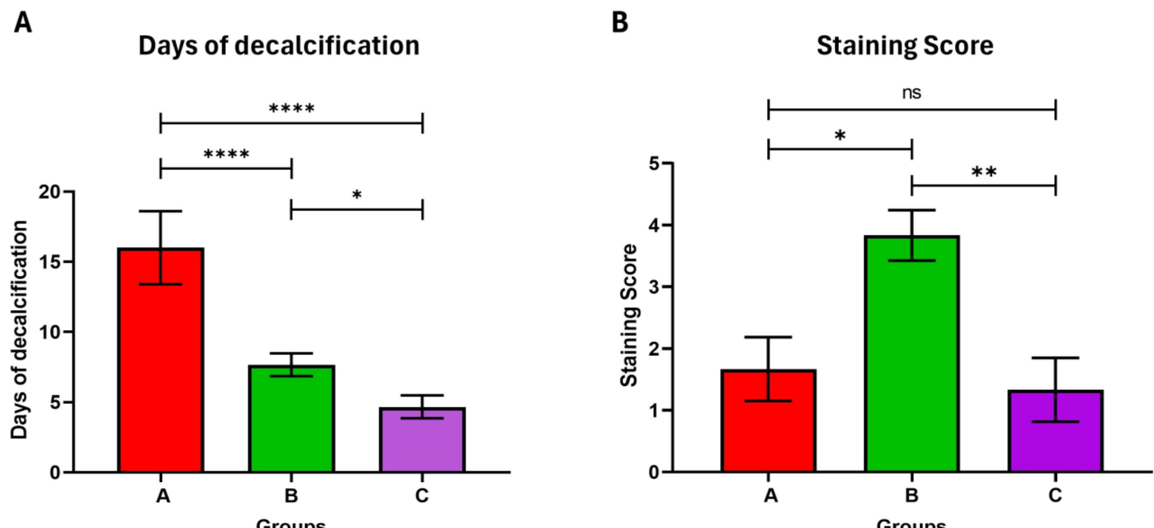

Figure 1 (A) The time for decalcification varied notably among the groups, with group B showing considerable variation compared to both groups C and A. Group A required significantly more days to achieve decalcification. In contrast, group C achieved it significantly faster than group B; (B) The staining score notably differed among the groups, with group B showing significantly higher scores than groups A and C, indicating that the 10% formic acid decalcified bone samples in group B achieved a better staining quality than those in groups A and C.

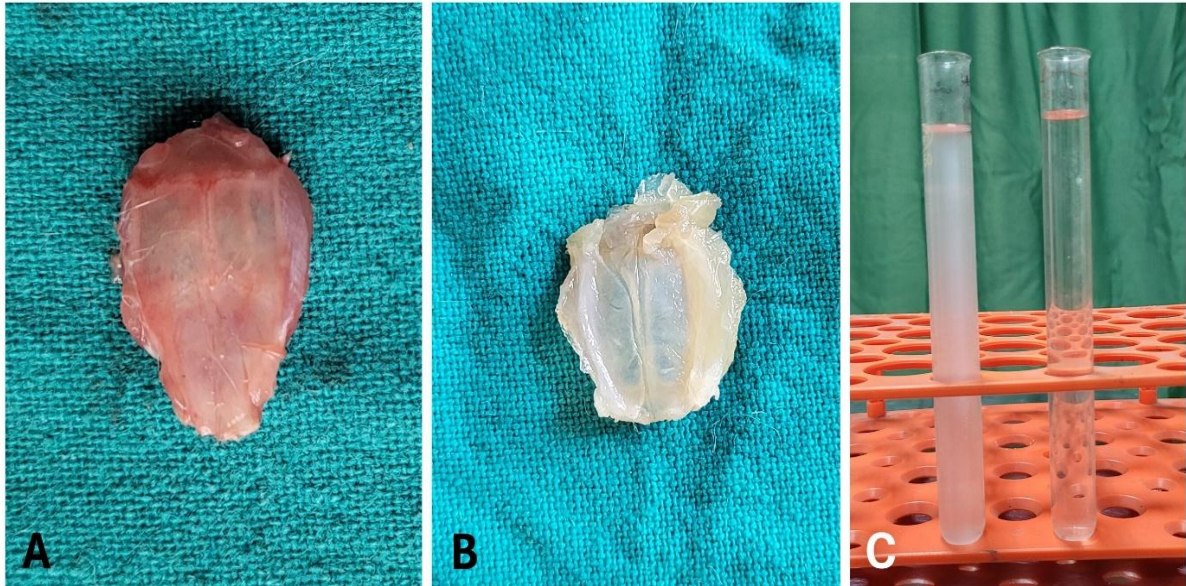

Figure 2 Upon decalcification, the gross observation of rat calvarial bone revealed, (A) Initially, the bone exhibits a rigid, opaque structure, while after the process, (B) it undergoes a discernible shift towards translucency and a softer texture due to the reduction in mineral content, (C) The presence of a white precipitate strongly suggests the residual presence of calcium, indicating incomplete decalcification (left), and the absence of residue, indicating complete decalcification (right).

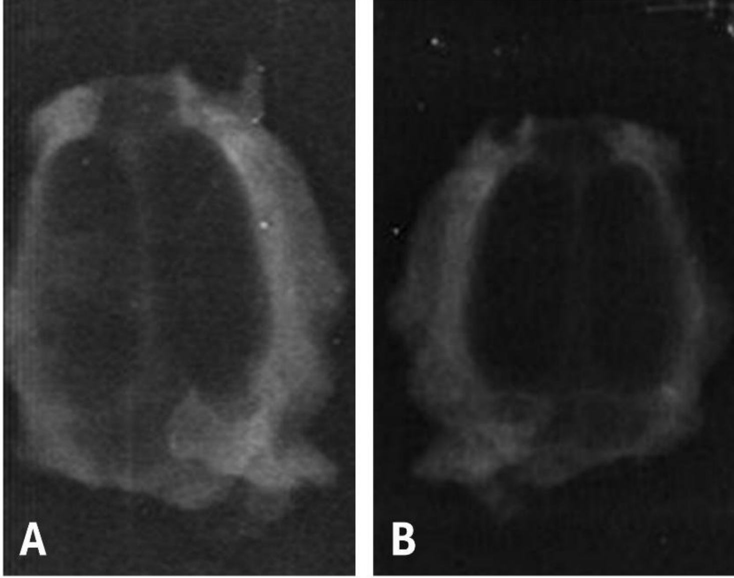

Figure 3 The detection of mineral loss in radiographic imaging from the initiation of decalcification solution application on day 1 (A) and until the complete decalcification (B).

Decalcification altered the appearance of rat calvarial bone, shifting it from a rigid, opaque structure to a translucent, softer form as mineral content diminishes (Figures 2A and 2B). This process modifies the texture of bone, making it more pliable while compromising its structural integrity, rendering it susceptible to bending or breakage under minimal stress. Consistently, the specimens treated with a 10% formic acid solution demonstrated the desired endpoint for decalcification. This was validated through rigorous daily chemical analyses, the calcium oxalate test, and radiographic imaging. The absence of any white precipitate in the calcium oxalate test and the clear observable reduction in mineral content in radiographic imaging confirmed the thoroughness and completeness of the decalcification process facilitated by the 10% formic acid solution (Figures 2C and 3).

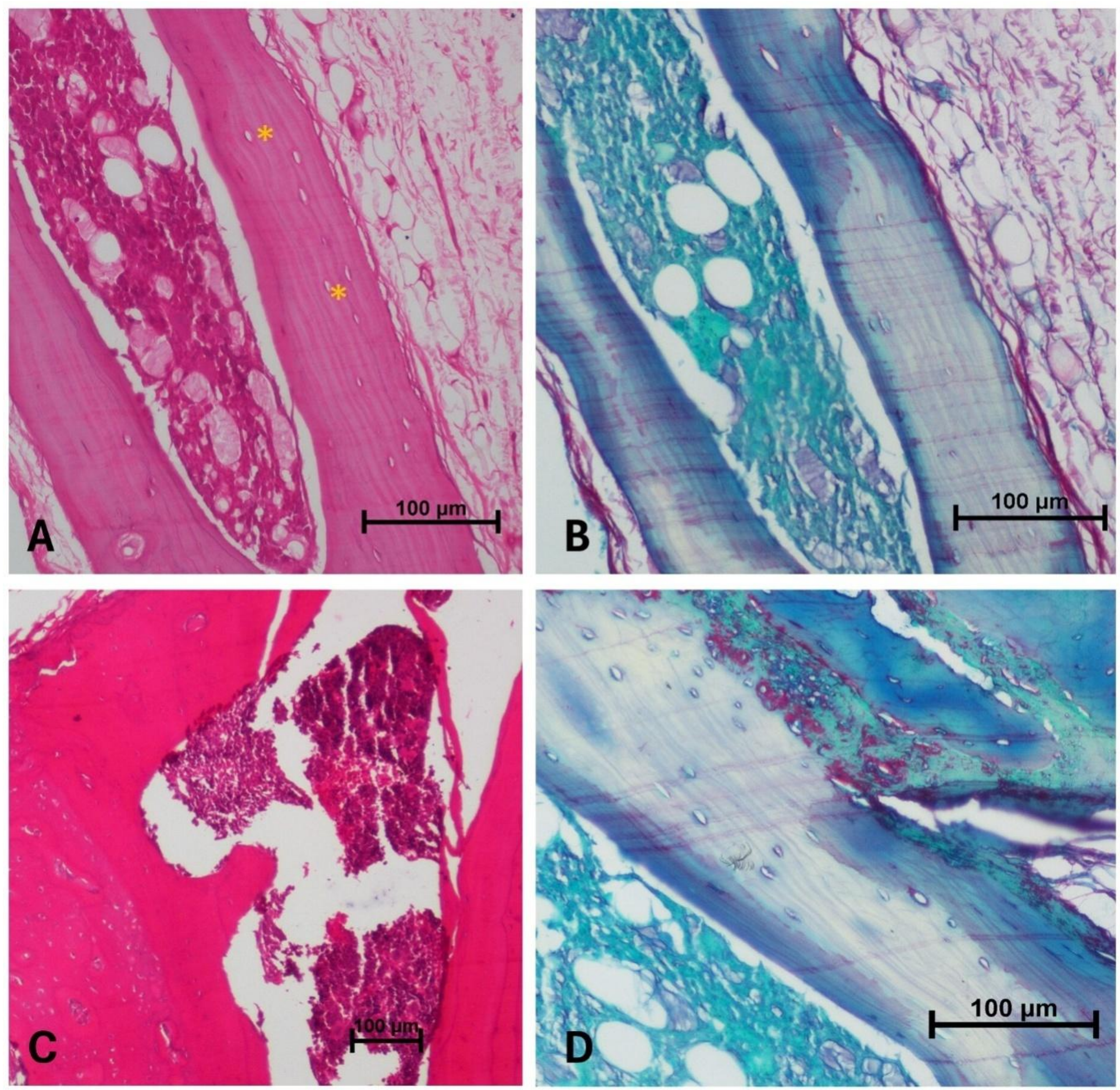

Figure 4 The effectiveness of the 10% formic acid concentration in maintaining tissue integrity while ensuring the integrity of the overall sample during (A and C) standard staining (H & E) and (B and D) specialized staining (RGB trichrome), (A) Osteocytes, are housed within small cavities called lacunae (orange colour) and appear as dark dots. The bone matrix appears pink due to its affinity to the eosin stain. (B) Red could potentially highlight osteoids and areas of active bone formation. Green emphasizes the bone matrix. Blue could represent areas with a high blood vessel density, indicating vascularization regions and nutrient supply within the bone tissue. (C) The section displayed a notable loss of tissue definition, hindering a clear distinction of structural components in samples due to the utilization of 20% formic acid for decalcification (D) The sections seemed inadequately stained, leading to diminished clarity in discerning the varied RGB grades, potentially causing a misjudgment or misinterpretation of the result in samples due to the utilization of 20% formic acid for decalcification.

Histological analysis, encompassing both standard staining (H&E) and specialized staining (RGB Trichrome), validated the efficacy of 10% formic acid. This concentration effectively maintained tissue integrity while preserving the overall bone sample structure (Figure 4A). The staining score notably differed among the groups, and among the tested groups, group B showed significantly higher scores than groups A and C. This indicates that the 10% formic acid decalcified bone sample in group B achieved better staining quality than those in groups A and C (Figure 1A). Upon H&E staining, decalcified rat calvarial bone revealed a dynamic microcosm of interconnected structures. Osteocytes, nestled within lacunae, punctuate the bone matrix. Trabecular and cortical bone

regions showcase distinct densities and arrangements, with trabecular bone forming a latticed network and cortical bone manifesting as denser outer layers. Interspersed blood vessels, vital for nourishment, course through the tissue, appearing as channels within the bone (Figure 4A).

Upon RGB staining, red coloured region could potentially highlight osteoids and areas of active bone formation, illustrating regions rich in collagenous matrix. The green hue emphasizes the matrix, and blue could represent areas with a high density of blood vessels, indicating regions of vascularization and nutrient supply within the bone tissue (Figure 4B). Conversely, the significant degradation of samples resulting from the use of 20% formic acid for decalcification notably compromised the quality of histological sections during staining, visibly impacting both standard staining (H&E) and specialized staining (RGB Trichrome) (Figures 4C and D). Statistical analysis of the collected data emphasized the significance of the 10% formic acid concentration, establishing it as the optimal condition for the efficient and effective decalcification of rat calvarial bone samples among the tested concentrations (5%, 10%, and 15%).

4 Discussion

Decalcification is performed in bone healing studies to make the bone tissue transparent or softer for better visualization and analysis under a microscope (El Khassawna et al. 2017). Bones naturally contain calcium salts, which make them hard and opaque, hindering the examination of cellular structures and detailed analysis of the healing process (Barrère et al. 2006). During decalcification, chemicals like ethylenediaminetetraacetic acid (EDTA) or hydrochloric acid remove calcium salts from the bone tissue. This process softens the bone and allows researchers to study the cellular and structural changes during bone healing, such as the formation of new bone tissue, blood vessels, and the behaviour of different cell types involved in the healing process (Choi et al. 2015). This helps in understanding the mechanisms of bone repair and aids in developing better treatments for bone injuries and diseases.

Common agents used for bone decalcification include EDTA, hydrochloric acid (HCl), formic acid, nitric acid, etc. (Khangura et al. 2021). EDTA is a widely used chelating agent that binds calcium ions, effectively decalcifying bones while preserving tissue structures for histological analysis. It is gentle, preserving antigenicity for immunohistochemistry and enabling a wide range of stains. However, it might prolong the decalcification process (several weeks) and can cause tissue shrinkage (Chow et al. 2018). Strong acids like HCl offer quicker decalcification but can damage tissue structures, impacting the quality of histological sections (Lindner et al. 2020). Formic acid is faster than EDTA and less damaging than HCl but might compromise some stains (Bogoevski et al. 2019). Each agent has its advantages and limitations, demanding a balance between speed, preservation of tissue structures, and the desired analyses in bone decalcification for research or clinical purposes. The speed of decalcification and the impact of decalcifying agents on tissue and staining properties are crucial factors that affect the choice of decalcification solutions (Sanjai et al. 2012). Faster agents such as nitric acid can damage tissue, affecting staining techniques. Hence, it is crucial to limit the exposure of tissue to decalcifying solutions as much as possible (Bancroft and Gamble 2008).

Several factors can influence the decalcification process in bone tissue studies. The primary factors include the choice of decalcification agent, the size and thickness of the bone specimen, temperature, pH of the decalcifying solution, and agitation (Dey 2023). The type of decalcification agent used significantly affects the rate and quality of decalcification. Stronger acids work faster but can damage tissue structures, while milder agents like EDTA preserve tissue integrity better but may take longer (Khangura et al. 2021; Chow et al. 2018; Lindner et al. 2020). The size and thickness of the bone specimen impact decalcification time. Smaller and thinner sections tend to decalcify faster due to increased surface area exposure to the decalcifying solution (Chow et al. 2018). Temperature plays a role as higher temperatures can speed up the decalcification process but may also degrade tissue structures or antigens of interest. Maintaining the appropriate pH of the decalcifying solution is crucial for effective decalcification. pH extremes can affect the process and the quality of subsequent histological analysis (Kapila et al. 2015). Agitation or stirring of the decalcifying solution can enhance the process by ensuring a consistent distribution of the decalcifying agent around the bone specimen, thereby speeding up the decalcification process (Dey 2023).

Monitoring the endpoint of bone decalcification is critical to prevent tissue damage or inadequate decalcification. To ensure optimal outcomes, regular visual inspections under a microscope track changes in tissue transparency and texture (Skinner 2003). Periodic X-rays assess the reduction in bone radiopacity as mineral content decreases. Chemical tests, such as calcium detection assays, gauge residual calcium levels in the solution, guiding the decalcification endpoint. Additionally, monitoring tissue hardness through gentle probing helps assess the progression of decalcification. These combined approaches allow adjustments in decalcification duration, solution concentration, or agent choice, ensuring precise control and stopping at the ideal endpoint for subsequent histological analysis while preserving tissue integrity (Dey 2023).

This study established a standardized decalcification protocol for rat calvarial bone histology using formic acid-formalin solutions. Among the tested concentrations, 10% formic acid emerged as the

most effective, achieving thorough mineral content removal within 8 days while preserving the specimen's structural integrity and texture. This concentration consistently demonstrated the desired endpoint, as confirmed by daily chemical analyses, calcium oxalate tests, and radiographic imaging. Histological analyses, including standard and specialized staining, further supported the efficacy of the 10% formic acid concentration in maintaining tissue integrity without compromising sample structure. Statistical analysis underscored the significance of this concentration, establishing it as the optimal condition for efficient and effective decalcification. However, further study should investigate the impact of the 10% formic acid concentration on specific cellular or molecular markers related to bone healing or pathology.

Conclusion

The study effectively standardized a decalcification protocol for rat calvarial bone using formic acid-formalin solutions, with 10% formic acid proving the most effective, ensuring comprehensive mineral removal within eight days while preserving tissue integrity. This standardized protocol offers a reliable method for researchers studying rat calvarial bone histology, ensuring consistent and high-quality results. Additional research investigating its applicability to other bone types or species may broaden its scope and utility in diverse research endeavours.

Ethical approval

Not applicable.

Data statement

The authors confirm that the data supporting the findings of this study are available within the article.

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Declaration of Interest

All authors declare that no commercial or financial relationships exist that could, in any way, lead to a potential conflict of interest.

Authors' contribution

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

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AN ASSESSMENT OF PESTICIDE POISONING INCIDENCES PRESENTED AT HEALTH CARE FACILITIES IN MASHONALAND CENTRAL PROVINCE, ZIMBABWE

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KEYWORDS

HHPs

HCF

HCW

Poisoning

Incidence

ABSTRACT

Acute Pesticide Poisoning (APP) is a significant problem in developing countries, causing loss of productivity and fatalities in the agricultural sector. However, limited information on the connection between pesticide use, APP, and deaths in Zimbabwe is available. This study aimed to evaluate incidents that resulted in APP and identify the specific pesticides that caused them at Health Care Facilities (HCFs) in Mashonaland Central Province. Researchers conducted a survey asking standardized questions to gather information on APP cases from 93 HCFs. Descriptive statistics and chi-square association tests for APP cases in the targeted HCFs were calculated using IBM SPSS version 22. The study found that 43% of APP cases were due to pesticides belonging to the World Health Organization (WHO) class II acute toxicity category, while 26.1% were caused by Highly Hazardous Pesticides (HHPs). Most of the cases were due to intentional poisoning (87.1%), with the majority (55.9%) of APP incidents being males. The highest APP cases were recorded in the 21-30 age group (38.8%), followed by the 31-40 age group (32.8%). These results emphasize the significant impact of intentional poisoning by WHO class II type pesticides, particularly HHPs, on the incidence of APP in Zimbabwe. To mitigate the impact of HHPs on human health, it is recommended that the government of Zimbabwe consider pesticide risk reduction measures, such as stricter pesticide registration criteria, import

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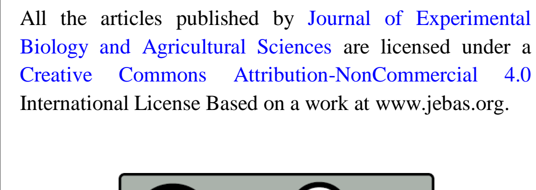

restrictions, and the promotion of less toxic alternatives. These findings highlight the urgent need for policymakers, researchers, and other stakeholders in the agricultural sector to work collaboratively towards creating a safer and more sustainable farming environment in Zimbabwe.

1 Introduction

Using pesticides in modern agriculture has greatly increased food production and security (Zimba and Zimudzi 2016; Tudi et al. 2021). However, the inappropriate handling and misuse of these chemicals have harmed human health and the environment (Ntzani et al. 2013; Pathak et al. 2022). The number of pesticide poisonings in farms worldwide has increased significantly from around 25 million cases in 1990 to 385 million in 2018 (Boedeker et al. 2020). Alarmingly, approximately 44% of the global population working in agriculture, including 860 million farmers and agricultural workers, are exposed to pesticide poisoning each year (Boedeker et al. 2020). Highly Hazardous Pesticides (HHPs), a small group of pesticides classified as acutely or chronically hazardous to human health or the environment, are responsible for most of these poisonings. HHPs are identified based on the Food and Agriculture Organisation of the United Nations (FAO)/World Health Organisation (WHO) Joint Meeting on Pesticide Management's (JMPM) 8-point criteria for HHPs identification (FAO/WHO 2016). These statistics emphasize workers' significant risks in the agricultural industry and the urgent need to prioritize implementing adequate safety measures to protect them. The increasing evidence about the harmful effects of pesticides underscores the importance of adopting alternative methods of agriculture that reduce the reliance on these chemicals.

It's important to understand that pesticide poisoning cases can arise from accidental mishaps and intentional acts. While accidental poisoning accounts for a smaller percentage of deaths related to pesticide exposure (Jørs et al. 2018; Eizadi-Mood et al. 2023), intentional self-poisoning unfortunately accounts for most of the cases. In fact, it makes up at least one in seven suicides worldwide (Mew et al. 2017; Eizadi-Mood et al. 2023). Despite this, the true extent of the problem and the global distribution of deaths are not accurately reported. It's worth noting that low- and middle-income countries in sub-Saharan Africa account for 3.5% of global pesticide-related self-poisoning fatalities (Mew et al. 2017).

It is difficult to obtain accurate data on pesticide poisoning cases in Africa due to inadequate notification records maintained by health authorities (Rao et al. 2005; Brassell et al. 2022). Underreporting incidents in health facilities is also a significant issue, as many country-specific reporting systems lack a central reporting point or legal mechanism requiring incident reporting (Boedeker et al. 2020). Studies indicate that the epidemiology of poisoning in Kenya, Malawi, South Africa, and Zimbabwe, as well as in other African countries, is not well established (Tagwireyi et al. 2016; Brassell et al. 2022). According to the WHO, unintentional poisoning caused eight deaths per 100,000 people in Zimbabwe in 2004 (Tagwireyi et al. 2016).

Toxic vigilance is crucial in hospitals as it provides critical epidemiological information to design targeted interventions (Razwiedani and Rautenbach 2017). Unfortunately, most healthcare workers (HCWs) in sub-Saharan African countries lack adequate training in occupational health and toxicology, which limits their ability to diagnose signs and symptoms of acute pesticide poisoning (APP) or identify the appropriate chemical groups and WHO pesticide hazard class (Lekei et al. 2017; Ssemugabo et al. 2020). As a result, diagnosing and managing pesticide poisoning cases is challenging and often leads to poor patient outcomes (Sibani et al. 2017; Ssemugabo et al. 2020). Inadequate diagnosis and management also impede epidemiological data collection, which is essential for identifying problematic pesticides that need regulation.

Zimbabwe's agricultural sector significantly contributes to the country's economy, accounting for 14% of the Gross Domestic Product (GDP) and employing 70% of its population, directly or indirectly. Smallholder farmers, who own 50% of the country's land, play a vital role in agricultural production (Kuhudzayi and Mattos 2018). However, farmers in low and middle-income countries, such as Zimbabwe, face a high risk of adverse health effects due to pesticide exposure (Jørs et al. 2018). Despite the risks associated with pesticide use, there is an increase in farmers' dependence on pesticides in Zimbabwe (Maumbe and Swinton 2003; Foti and Chikuvire 2005; Mutami 2015). Unfortunately, Zimbabwe lacks national data on the incidence of APP or the pesticides that cause deaths, which is crucial for developing effective regulations for problematic pesticides. Therefore, this study aims to characterize the incidents and severity of pesticide poisoning cases presented to healthcare facilities (HCFs) in Mashonaland Central, Zimbabwe. It also aims to identify the pesticide formulations commonly associated with pesticide poisoning and determine the events that lead to pesticide poisoning incidences.

2 Materials and Methods

2.1 Study setting and design

This research aims to investigate the occurrence and treatment of Acute Pesticide Poisoning (APP) by Healthcare Workers (HCWs) in seven districts, namely Bindura, Muzarabani, Guruve, Mazowe, Rushinga, Shamva, and Mount Darwin of Mashonaland Central Province in Zimbabwe. The study used a cross-sectional design to

collect qualitative and quantitative data by distributing questionnaires to HCWs handling APP cases. The province was selected based on its high agricultural production statistics, which suggest high pesticide usage. A total of 93 healthcare facilities were visited out of 140 identified using the District Health Information Software (DHIS) due to limited accessibility, time, and resources. 99 HCWs were interviewed, with six of the health care facilities having two respondents per facility instead of one. Their responses provided valuable insights into the management of APP among healthcare workers in the region.

2.2 Data collection

In this study, a semi-structured questionnaire was used to collect data on Acute Pesticide Poisoning (APP) incidences. To ensure the questionnaire's effectiveness and accuracy, we pilot-tested it in July 2021 with a small sample of 10 healthcare workers (HCWs) from selected facilities in Mashonaland Central. Based on the pilot test results, some necessary refinements were made to the questionnaire to align it with the study's objectives. The questionnaire was administered at the Outpatient Department (OPD) of health facilities, where most APP incidences are received and recorded. In some cases, it was administered in the records section of the health facility. We primarily interviewed Doctors, Nurses, and Environmental Health Technicians who directly provide care to patients suffering from APP. Eight (8) enumerators were trained for two days on administering the questionnaire and the study's objectives. The data collection process took place in September 2021.

The questionnaire gathered data on respondents' demographics, such as their age, gender, profession, and experience, as well as data on the number of pesticide poisoning incidents recorded, events leading to pesticide poisoning, the outcome of the poisoning, the pesticides responsible for acute poisoning, the pesticide poisoning record-keeping system, and healthcare workers' training on APP management. While the data collected using semi-structured questionnaires were primarily quantitative, we also collected qualitative data through further probing HCWs with open-ended and follow-up questions.

2.3 Statistical analysis

Quantitative data were collected and organized in Microsoft Excel. The collected data were coded and verified to eliminate errors, and IBM SPSS version 22 was used to calculate frequency, relative frequency, and chi-square (v2) tests for association at a 5% significance level. Qualitative data were organized thematically and then triangulated with quantitative data to validate it. This approach ensured the reliability and accuracy of our study findings. The pesticides responsible for APP were classified according to the WHO Hazard Classifications and functional groups. Descriptive statistics were used to analyze the data (WHO 2020).

2.4 Ethical considerations

The study protocol was approved by both the Medical Research Council of Zimbabwe (MRCZ) and the Ministry of Health and Child Care. The study adhered to the Covid-19 prevention and containment measures recommended by the Government of Zimbabwe and the World Health Organization (WHO). Healthcare workers (HCWs) were informed about the study's aim and were given the option to participate or decline without any negative consequences. Oral and written consent was obtained from the participants after that. The confidentiality of the HCWs was maintained, and their information was kept anonymous.

3 Results

3.1 Demographic characteristics of Healthcare Facilities visited

As part of this study, 93 healthcare facilities (HCFs), including rural health centres, clinics, private hospitals, provincial referral hospitals, district hospitals, rural hospitals, and private surgeries were interviewed. The results related to these participants are mentioned in Table 1. During the study, a total of 93 healthcare facilities were visited, on six of the facilities two respondents per facility were interviewed, resulting in 99 healthcare workers being interviewed (Table 2). Out of all the participants who responded, 85.9% were nurses, 7.1% were Environmental Health Technicians, and 5% were doctors. The largest group of respondents (38.4%) had 11-15 years of work experience, followed by those with 1-5 years of experience (28.3%) (Table 2). However, none of the participants had received on-the-job refresher training on APP management.

3.2 Socio-demographic characteristics of APP cases

Out of the 99 respondents interviewed, 22 APP cases were excluded from the data analysis as they lacked information on the circumstances that led to the APP incident. Among the remaining 77 APP cases, most individuals affected (55.9%: n=77) were males, while only 44.1% were females. The association between gender and poisoning was significant ($p<0.05$). In cases of intentional poisoning, males (48.1%) were affected more than females (39%). In terms of age, the highest number of intentional poisoning cases (38.8%: n=67) were recorded in the 21-30 age range, followed by the 31-40 age range (32.8%). The association between age and poisoning was also significant ($p<0.05$) (Table 3).

3.3 Pesticides responsible for acute poisoning cases- incidences presented at HCFs

The studied Healthcare Facilities (HCFs) reported poisoning cases caused by 16 pesticides. The most frequent causes of poisoning were Dimethoate (14.3%), Lambda Cyhalothrin (14.3%), Aldicarb (13%), Aluminium Phosphide (13%), and unknown substances (15.3%).

Table 1 Health Care Facilities visited during the study (N=93)

Category	Owner	HCFs Interviewed
Clinic	Municipality	2
Clinic	Government	10
Clinic	Rural District Council	10
District Hospital	Government	5
Mission Hospital	Private/Church related	4
Private Hospital	Private	1
Private Surgery	Private	11
Provincial Hospital	Government	1
Rural Health Center	Rural District Council	26
Rural Health Center	Government	21
Rural Hospital	Government	2
Total Respondents		93

Table 2 Health Care Workers interviewed and years of experience (N=99)

Health Care Workers interviewed	Frequency (N)	Percentage (%)
Doctor	5	5.0
Nurse	85	85.9
Environmental Health Technician	7	7.1
Information Officer	1	1.0
Clinic Officer	1	1.0
Respondents' working experience in years		
1-5	28	28.3
6-10	16	16.1
11-15	38	38.4
16-20	9	9.1
20+	8	8.1

Table 3 Pesticide poisoning cases categorized by age and gender (n=77)

Age	Intentional poisoning		Unintentional poisoning	
	Frequency (n=67)	Percentage	Frequency (n=10)	Percentage
Less than 20	8	11.9	5	50
21- 30	26	38.8	0	0
31 – 40	22	32.8	4	40
41 – 50	4	6.0	1	10
51 – 60	2	3.0	0	0
61 – 70	1	1.5	0	0
Over 70	4	6.0	0	0
Gender				
Male	37	55.2	6	60
Female	30	44.8	4	40

Table 4 Pesticides reported to be responsible for acute poisoning in the case studies (n=77)

Pesticide Name	Pesticide functional group	WHO Hazard Chemicals Classification	Respondents (%)
Lambda Cyhalothrin	Pyrethroid	II	14.3
Fenvalerate	Pyrethroid	II	1.3
Aldicarb[a]	Carbamate	1a	13
Carbaryl	Carbamate	II	2.6
Aluminium Phosphide	Inorganic compound	FM	13
Imidacloprid+ Beta-cyfluthrin	Neonicotinoid+ Pyrethroid	1b[b]	1.3
Lambda Cyhalothrin + Acetamiprid	Neonicotinoid+ Pyrethroid	II	2.6
Paraquat[a]	Bipyridyl	II	4
Copper Oxychloride	Inorganic compound	II	1.3
Dimethoate	Organophosphate	II	14.3
Methamidophos[a]	Organophosphate	1b	6.5
Chlorpyrifos	Organophosphate	II	1.3
Dichlovos[a]	Organophosphate	1b	1.3
Glyphosate	N-Glycine	III	4
Triadimenol[a]	Triazole	II	1.3
N-Decanol	Fatty Alcohol	U	2.6
Not known	Not known	Not known	15.3

[a]Highly Hazardous Pesticide; [b]Based upon the more toxic active ingredient; FM-Fumigant, Fatal if inhaled

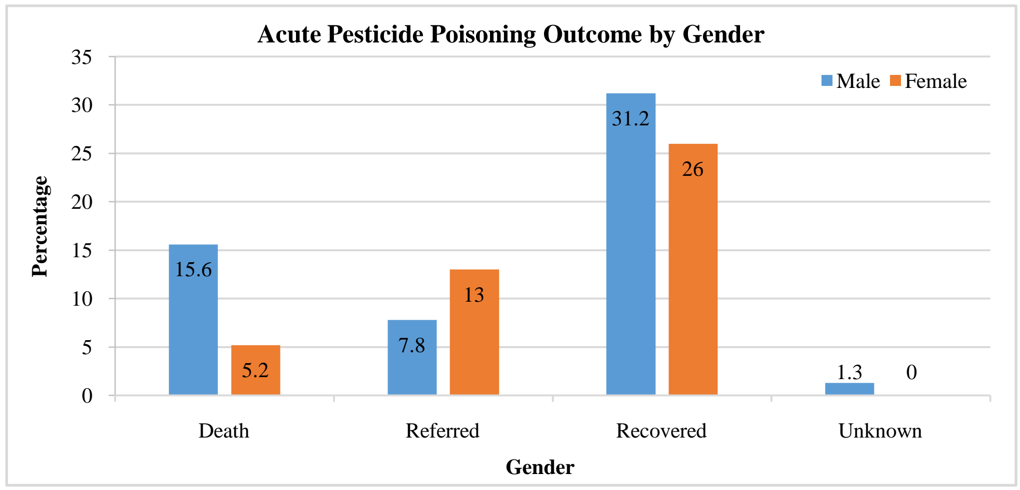

Figure 1 Acute Pesticide Poisoning Outcome by Gender

Other pesticides like Methamidophos (6.5%), Glyphosate (3.9%), and Paraquat (3.9%) had frequencies of less than 10%. Among all the cases, 26.1% (n=77) were caused by highly hazardous pesticides (HHPs), as mentioned in Table 4. The pesticides responsible for poisoning belonged to different functional groups, including organophosphates (25%), carbamates (12.5%), pyrethroids (12.5%), neonicotinoids (12.5%), and inorganic compounds (12.5%). Despite the banning of Aldicarb, Paraquat, and Methamidophos in Zimbabwe, these pesticides were still found to be responsible for some of the poisoning cases.

3.4 Outcomes of APP incidences presented at HCFs

Out of all the recorded cases of APP, 56.1% of them recovered from their illness, while 21.8% of them unfortunately passed away. About 20.8% of the patients were referred to another healthcare provider, and the status of the remaining 1.3% is unknown (Table 5).

A significant proportion (15.6%: n=77) of deaths were recorded in males, while 5.2% were females (Figure 1). According to the recorded data, the highest mortality rate of 10.8% was caused by

Table 5 Poisoning outcome by pesticide recorded in Mashonaland Central Province (n=77)

Pesticide name	% Mortality	% Recovered	% Transferred	% Missing data
Aldicarb	2.7	7.8	2.5	0
Aluminium Phosphide	10.8	0	2.2	0
Lambda Cyhalothrin+ Acetamiprid	0	2.6	0	0
Carbaryl	1.4	1.3	0	0
Chlorpyrifos	0	0	0	1.3
Copper oxychloride	0	1.3	0	0
Dichlorvos	0	1.3	0	0
Dimethoate	0	7.8	6.5	0
Fenvalerate	0	1.3	0	0
Glyphosate	0	2.6	1.4	0
Lambda Cyhalothrin	2.7	10.3	1.2	0
N-Decanol	0	2.6	0	0
Paraquat	1.4	0	2.6	0
Methamidophos	1.4	3.9	1.2	0
Imidacloprid+ beta-cyfluthrin	0	1.3	0	0
Triadimenol	0	1.3	0	0
Not Known	1.4	10.4	3.5	0
Total	21.8	55.8	21.1	1.3

Aluminium phosphide poisoning, followed by Lambda Cyhalothrin (2.7%) and Aldicarb (2.7%). On the other hand, Paraquat, Methamidophos, and Carbaryl had a mortality rate of 1.4% each. Lambda Cyhalothrin recorded the highest percentage of recovered cases (10.3%), followed by Aldicarb and Dimethoate, which recorded (7.8%) each. In addition, 10.4% of APP cases recovered from unknown pesticides, and 1.3% of the poisoning cases recorded had unknown outcomes because of missing information. Dimethoate had the highest number of referred cases (6.5%) recorded during the survey.

4 Discussion

According to the demographic information, most healthcare workers (HCWs) have more than ten years of experience, but none have received any on-the-job training on managing Acute Pesticide Poisoning (APP). Although the HCWs have vast working experience and have handled many APP cases, the lack of refresher training has resulted in gaps in their knowledge of APP management. A study conducted on HCWs with working experience ranging from 1 to 24 years in Tanzania revealed that they had poor knowledge of pesticide poisoning management and were unfamiliar with the adverse health effects of pesticides (Lekei et al. 2017). Knowing the general pattern of APP is crucial, which can lead to early diagnosis and control of poisoning, resulting in reduced morbidity and mortality rates (Koulapur et al. 2015).

According to recent data, most cases (87%) of APP (Acute Pesticide Poisoning) reported in Zimbabwean Health Care Facilities (HCFs) are caused by intentional poisoning. In contrast, a previous study conducted in Zimbabwe over two years suggested a lower figure of 32% due to intentional poisoning (Tagwireyi et al. 2002). The difference between these two studies can be attributed to the increased use of pesticides in Zimbabwe during the last two decades (Zinyemba et al. 2021). Several studies in Low to LMIC countries like Iran, Sri Lanka and India have reported that the intentional use of pesticides for poisoning is a significant challenge associated with pesticide use (Jesslin et al. 2010; Razwiedani and Rautenbach 2017; Noghrehchi et al. 2022; Chan et al. 2023). It has been observed that intentional pesticide poisoning is the cause of one in five suicides worldwide (FAO/WHO 2019). This has led to a global call to reduce and eliminate highly hazardous pesticides and replace them with lower-risk alternatives (Soko 2018; Chan et al. 2023; Ter Horst et al. 2023). It is noted that while a study conducted in Uganda found that 54.7% of all APP cases were caused by non-intentional poisoning, this study discovered that only 13% of cases were due to unintentional poisoning (Sekabojja et al. 2020). However, it is

important to consider that the official records of APP may not be entirely accurate as many cases in small communities, farms, and homes go unreported. Farmers who experience mild to moderate pesticide poisoning symptoms often do not seek medical attention unless the condition is severe or life-threatening (Ssemugabo et al. 2017; Tessema et al. 2022). This means the actual number of APP cases is likely higher than officially reported.

The 21-30 age group had the majority (38.8%) of intentional poisoning cases, followed by the 31-40 age group (32.8%). The findings of this study confirmed the WHO, which suggested that pesticides are the most common method of suicide worldwide, and the age group of 15-29 is the most vulnerable (WHO 2022). In another study in Zimbabwe, 42% of intentional pesticide poisoning cases were found to occur in the 21-30 age range (Kasilo et al. 1991). The findings where most APP poisoning incidences were in the 19-29 age range were reported several times in the literature (Jesslin et al. 2010; Pedersen et al. 2017; Samaria et al. 2024; Teym et al. 2024). Domestic disputes, especially among married couples, have also been reported to cause the occurrence of intentional poisoning cases for this age group (Tagwireyi et al. 2002; Chan et al. 2023). Other reasons have also been reported as a result of problems in family, studies, life settlement and employment, which result in stress and may make them attempt suicide (Jesslin et al. 2010; Samaria et al. 2024).

The results of the current study, in which 72.7% of those poisoned were under the age of 40 years, corroborated with a study in South Africa by Razwiedani and Rautenbach (2017), where 79.8% of those poisoned by organophosphates were under the age of 40. Most of the APP incidences were males, and most of them were intentionally poisoned. The incidences of APP by males being much more than by females have been reported in many studies (Tagwireyi et al. 2002; Razwiedani and Rautenbach 2017; Sekabojja et al. 2020; Chan et al. 2023; Samaria et al. 2024). The high number of male cases of poisoning by pesticides has been attributed to the majority of them being engaged in rigorous agricultural works such as spraying and other non-farm related risks of exposure such as stress, family overload and domestic violence (Noghrehchi et al. 2022; Eizadi-Mood et al. 2023). Males also select more lethal methods for deliberate self-harm; they are less likely to seek help for depression and also express their depression differently to women. This results in men more likely to behave impulsively (including suicide) and less likely to be diagnosed and effectively treated (Mergl et al. 2015; Freeman et al. 2017; Eizadi-Mood et al., 2023).

According to a report by Slabbert and Smith (2011), some cases of Acute Pesticide Poisoning (APP) were transferred to referral hospitals, even though these hospitals have a better capacity to handle APP. Referrals to secondary hospitals are made when patients require special care, surgery, high or intensive care management, and remarkable investigations unavailable at the referring facility (Slabbert and Smith 2011; Pedersen et al. 2017).

According to a study, most poisoning cases were caused by WHO class II pesticides, namely Lambda Cyhalothrin and Dimethoate, followed by WHO class 1a Aldicarb and an unclassified highly toxic Fumigant Aluminium Phosphide. These products are categorized as extremely hazardous (1a), highly hazardous (1b) or moderately hazardous (II), and their association with poisoning at the HCF is consistent with their acutely toxic characteristics (World Health Organization 2020; Waktola et al. 2023; Samaria et al. 2024). A similar outcome was reported by HCWs in northern Tanzania, where 71% of the pesticides causing poisoning were WHO class II (Lekei et al. 2017).

It has been reported that in Kenya, the majority of the cases of acute poisoning among healthcare workers were caused by Dimethoate and Lambda Cyhalothrin (Marete et al. 2021). Studies conducted in India have suggested that organophosphates are the primary cause of most Acute Pesticide Poisoning (APP) cases, followed by Pyrethroids (Samaria et al. 2024; Hurtado et al. 2024). Lambda Cyhalothrin is responsible for most poisoning cases in Asia, Africa, and Latin America. Dimethoate has been listed among the top five pesticides responsible for acute poisoning in Zimbabwe between 1970 and 1990 (Nhari 1996). The Rotterdam Convention, Multilateral Environmental Agreement has categorized Lambda Cyhalothrin 5EC and Dimethoate 40EC formulations as Severely Hazardous Formulations due to APP incidents (Rotterdam Convention 2024). As they are cheap and readily available, Lambda Cyhalothrin and Dimethoate are used extensively in the cultivation of various crops in the region and, thus, are the most likely cause of APP.

Aluminium phosphorus poisoning was responsible for most APP cases presented at the HCFs. It has also been reported to be responsible for the majority (over 80%) of deliberate acute poisoning and deaths in Iran and Ethiopia (Navabi et al. 2018; Dorooshi et al. 2021; Waktola et al. 2023). In India, acute APP with Aluminium Phosphide is ubiquitous (68%) and has up to 60% mortality rates (Sarkar et al. 2022). It emerged as a poison of suicidal deaths as it has no effective antidote, is cheap, freely available, and is a 'sure agent of death (Yatendra et al. 2014).

According to a study, Aldicarb was associated with low mortality rates despite being classified as WHO class 1a due to its high acute toxicity. Lambda-cyhalothrin had the highest recovery rate but moderate mortality rates, possibly because mammals quickly metabolize pyrethroids to non-toxic metabolites (Bradberry et al. 2005). Paraquat and Dimethoate were reported to be the most acutely toxic pesticides responsible for deaths from 2002 to 2019 in Sri Lanka (Manuweera et al. 2008; Buckley et al. 2021). Paraquat poisoning has been found to cause many cases of acute

pesticide poisoning, with most outcomes being fatal. After the product ban, the use of Paraquat for acute pesticide poisoning also significantly decreased, with an overall reduction in case fatality (Kim et al. 2017; Chan et al. 2023). Deliberate self-poisoning through the ingestion of Paraquat is a significant cause of illness and mortality in the Asia-Pacific region (Tajai and Kornjirakasemsan 2023).

Mashonaland Central is a region known for intensive farming. As a result, pesticides are easily accessible to farmers. The choice of pesticides farmers use is based on availability, capital, and the range of crops grown in the area (Oesterlund et al. 2014; Bar et al. 2022). Some of the banned pesticides in Zimbabwe, such as Aldicarb, Paraquat and Methamidophos, have still been detected in the area, suggesting that they may have been smuggled into the country. However, the issue of banned pesticides is not unique to Zimbabwe, as it has also been reported in other countries such as Malawi, Kenya, Morocco, Brazil, Taiwan, Costa Rica, Nicaragua, India and Kazakhstan. Illegal pesticides often enter these countries through porous borders, making it difficult to control their circulation (Loha et al. 2018; Kosamu et al. 2020; Benaboud et al. 2021; Bayoumi 2022). Developed countries are also not spared from the illegal pesticide trade. In the first four months of 2020, illegal pesticides worth 94 million were seized in the EU, USA, and Colombia (Bar et al. 2022).

Conclusions and Recommendations

In this study, we analyzed the demographic characteristics of the healthcare facilities visited to identify gaps in knowledge regarding Acute Pesticide Poisoning (APP) management. The results showed that most respondents were nurses (85.9%) with over ten years of experience. However, none of them had received any on-the-job refresher training on APP management, indicating a need for training programs to bridge the knowledge gap. Intentional poisoning was identified as the leading cause of APP cases, with males representing the majority of cases (48.1%). The 21-30 age range had the highest percentage (38.8%) of intentional poisoning cases. The study identified the pesticides responsible for most poisoning cases, including Lambda Cyhalothrin, Dimethoate, Aldicarb, Aluminium Phosphide, and unknown. The highest number of deaths were reported to be as a result of Aluminium Phosphide poisoning. The outcomes resulting from APP were noted as death (21.8%), recovery (56.1%), patient transferred to a referral hospital (20.8%), and unknown (1.3%). The study recommends that healthcare professionals undergo on-the-job refresher training on APP management. Moreover, the government should regulate the use of highly toxic pesticides, including banning highly hazardous ones and replacing them with less toxic alternatives. Finally, there is a need for more research to evaluate the effectiveness of current APP management strategies to improve outcomes for APP cases.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Potential effect of fruit and flower extracts of *Arbutus unedo* L. on *Tetrahymena pyriformis* exposed to a cobalt-60 source

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Tetrahymena pyriformis

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Antioxidant

ABSTRACT

Exposure of *Tetrahymena pyriformis* cultures to cobalt-60 for 72 h significantly impacted the cells' growth, appearance, and physiology. This study aims to investigate the protective effects of *Arbutus unedo L* flowers and fruit extracts on *T. pyriformis* against gamma radiation. Initially, aqueous and 50% ethanolic extracts of the fruits and flowers were prepared, and their cytotoxicity on the ciliate was evaluated. The irradiated ciliate's cellular viability and morphological aspect improved when a non-toxic concentration of 25 µg/mL was added to the growth medium. The addition of extracts restored glyceraldehyde-3-phosphate dehydrogenase and succinate dehydrogenase activities to their initial levels, similar to non-irradiated cells. In addition, the extracts reduced oxidative stress markers, such as lipid peroxidation, and decreased the activities of antioxidant defence enzymes, catalase, and superoxide dismutase. This may be attributed to the antioxidant properties of the extracts. Results of this study revealed that the flower extracts exhibited better protective effects than the fruit extracts, with superior antioxidant activity in the in-vitro DPPH scavenging assay. These results suggest that *A. unedo* flower extracts may have potential as exogenous radioprotective agents.

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1 Introduction

The use of plant extracts for preventive and therapeutic purposes has attracted increasing research interest in recent years, as extracts are composed of complexes of plant metabolites, and their benefits have been recognized (Başaran et al. 2022). *Arbutus* species (Ericaceae) are traditionally used to treat various diseases and are known for their antioxidant, antiseptic, antidiabetic, diuretic, anti-inflammatory, and other properties (Mrabti et al. 2021). The strawberry tree (*Arbutus unedo* L.) is a small tree of the Ericaceae family native to the Mediterranean region that prefers regions with warm summers and temperate winters and is found in Europe (Portugal, Spain, France, Italy etc.), the Canary Islands, North Africa (Morocco, Algeria, Tunisia), and Western Asia (De Santis et al. 2023). *A. unedo* is a shrub with high resistance to difficult climatic and soil conditions, with rapid regeneration after forest fires, which is particularly important for faunal diversity and prevention of soil erosion (Helluy et al. 2021). The red and edible fruits of the strawberry tree, which generally coexist with the pinkish-white flowers that appear in winter, are part of the Mediterranean diet (Sanna et al. 2023). The plant's fruit has been used in several countries for therapeutic purposes in traditional medicine. In Morocco, different parts (fruit, root, leaves, and flowers) of *A. unedo*, also known as "sasnou", are often used traditionally to treat hypertension, diabetes, heart disease, high cholesterol, digestive problems, and as a diuretic, antidiarrheal, and anti-inflammatory (El Haouari et al. 2021). Because of its nutritional and medicinal values, several studies have investigated the composition of different extracts of *A. unedo*. The plant is an important source of phytochemicals, bioactive compounds (phenols, anthocyanins, triterpenes, iridoids, sterols), vitamins, minerals, essential fatty acids, and dietary fiber, which gives it interesting biological activities such as antioxidant, anti-inflammatory, antimicrobial and other activities (Wahabi et al. 2023).

Extracts of this plant are considered an important source of antioxidants, flavonoids, acid derivatives, anthocyanins, carotenoids, vitamins, etc. (Bajoub et al. 2023); it would be interesting to explore these properties against the prevention of reactive oxygen species (ROS) generated indirectly by ionizing radiation (IR). In fact, IR such as gamma radiation contributes indirectly, through the radiolysis of water, to the appearance of ROS such as superoxide ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet}$), hydrogen radicals ($H^{\bullet}$), and hydrogen peroxide (H_2O_2) (Şolpan et al. 2022). An increase of these free radicals causes a cellular imbalance, leading to the appearance of oxidative stress by altering the activities of enzymes, membrane structure, and DNA, and consequently, the development of various pathologies (Ponnampalam et al. 2022; Pardillo-Díaz et al. 2022; Sadiq 2023).

Previous work has shown the damage caused by ROS generated after exposure to gamma radiation on a ciliated protozoan *Tetrahymena pyriformis* (Ziyadi et al. 2022b). The use of this ciliate as a model in various studies presented several advantages, such as being unicellular with a short life cycle, easy to culture under laboratory conditions, and the possibility of analyzing the toxic effects of substances over several generations (Lim 2022; Sabraoui et al. 2022). Previous study showed that IR affected the growth, morphology, and activities of metabolic enzymes, increased the production of ROS and their damage, and activated the antioxidant system of the protozoa after 72 h of exposure to a radioactive source of cobalt-60 (^{60}Co)(Ziyadi et al. 2022b).

In this context, protection against these IR-induced cellular perturbations by evaluating the radioprotective and antioxidant effects of natural plants known for their therapeutic virtues will be an interesting area of research (Ziyadi et al. 2022a). Therefore, this study aimed to evaluate the ability of aqueous and ethanolic extracts of the two plant parts of *A. unedo* (flowers and fruits) to reduce the effects observed in *T. pyriformis* after exposure to a ^{60}Co source. The study included growth (number and time of generation), morphological aspect, metabolic enzymes (glyceraldehyde-3-phosphate dehydrogenase-GAPDH and succinate dehydrogenase-SDH), as well as oxidative markers such as lipid peroxidation and antioxidant enzymes (catalase-CAT and superoxide dismutase-SOD).

2 Material and methods

2.1 Plant harvest and extract preparation

The flowers and the fruits of the strawberry tree (*A. unedo*) were collected in January 2022 (the tree produces flowers at the same time as the fruit ripens) from the "Forest of El Harcha" site in the "Rabat-Salé-Kénitra" region, Morocco (33°28'31.296" N latitude and 6°8'50.568" O longitude). Samples were immediately frozen at -20°C and then were dried using a lyophilization system (Labconco). Lyophilized samples were ground to a fine powder. The aqueous extract was obtained by decoction under reflux (30 min) of 25 g of powder with 250 mL of HPLC-grade water. After filtration and evaporation at 60°C, the extract obtained was frozen and finally dried by lyophilization. The 50% ethanolic extract was obtained by hydroalcoholic maceration (5 g of lyophilized powder /100 mL (w/v)) for 48 h. The mixture was filtered and evaporated at 65°C, dried through a desiccator, and stored at -20°C.

2.2 Antioxidant activity

The antioxidant activity of the extracts was evaluated according to the DPPH (2,2-diphenyl-1-picrylhydrazyl) test described by Tenuta et al. (2020). The *A. unedo* extracts were dissolved in methanol to obtain 25, 50, 100, 200, and 300 µg/mL concentrations. Subsequently, 200 µL of each prepared concentration was added to 800 µL of 100 µM DPPH solution. The mixture was incubated for 30 min in the dark at 25°C, and the

absorbances were determined by spectrophotometry at 517 nm. The percentage of antioxidant activity was calculated as $\% AA = \frac{(A0-A1)}{A0} \times 100$, where A0 is the absorbance of the control (methanol) and A1 is the absorbance of the extract. Plotting the curve of % AA as a function of extract concentration allowed the determination of the EC_{50} (concentration to obtain a 50% antioxidant effect).

2.3 Growth conditions of *T. pyriformis*

The culture medium for the ciliated protozoan *T. pyriformis* ATCC 30005 strain E was a PPYE medium consisting of 0.6% proteose peptone and 0.1% yeast extract (Rodrigues-Pousada et al. 1979). Culture was performed under aerobic conditions at 28°C for 72 h without shaking. Cell viability and morphology were assessed by optical microscope (Optika B-600Ti). Viability was estimated by counting the number of cells/mL using the Malassez counting chamber (0.0025 mm^2, 0.2 mm deep) after fixation with 10% glutaraldehyde in the PBS buffer at pH 7. Generation number (n) and generation time (g) were estimated according to Ziyadi et al. (2022b). The generation number was calculated using the formula: $n = \frac{\log N_1 - \log N_0}{\log 2}$, where N_1 is the cell number at 72 h and N_0 is the cell number at T_0. The generation time was calculated as: $g = \frac{\text{time of growth}}{n}$. For morphology, photographs of cells in a microscope slide were taken with a camera (Optika M-699), and the ratio shortest (W)/longest (L) axis of the cell was calculated using Optika Vision 3.4 software.

2.4 Cytotoxicity of *A.unedo* extracts

The influence of aqueous and ethanolic extract of *A. unedo* fruits and flowers on the viability of *T. pyriformis* was evaluated at different concentrations of extracts ranging from (2 to 1000 µg/mL). The concentrations of extracts were prepared in 10 mL tubes of PPYE medium and inoculated with fresh pre-culture of the protozoa. Cells were counted in the exponential phase (72h). The probit analysis (Bliss 1935) determined the IC_{50} (inhibitory concentration at 50%).

2.5 Gamma exposure of cultures

The protozoan cultures (100 mL) were exposed to gamma radiation using a ^{60}Co source (North American Scientific, Chatsworth, CA) at 20 cGy/h for 72 h according to the protocol described by Ziyadi et al. (2022b). This exposure was performed in the absence of plant extracts (to evaluate radiation damage on *T. pyriformis*) and in the presence of extracts (to evaluate their protective effect on *T. pyriformis*).

2.6 Preparation of crude cell-free extract

T. pyriformis cells were harvested by centrifugation (5000 × g for 15 min at 4°C), washed twice with 20 mM Tris-HCl buffer (pH 7.5) and resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 2 m Methylenediamine tetraacetic acid (EDTA), 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol and 1% (v/v) glycerol at a rate of 3 mL/g cell weight. The suspension was sonicated using a Bandelin Sonopuls sonicator (40 s, 90%, 10×) in an ice bath with 1 min rest between each sonication cycle, followed by centrifugation at 15,000 × g for 45 min at 4°C to obtain a soluble protein fraction. The Bradford method (Bradford 1976) with BSA as a standard was used to determine the protein concentration of the crude cell-free extract.

2.7 Lipid peroxidation assessment

Lipid peroxidation was estimated as described by Samokyszyn and Marnett (1990) by mixing 1 mL of the crude cell-free extract and 1 mL of thiobarbituric acid (TBA) prepared in 0.25 M HCl with 30% trichloroacetic acid. After incubation at 100°C for 15 min, the reaction was stopped by introduction into an ice bath and centrifuged at 1000 × g for 10 min. In this reaction, TBA reacts with the peroxidation product (malondialdehyde (MDA)) to form thiobarbituric acid reactive substances (TBARS). TBARS were measured by a spectrophotometric reading of the supernatant at 535 nm and converted to nmoles MDA/mg protein.

2.8 Enzymes activities

The activities of GAPDH, SDH, CAT, and SOD were measured by monitoring the reaction products with a spectrophotometer, and the conditions of the reactions are grouped in Table 1. The activities of the enzymes were expressed in µmole/min/mg of protein using the extinction coefficient of each substrate.

2.9 Statistical analysis

All assays were performed in three different experiments, and results were determined as mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA analysis and Tukey's multiple comparison test using IBM SPSS Statistics (version 25). The 5% significance level was used.

3 Results

3.1 Extraction and cytotoxicity of extracts on *T. pyriformis*

Aqueous and 50% ethanol extractions were performed using freeze-dried fruits and flowers of the strawberry tree. The aqueous extract yielded 33.88 and 33% for fruits and flowers, respectively. In contrast, the yield of ethanolic extract was 63.4 and 46.6% for flowers and fruits, respectively. As a working solution, the dry extracts were taken up in distilled water at a 20 mg/mL rate.

The effect of aqueous and ethanolic extracts of fruits and flowers of *A. unedo* on the growth of *T. pyriformis* was studied by

Table 1 Enzymatic reaction conditions for GAPDH, SDH, CAT, and SOD

Enzyme	Reaction mixture	Absorbance length	According to
GAPDH	10 mM sodium arsenate, 1 mM NAD^+, 1 mM D-G3P in 50 mM tricine buffer (pH 8.5). The reaction is started by 10 µL of the crude extract.	340 nm	Iddar et al. 2002
SDH	0.053 mM dichloroindophenol, 0.3 mM EDTA, and 50 µg of protein in 100 mM potassium phosphate buffer (pH 7.4). The reaction starts with 50 µL of KCN Succinate (3.25 mg/mL of KCN in 0.5 M succinate).	625 nm	King 1967
CAT	7 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.5). The reaction is started by 10 µL of the crude extract.	240 nm	Aebi 1984
SOD	3.5 mM 2-mercaptoethanol, 2.5 mM $MnCl_2$, 5 mM EDTA, and 10 µL of the crude extract in 50 mM potassium phosphate buffer (pH 7). The reaction was started by 0.27 mM of NADH.	340 nm	Paoletti et al. 1986

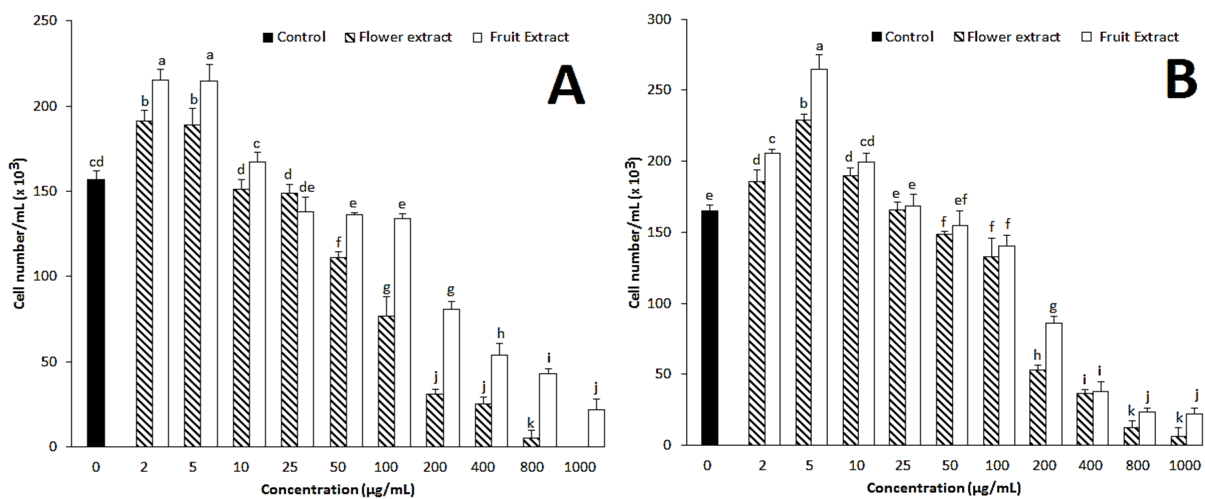

Figure 1 Cytotoxicity of flower and fruit extracts of *A. unedo* (A) Aqueous extracts, (B) Ethanolic extracts, Data are reported as mean ± SD of 3 independent experiments and 3 measurements, Letters indicate differences between values using the Tukey test at $P< 0.05$.

culturing the protozoan in the presence of each extract at different concentrations (2, 5, 10, 25, 50, 100, 200, 400, 800 and 1000 µg/mL). Cells were counted in the exponential growth phase (72 h) and compared to the control (*T. pyriformis* grown in PPYE medium without extracts). The cytotoxicity of the extracts is shown in Figure 1, and the result showed that at low concentrations, stimulation of growth compared to the control was observed with the addition of all the plant extracts, and it was up to 5 µg/mL for aqueous extracts and up to 10 µg/mL for ethanolic extracts. Inhibition of growth was observed above 50 µg/mL for the aqueous and ethanolic extracts, and it depends on the increasing concentration of the extracts, with a more pronounced inhibition for flower extracts compared to fruit extracts (Figure 1). Probit analysis was used to calculate the probable IC_{50} for the studied extracts, and the results show IC_{50} of 107.3, 321.6, 166.4, and 260.9 µg/mL for aqueous extract of flowers, fruits, ethanolic extract of flowers, and fruits, respectively. Moreover, the effect of *A. unedo* extracts on the appearance of *T. pyriformis* was determined by observations through an optical microscope, and no change in the appearance of the protozoan was observed at 50 µg/mL for aqueous extracts and 100 µg/mL for ethanolic extracts. Cell shrinkage was observed at high concentrations of the plant extracts (data not shown). For these reasons, 25 µg/mL concentration for aqueous and ethanolic extracts was chosen to study the protective effect against gamma radiation. These concentrations allowed for maintaining approximately the initial number of cells without affecting their appearance.

3.2 Assessment of the effect of *A. unedo* extracts against gamma radiation

3.2.1 influence of extracts on growth and morphology of irradiated cells

The effect of ^{60}Co source (20-cGy/h for 72 h) exposure on the growth of *T. pyriformis* was evaluated. The viability of *T. pyriformis* was estimated by measuring the generation number and time. The results, presented in Figure 2, showed a decrease in the generation number and an increase in the generation time, indicating a lower growth in the presence of a ^{60}Co source (the exposed cells completed 6.26 ± 0.15 cell generations, while the

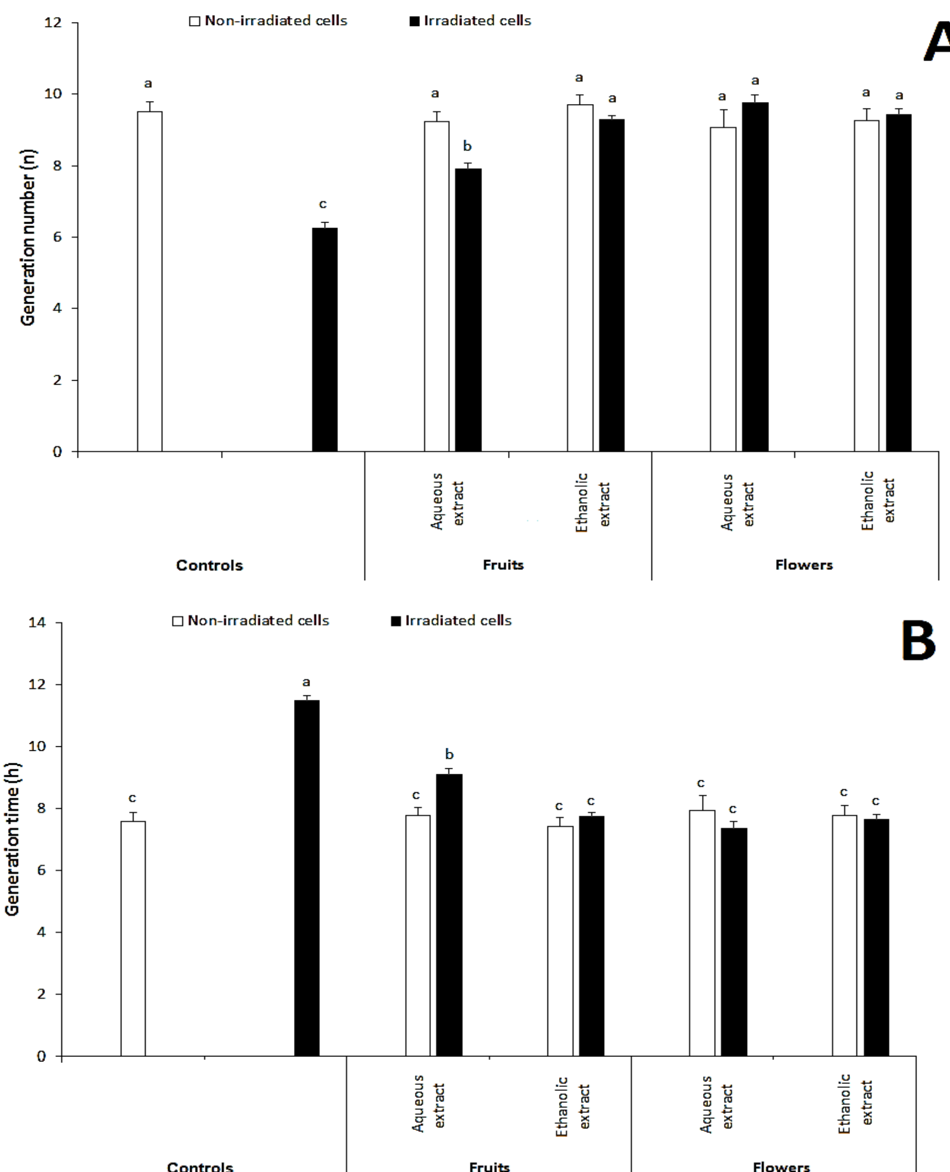

Figure 2 Effect of fruit and flower extracts of *A. unedo* on parameters of growth (A) Generation number, (B) Generation time; Data are reported as mean ± SD of 3 independent experiments and 3 measurements; letters indicate differences between values using Tukey test at $P< 0.05$.

control cells completed 9.5 ± 0.29 generations and the time required for one generation in the exposed cells was 11.5 ± 0.17 h, compared with 7.6 ± 0.22 h in the control cells). The exposed cells completed 6.26 ± 0.15 cell generations, while the control cells completed 9.5 ± 0.29 generations. Additionally, the time required for one generation in the exposed cells was 11.5 ± 0.17 h, compared with 7.6 ± 0.22 h in the control cells. On the other hand, the protective effect of aqueous and ethanolic extracts (25 µg/mL) of the flowers and fruits of the strawberry tree added to the ciliate culture medium against radiation growth inhibition was also evaluated.

Figure 2 shows the improvement in growth under the effect of plant extracts compared to the exposed and untreated cells. Adding flower and fruit extracts allowed the complete or partial recovery of growth. The ethanolic extracts were better than the aqueous extracts since both the ethanolic extracts of flowers and fruits made it possible to find the initial values of the generation time and the generation number when the protozoan was cultured under irradiation. Only the aqueous extract of the flower was able to restore normal growth, and the aqueous extract of the fruit restored about 80% of number and time generation values (generation number = 7.9 ± 0.18 and generation time = 9.11 ± 0.15 h) (Figure 2).

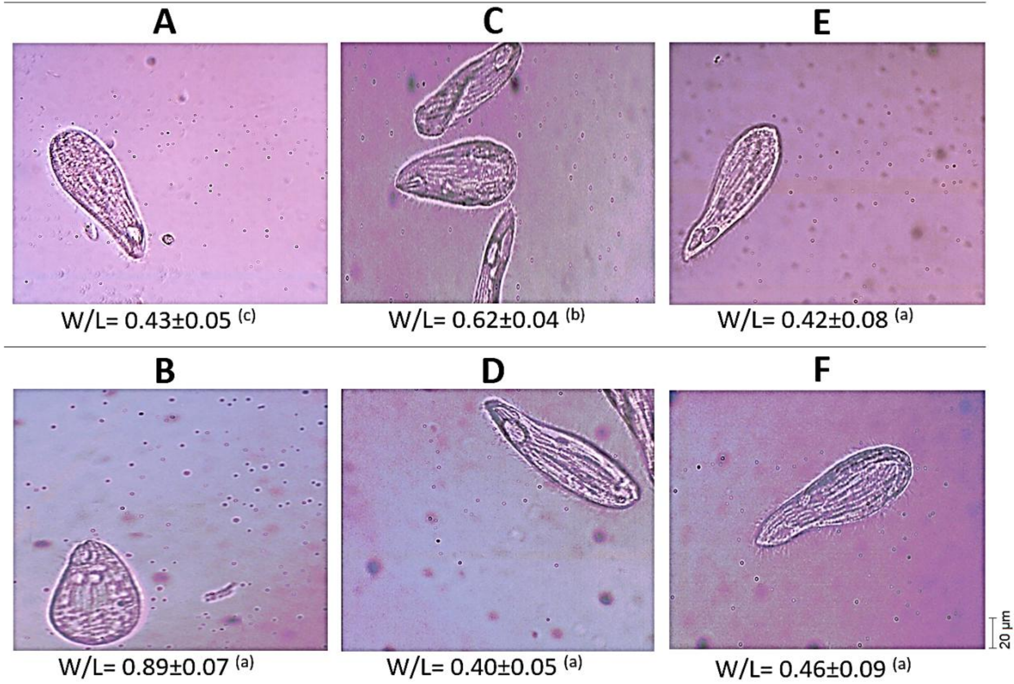

Figure 3 Effect of fruit and flower extracts of *A. unedo* on *T. pyriformis* morphology using microscope images with magnification × 40, (A) Control, (B) Exposure conditions without extracts, (C) Exposure conditions with aqueous fruits extract, (D) Exposure conditions with aqueous flowers extract, (E) Exposure conditions with ethanolic fruits extract, (F) Exposure conditions with aqueous flowers extract, Cell shape (W/L) was calculated for 50 cells in 3 independent experiments, Tukey test $P < 0.05$ was used to indicate significant differences between means (small letters in the figure).

For the morphology of *T. pyriformis*, photographs of cells in different conditions (irradiated cells with and without plant extracts) were taken with a microscope camera (Optika) and compared to the control (non-irradiated cells) (Figure 3). The shortest (W)/longest (L) ratio was calculated using Optika Vision 3.4 software. The W/L ratio was increased in *T. pyriformis* cells grown in a ^{60}Co source (W/L= 0.89±0.07 in irradiated cells compared to 0.43±0.05 in control cells) (Figure 3). The shape of irradiated cells grown with the ethanolic extracts was completely restored (W/L= 0.42±0.08 for fruits and 0.46±0.09 for flowers), compared to the shape of the irradiated cell without extracts (W/L= 0.89±0.07). The aqueous extract of the fruits also showed a potential protective effect against the effects of radiation on *T. pyriformis* cell morphology, significantly restoring part of the cell shape (W/L= 0.62±0.04), while the aqueous extract of the flowers made it possible to completely restore the initial shape of the exposed cells (W/L= 0.40±0.05).

3.2.2 influence of extracts on lipid peroxidation in the irradiated cells

As shown in Table 2, exposure to the ^{60}Co source significantly increased lipid peroxidation in *T. pyriformis* after 72 h of growth with an MDA production value of 3.55±0.12 nmol/mg compared to the control (0.17±0.05 nmol/mg). Adding the aqueous extract of the fruits at 25 µg/mL to the culture medium reduced the production of MDA in the irradiated cells to 1.75±0.09 nmol/mg. All the other extracts (aqueous extract of flowers and ethanolic extracts of flowers and fruits) made it possible to restore the initial value (control) of the MDA level of *T. pyriformis* cells under irradiation conditions (Table 2).

3.2.3 influence of extracts on some enzyme activities of the irradiated cells

The enzymatic activities of GAPDH, SDH, CAT, and SOD were determined in crude extracts of *T. pyriformis* grown under irradiation conditions with and without plant extracts and compared with non-irradiated cells with and without plant extracts. Table 2 summarizes the results of the analyses and shows that adding aqueous and ethanolic extracts of flowers and fruits of *A. unedo* did not affect the tested enzymatic activities when *T. pyriformis* was grown under normal conditions, and these activities were considered as controls. When *T. pyriformis* was grown in the presence of a ^{60}Co source, metabolic enzymes GAPDH and SDH activities were inhibited. Under irradiation conditions, GAPDH and SDH activities in the protozoa retained about 40 to 45% of the residual activities compared to the control (3.55±0.12 and 25.67±2.11 instead of 0.846±0.041 and 59.87±4.04 µmol/min/mg of protein for GAPDH and SDH, respectively). Adding aqueous

Table 2 Effect of irradiation with ^{60}Co source on lipid peroxidation (MDA production) and some enzyme activities (GAPDH, SDH, CAT, and SOD) of *T. pyriformis* grown with and without *A. unedo* extracts.

Growth conditions	MDA (nmol/mg)	GAPDH (µmol/min/mg)	SDH (µmol/min/mg)	CAT (µmol/min/mg)	SOD (µmol/min/mg)
Control*	0.17±0.05^{c}	0.846±0.041^{a}	59.87±4.04^{a}	0.354±0.037^{d}	0.288±0.036^{c}
Irradiation	3.55±0.12^{a}	0.376±0.027^{b}	25.67±2.11^{d}	1.965±0.191^{a}	1.99±0.097^{a}
Irradiation + fruit aqueous extract	1.75±0.09^{b}	0.814±0.030^{a}	41.27±1.01^{c}	1.017±0.093^{b}	0.482±0.055^{b}
Irradiation + flower aqueous extract	0.18±0.07^{c}	0.861±0.046^{a}	56.24±1.24^{a}	0.424±0.038^{cd}	0.281±0.031^{c}
Irradiation + fruit ethanolic extract	0.21±0.04^{c}	0.835±0.039^{a}	49.08±2.85^{b}	0.508±0.059^{c}	0.435±0.056^{b}
Irradiation + flower ethanolic extract	0.19±0.07^{c}	0.831±0.017^{a}	62.13±3.37^{a}	0.352±0.018^{d}	0.334±0.029^{c}

* No significant difference between MDA production and enzyme activities was observed in controls (non-irradiated *T. pyriformis*) without and with the plant extracts.

and ethanolic extracts of the flowers and fruits of *A. unedo* to the culture of irradiated cells of *T. pyriformis* made it possible to recover the totality of GAPDH activity (Table 2). For SDH, the activity was completely restored for *T. pyriformis* irradiated only in the presence of flower extracts (aqueous and ethanolic). The aqueous and ethanolic extracts enabled the recovery of 69 and 82%, respectively, compared to the initial activity. The control had an activity of 59.87±4.04 µmol/min/mg, while the aqueous and ethanolic extracts had activities of 41.27±1.01 and 49.08±2.85 µmol/min/mg, respectively (Table 2). CAT and SOD, involved in the antioxidant defence system, were also monitored in this study. Radiation increased the activity of these two enzymes, and their respective activities became 1.965±0.191 and 1.99±0.097 µmol/min/mg compared to the control with and without extract, which are 0.354±0.037 and 0.288±0.036 µmol/min/mg for CAT and SOD, respectively (Table 2). The initial activity found in the control was significantly recovered in *T. pyriformis* irradiated in the presence of aqueous and ethanolic extracts of flowers (CAT: 0.424±0.038 and 0.352±0.018; SOD: 0.281±0.031 and 0.334±0.029 µmol/min/mg for the aqueous and ethanolic extracts, respectively). The fruit extracts enabled the recovery of part of the activities compared to the control. The CAT activity was 1.017±0.093 µmol/min/mg, and SDH activity was 0.482±0.055 µmol/min/mg for the aqueous extract. For the ethanolic extract, CAT activity was 10.508±0.059 µmol/min/mg, and SDH activity was 0.435±0.056 µmol/min/mg (Table 2).

3.3 Evaluation of the *in vitro* antioxidant activity of *A. unedo* extracts

The antioxidant activity of aqueous and ethanolic extracts of *A. unedo* was evaluated in vitro using the DPPH scavenging test. The antioxidant activity of the flower extracts was better than that of the fruit extracts, with lower concentrations required to achieve a 50% antioxidant effect (EC_{50}) (Figure 4). The EC_{50} for the aqueous flower extract was significantly higher than that for the ethanolic

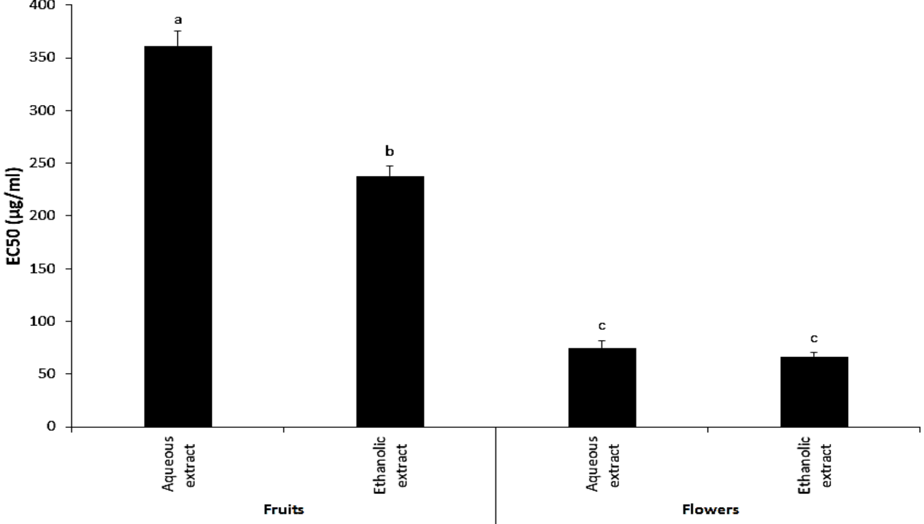

Figure 4 Effect of fruit and flower extracts of *A. unedo* on DPPH scavenging; Data are reported as mean ± SD of 3 independent experiments and 3 measurements; Letters indicate differences between values using the Tukey test at $P < 0.05$

flower extract, and the scavenging power of the aqueous and ethanolic flower extracts was similar. The EC_{50} values for aqueous fruit extract, ethanolic fruit extract, aqueous flower extract, and ethanolic flower extract were 360.85 ± 15.053, 237.43 ± 10.08, 71.13 ± 7.15, and 65.97 ± 4.13 µg/mL, respectively (Figure 4).

4 Discussion

In their previous work, Ziyadi et al. (2022b) investigated the impact of irradiation on the growth of the ciliate protozoa *T. pyriformis*. The results indicated that exposure to doses greater than 6 cGy/h using cobalt-60 (^{60}Co) or cesium-137 (^{137}Cs) sources had a negative effect on the cells, retarding their growth, inhibiting their metabolism, and altering their morphology. Our study found similar results, as exposing *T. piriformis* cultures to a ^{60}Co source at 20 cGy/h during the exponential phase reduced cell growth by decreasing the number of generations in 72 h and increasing the generation time. Additionally, radiation altered the morphology of the cells, causing them to become more rounded. This rounding aspect is a characteristic of *T. pyriformis* under various stress conditions (Suryanto et al. 2022). Radioactivity also affects certain metabolic enzymes, such as GAPDH and SDH, which are key enzymes in cellular metabolism and have important physiological roles (Wei et al. 2022; Wang et al. 2023). In addition, exposure to a ^{60}Co source indirectly affected cells by increasing the amount of ROS, thereby increasing lipid peroxidation, which is one of the damages caused by ROS. These free radicals can interact with biomolecules such as lipids, proteins, and DNA, leading to cellular dysfunction (Gebicki and Nauser 2021). Radiation exposure can cause an increase in ROS due to the radiolysis of water in the cell. The irradiated cells triggered the antioxidant defence system, resulting in increased activity of CAT and SOD in response to the increase of ROS in the cell. This phenomenon has been observed in microorganisms exposed to ROS and radiation (Iddar et al. 2022).

Various plant extracts have been tested for their antioxidant properties and ability to protect against the harmful effects of radiation (El-Shawi et al. 2023; Wu et al. 2023). In another study by Ziyadi et al. (2022a), the protective effects of certain pure essential oils (*Rosmarinus officinalis*, *Origanum compactum*, *Lavandula angustifolia,* and *Eucalyptus globulus*) on *T. pyriformis* against irradiation were evaluated. In this study, we assessed the ability of strawberry tree extracts from the El Harcha region of Morocco to protect *T. pyriformis* against the effects of radiation. We tested two types of extracts from the flowers and fruits of the tree: decoction and ethanol maceration. These methods are widely recognized as the simplest and most effective for extracting bioactive compounds from plant materials (Lezoul et al. 2020). The chemical defence compounds in plant extracts can be toxic to microorganisms (Zaynab et al. 2021). Therefore, we tested *A. unedo* extracts at a non-cytotoxic concentration of 25 µg/mL to evaluate their protective effect against radiation in *T. pyriformis*.

Adding the aqueous and ethanolic extracts of both flowers and fruits of *A. unedo* to the culture of irradiated cells resulted in the recovery of growth, similar to cells in normal conditions. However, the aqueous extract of the fruits only allowed for a partial recovery of growth at 80%. These extracts were also shown to protect the growth of *T. pyriformis* against the effects of radiation on metabolic enzymes essential for cell survival, such as GAPDH and SDH. For GAPDH, 100% of its activity was found in cells irradiated with all tested extracts (Table 2). As for SDH, only the aqueous and ethanolic extracts of flowers allowed for the recovery of 100% of its activity. The fruit extracts allowed for the recovery of 69 and 82% for aqueous and ethanolic extracts, respectively. Numerous studies have demonstrated the benefits of using specific plant extracts and natural products as radioprotectants in experimental models (Dowlath et al. 2021). In comparison, using non-toxic concentrations of pure essential oils (5 µg/mL for *R. officinalis* and *E. globulus* and 10 µg/mL for *O. compactum* and *L. angustifolia*) in the culture of *T. pyriformis* exposed to ^{60}Co at 20 cGy/h restored over 90% of the growth and metabolic enzyme activities (Ziyadi et al. 2022a).

Gamma radiation can cause radiolysis of water, resulting in the formation of ROS and cellular damage (Obrador and Montoro 2023). Irradiation-induced oxidative damage indirectly affects cellular proteins and lipids, leading to reactions such as thiolation and carbonylation under ROS (Dowlath et al. 2021). The *A. unedo* extracts effectively reduced lipid peroxidation in the irradiated protozoa, with the aqueous fruit extract having a slightly lesser effect (Table 2). This plant is rich in polyphenol and flavonoid compounds (Wahabi et al. 2023), which possess antioxidant and radioprotective properties (Dowlath et al. 2021). The DPPH test confirmed that the extracts had considerable scavenging activity, with the flower extracts exhibiting higher activity than the fruits. Finally, the antioxidant properties of the extracts also enabled the enzymes CAT and SOD, which are involved in the antioxidant defence system, to recover their initial levels before irradiation partially. The aqueous and ethanolic extracts of the flowers restored the enzymes to their initial activities, while the fruit extracts allowed for partial recovery of the activity compared to the control (Table 2). Previous studies have shown that adding exogenous antioxidants can support the enzymatic defense system by scavenging ROS (Supruniuk et al. 2023). The results of this work describe, for the first time, the use of *A. unedo* extracts as protective agents against the negative effects of irradiation in the *T. pyriformis* cell model.

5 Conclusion

Aqueous and ethanolic extracts of the strawberry tree (*A. unedo*) from the El Harcha region, Morocco, protected *T. pyriformis*

against the negative effects of exposure to a ^{60}Co radioactive source. Compared to the non-irradiated cells, the extracts completely or partially restored the protozoan's growth, morphology, and physiology in the irradiated cells. Flower extracts showed a higher protective potential and antioxidant activity (protection of cells against radiation-induced ROS) than fruit extracts. Also, ethanolic extracts proved more effective than aqueous extracts in protecting *T. pyriformis* against irradiation. These results may open prospects for using *A. unedo* flower extracts as exogenous radioprotective agents.

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Conflicts of interest

The authors reported no potential conflict of interest.

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Characterization of herbicide use practices in cereal agroecosystems in western Burkina Faso

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Agricultural practices

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ABSTRACT

The use of pesticides often leads to environmental contamination above acceptable levels. The level of contamination is related to poor pesticide application practices, in addition to the properties of pesticides and soil characteristics. The primary objective of this study was to characterize herbicides and their application practices in cereal crops in the regions of Hauts-Bassins, Sud-Ouest, Cascades, and Boucle du Mouhoun in Burkina Faso. A semi-structured questionnaire was used to collect and extract data from 617 cereal farmers in the four regions. During the survey, the identified herbicides were characterized using pesticide property databases. The survey shows that most cereal farmers in the regions are illiterate (58.18%) and have not received any training in pesticide use (84.28%). Only a small percentage of farmers (1.3%) consult technical services for the selection of herbicides to be used. The survey also revealed that 60% of farmers leave empty herbicide containers in the wild, 39.93% use water sources to clean sprayers, and 17.83% use them to prepare the spray mixture. A total of 25 active ingredients were identified in 117 commercial herbicide products with a total use of 8100 litres and 280 kg. Of the listed herbicides, 45.37% were not approved by the Sahel Pesticide Committee (CSP). Among the non-approved herbicides, 27.78% contained paraquat, atrazine, and acetochlor, which the CSP bans. The

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study shows that farmers do not follow good practices when using herbicides, which can contaminate different environmental compartments and cause harmful effects to non-target organisms.

1 Introduction

Agriculture is the main activity for 56.2% of the population in Burkina Faso (INSD 2022), focusing on cereal crops. However, the country faces challenges meeting its food crop needs due to population growth and production constraints (Ouédraogo et al. 2009; MAAH 2018). Various pests associated with cereal crops are the main production constraints, causing losses of up to 40% per year (Mahmood et al. 2016). To combat this, farmers use synthetic chemical pesticides, which are applied uncontrolled and in large quantities yearly (Bernhardt et al. 2017). This is because of the increased cultivated area and productivity, the drain on farm labour (Gianessi 2013; Houngnihin et al. 2021), and the high cost of mechanical weeding (Olina Bassala et al. 2015). As a result, farmers are turning to chemical weed control with herbicides (Olina Bassala et al. 2015). However, the excessive use of phytosanitary products can cause harm to living organisms (MAAH 2020), the environment, and ecosystems (GRAAD 2017; Tchamadeu et al. 2017; Gbombele et al. 2019). Herbicide formulations contain highly toxic active molecules, and 80-90% of these active ingredients are transferred to the non-target environment during spraying (van der Werf 1997), leading to ecosystem degradation. Poor farming practices and non-compliance with pesticide use conditions have been identified (FAO 2002; Compaore et al. 2019). The most common poor practices used by farmers include repeated treatments, overdosing, mixing of several pesticides when spraying, washing of sprayers at water points, use of unregistered or banned products, and poor management of empty containers (Gomgnimbou et al. 2009; Tchamadeu et al. 2017; Bayili et al. 2019). Studies on pesticides have mainly focused on cotton-growing areas (Bayili et al. 2019), market gardening (Tarnagda et al. 2017; Ngakiama et al. 2019), and peri-urban dam reservoirs (Bèkouanan 2018). Limited data is available on herbicide use practices in cereal crops, which occupy most of the agricultural land in Burkina Faso. This study aims to characterize herbicides and their use practices in cereal crops in the Hauts-Bassins, Sud-Ouest, Cascades, and Boucle du Mouhoun regions of Burkina Faso.

2 Materials and Methods

2.1 Study Sites

These field studies were carried out in the Hauts-Bassins, Sud-Ouest, Cascades, and Boucle du Mouhoun regions in the western part of Burkina Faso (Figure 1). These regions cover both Sudanese and Sudano-Sahelian climates, with the latter being the wettest area in the country. These four regions account for 41% of

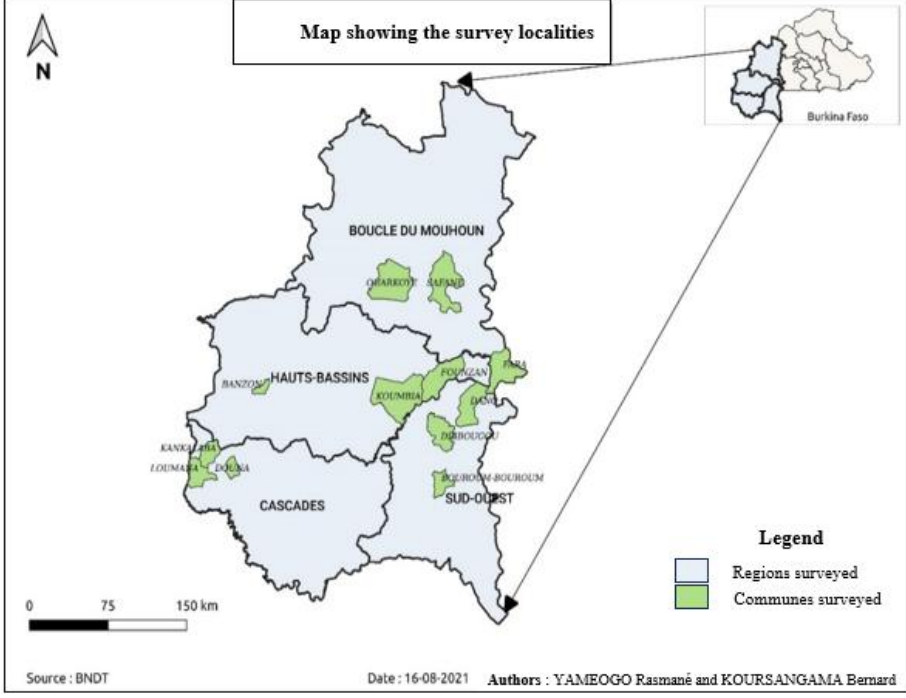

Figure 1 Indicative map of the study area

the country's total cultivated land (MAFAP 2013), which has led to a significant increase in the use of phytosanitary products. The survey covered three communes in each region, comprising 12 communes. During the study period (2020-2021), the communes in the study area received an average rainfall of 877 mm, varying from 640.5 mm to 1223.25 mm.

2.2 Human study population and inclusion criteria

The survey only included farmers who grow cereal crops. To be eligible, they must have at least one cereal field within the survey area and used herbicides in the 2020-2021 crop year. The selection process involved random selection from those who met the criteria and volunteered. In each of the 12 communes, a minimum of 50 farmers were selected.

2.3 Surveys of farming practices and characterization of herbicides used

This survey aimed to determine which herbicides are used in cereal growing areas and how they are applied. Each farmer was provided with a semi-structured questionnaire that covered various topics, including sociodemographic information, general farm data, herbicide identification, herbicide use conditions and practices, and environmental risk perception. To get a better understanding of the herbicides being used, a literature search was conducted using several sources, such as the list of pesticides registered by the CSP, the Pesticide Properties Database/Biopesticide Database (PPDB/BPDB) (Lewis et al. 2016), and pesticide directories from neighbouring countries like Ghana and Côte d'Ivoire, which don't have the same registration system as Burkina Faso.

2.4 Data Analysis

Survey data was collected using a paper questionnaire and entered into Microsoft Excel 2016. The analysis was descriptive, with frequencies and proportions calculated using R (version 4.0.1, 06-06-2020), and graphs were generated using Microsoft Excel 2016.

3 Results

3.1 Sociodemographic and farming characteristics

In total, 617 farmers were interviewed in the 12 communes, and 88% were men. The age of the respondents varied from 18 to 85 years, with a median of 41 years. The majority of respondents, about 67.25%, fell within the age range of 35 to 55 years. As for pesticide use practices, 84.28% of farmers received no training. Moreover, 58.18% of the farmers were illiterate, while 28.69% had only primary education as their highest level of education, as shown in Table 1. Regarding water availability, 77.8% of the farmers stated that they had at least one water point close to their farms, and the average distance from them was 50 meters. These water points included wells (24.79%) and streams (24.2%). Other water points identified included boreholes, rivers, gullies, puddles, and ponds, with proportions ranging from 4.8% to 19%. Cereal production was the most common among the crops grown in the study area. Among the major cereals, maize was grown on 1222 ha, sorghum on 552.9 ha, rice on 324.8 ha, and millet on 203.2 ha.

3.2 Agricultural Practices

The study analyzed the farming practices related to the use of herbicides. It focused on the source of supply, choice of herbicides, management of empty packaging, handling of residual spray liquid, mixing of several herbicides, sprayer used, where it is washed, resumption of treatment, and perception of the risk of environmental contamination. The survey revealed that 89.87% of cereal farmers purchase herbicides from the local market, 5.35% from authorized dealers, and 4.48% from crop phytopharmaceutical companies. Only 1.3% of farmers consult technical services while selecting herbicides. 79.58% of farmers prepare the spray mixture on the field, 17.83%

Table 1 Distribution of cereal farmers by sociodemographic characteristics

	Social status	Frequency (%)
Sex	Men	88
	Women	12
Training in the use of pesticide status	Untrained	84.28
	Trained	15.72
Education level	Illiterate	58.19
	Primary	28.69
	Secondary and above	13.13
Ages	18 to 35	21.87
	36 to 55	67.25
	Over 55	10.85

near water sources, and 2.59% at home. Moreover, 98% of farmers use a backpack sprayer to treat their fields. Regarding cleaning equipment, 39.93% of farmers clean it at water sources, 38.09% in the field, and 21.98% at home (Figure 2).

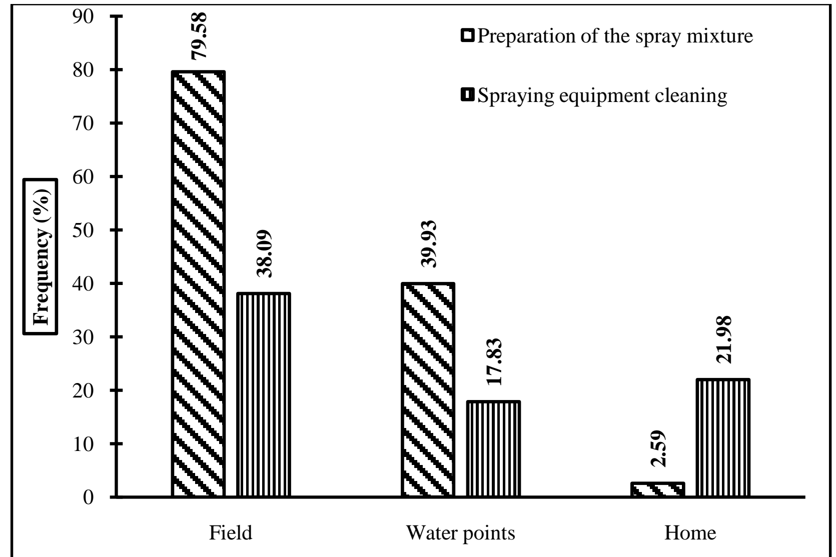

Figure 2 Distribution of farmers according to where the herbicide mixture is prepared and where the spraying equipment is washed

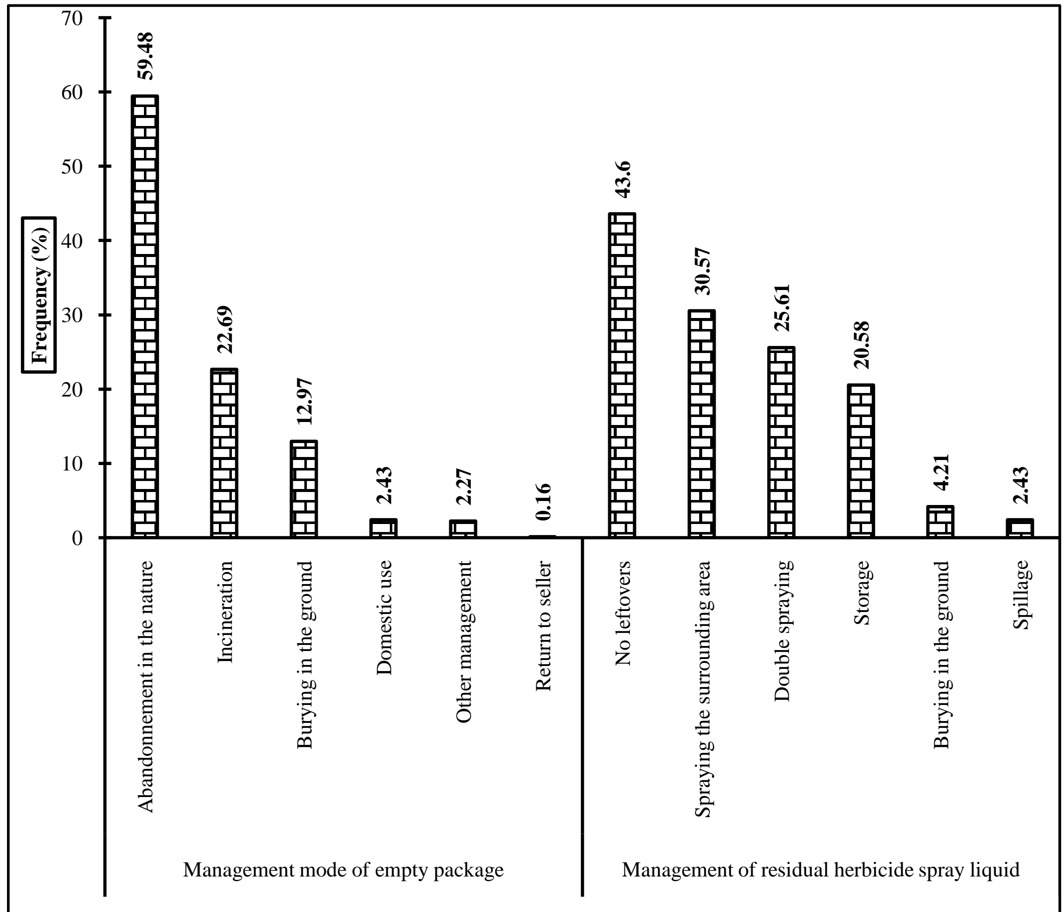

Figure 3 Distribution of farmers by management mode for empty packaging and residual herbicide spray liquid

After treatment, 60% of the farmers disposed of empty herbicide containers in the wild, 13% buried them, and 23% incinerated or used them for domestic purposes (Figure 3). In 20.58% of the cases, the remaining spray mixtures are stored for later use, and 25.61% of farmers do a second round of spraying (Figure 3). In terms of environmental risks, 70.18% of the farmers were aware of the herbicide contamination risk, while 30% said otherwise. Out of the surveyed farmers, 18.64% had mixed different herbicide formulations for spraying, and 48.69% had done so for efficacy reasons. During the campaign, 23.5% of farmers resumed spraying.

3.3 Knowledge about herbicides used

A study has identified 117 commercial herbicides formulated from 25 active ingredients in the four regions surveyed (Figure 4). Glyphosate, nicosulfuron, atrazine, and 2,4-D are the most commonly used active ingredients found respectively in 44.44%, 12.82%, 6.84%, and 5.98% of the formulations (Figure 4). Of these herbicides, 45.37% are not approved, and 27.78% of these non-approved herbicides contain active ingredients banned by the Sahel Pesticide Committee. Additionally, 91.22% of the herbicides are liquid, with the most common formulations being concentrated soluble (SL) at 59.23% and concentrated suspension (SC) at 17.53%. These herbicides are imported from eight countries. The study found that 51.96% of the commercial herbicide formulations were of Chinese origin, 24.73% were from France, and 9.84% were from Ghana (Figure 5). The herbicide formulations belong to classes III (66%), II (18%), and U (8%) of the WHO toxicity classification (Figure 6). Farmers surveyed used approximately 8100 L and 280 kg of herbicides during the study crop year.

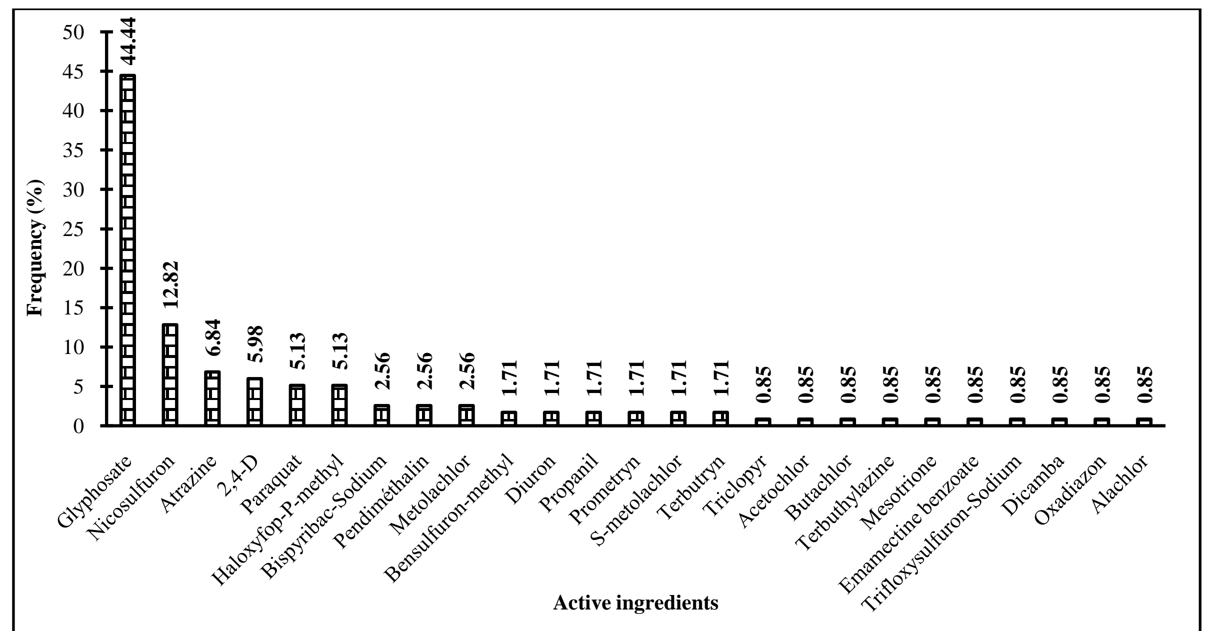

Figure 4 Distribution of herbicide commercial formulations by active ingredients

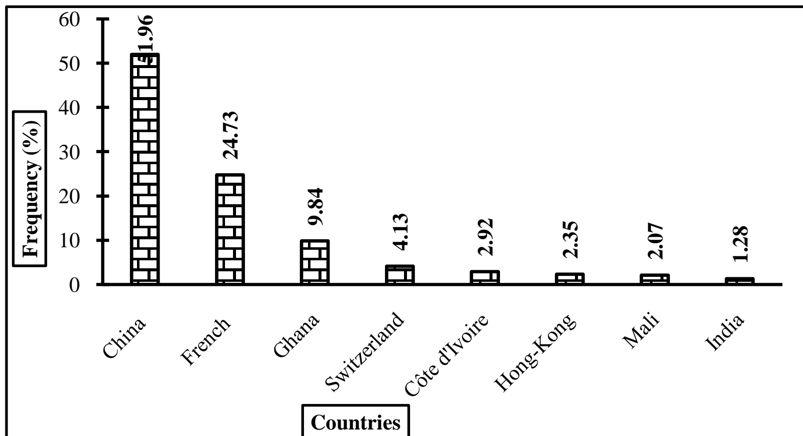

Figure 5 Distribution of herbicide commercial formulation by country of origin

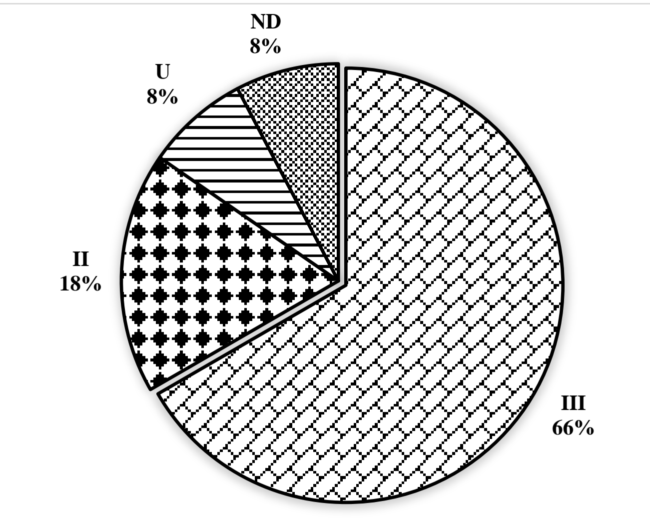

Figure 6 Distribution of herbicide commercial formulations by WHO class.

4 Discussion

According to a recent survey, adults with low literacy levels mostly engage in cereal farming. This finding is consistent with a study by Compaore et al. (2019) on the rice-growing lowlands of Dano in southern Burkina Faso, where they found that 61% of the survey population was illiterate. Other authors have made similar observations regarding illiteracy in non-cereal crops (Lelamo et al. 2023). The survey also revealed that many cereal farmers have not received any training on proper pesticide use. This differs from the findings of studies on cash crops like cotton and coffee, where almost all farmers are trained or have access to information on safe pesticide use (Ouédraogo et al. 2009; Lelamo et al. 2023). The lack of training for cereal farmers may be due to low funding for the food sector in favour of export crops. However, cereal farmers' illiteracy and lack of knowledge suggest a limited understanding of pesticide use instructions (Compaore et al. 2019). Maize and sorghum are the two main cereals grown in terms of area. However, the results obtained from the four regions vary slightly from the national agricultural statistics 2019, where the sorghum area is slightly higher than maize (ISND 2019). While maize cultivation increases the demand for pesticides (MAFAP 2013), sorghum is often cultivated without crop rotation, encouraging the proliferation of weeds. Moreover, using fertilizers tends to benefit the growth of weeds, increasing the need for herbicides (Aubertot et al. 2005).

According to this survey, cereal farmers receive little technical assistance when selecting herbicides. As most of these farmers are illiterate, they choose the herbicides themselves. This is also the case in vegetable production in the centre-north of the country, where farmers randomly choose pesticides or base their choices on their own experience (Ouédraogo et al. 2021). This practice is also prevalent in other member countries of CILSS, which is one of the reasons why pesticides are misused (Adjovi et al. 2021). According to Bayili et al. (2019), local pesticide dealers are not trained in the management and use of pesticides. These findings could explain why banned or unapproved products are used, and treatments are misused and resumed. These practices are potentially dangerous for the environment.

Many farmers dispose of their herbicide containers in the wild, while others apply a second dose if there are any residual herbicide preparations, and some mix different herbicide formulations for more efficient weed control. These practices, reported in cotton-growing areas previously (Tapsoba and Bonzi 2006), could result in an accumulation of pesticide active ingredients in the environment. This may cause toxic molecules to become readily available for transport through runoff to other environments. Since most farmers report having water retention areas around their fields, with a median distance of about 50 m, the aquatic environment is the primary destination for these herbicide surpluses. Earthworms and amphibians living in soil and water, respectively, could also be affected by the migration of herbicides into their habitats (Boileau 2015; Pelosi et al. 2021). Farmers washing sprayers on the ground in fields and near water reservoirs further increases the risk to aquatic and terrestrial organisms. This practice directly leaches many toxic molecules into the soil and water, habitats for various non-target organisms, including disease vectors. Agricultural pesticides have been identified by Hien et al. (2017) and Diabaté et al. (2002) as a contributing factor to the development of resistance in malaria vector mosquito populations in Burkina Faso.

One hundred and seventeen commercial herbicide specialities formulated from 25 active ingredients were identified in the four regions surveyed. These herbicides were sourced from eight countries, with China being the biggest source, followed by France

and Ghana. This diversity of origin could make the task of controlling herbicides more complicated. For instance, some unauthorized pesticides with questionable quality are fraudulently imported into Burkina Faso from neighbouring countries (Paré and Toé 2011). About half of the herbicides identified were unauthorized, and some contained active substances that the CSP banned due to their environmental toxicity, including paraquat, atrazine, and acetochlor (CSP 2014). Glyphosate was the most commonly used active ingredient in herbicide formulations, as observed in previous studies by Bayili et al. (2019) and Le Du-Carrée (2021). However, the ecotoxicological risk of glyphosate remains to be elucidated, as Le Du-Carrée (2021) suggests that it could disrupt embryonic development in fish following re-exposure to parental compounds. Additionally, the greater the number of molecules introduced, the greater the potential for environmental contamination (Mamy et al. 2008) and the greater the possibility of synergistic toxicity to non-target organisms.

Cereal farmers prefer liquid formulations because they are easier to mix, store, and transport (Fishel 2019). However, these formulations pose a relatively high risk of environmental contamination. This is because they are highly concentrated in active ingredients, and a simple dosing error could result in overdosing. They also have a high potential for drift and are difficult to target (Fishel 2019). Furthermore, spraying is almost exclusively done with backpack sprayers, which, according to the Food and Agriculture Organization of the United Nations (FAO), are used under high pressure, resulting in significant drift due to the production of fine droplets (FAO 1998).

Conclusion

This study in Burkina Faso identified several herbicides commonly used in the cereal-growing regions of Hauts-Bassins, Sud-Ouest, Cascades, and Boucle du Mouhoun. The investigation has found that the supply chain for these products is not entirely regulated due to their diverse origins. Local farmers and retailers often lack training and do not consult technical services, which leads to poor management of empty packaging and the use of unauthorized or banned products. Combined with the number and chemical characteristics of the herbicides used, these practices pose a risk of environmental contamination. This contamination could affect non-target organisms, including disease vectors whose life cycle partly occurs in contaminated waters.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Effect of different doses of nitrogen and inoculation with *Azospirillum brasilense* on the productive characteristics of maize

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Zea mays L.

Inoculation

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Yield

ABSTRACT

This study assessed the effects of different nitrogen doses on maize crops, with and without the inoculation of *Azospirillum brasilense*. The experiment was carried out during the 2020/2021 harvest season in the administrative department of Concepción, district of Horqueta, Paraguay, at the coordinates of 23°14'31.7" S and 56°53'05.9" W. The experiment followed a randomized complete block design (RCBD) arranged in a factorial design (4 X 2). Factor A included nitrogen doses (0, 40, 80, and 120 kg ha^{-1}), while factor B corresponded to the bacterial inoculation (with and without *A. brasilense*). The experiment was performed in three replications; each experimental unit (EU) was 22.5 m^2. The study evaluated the following characteristics: plant height, cob insertion height, cob length and diameter, weight of 1000 kernels, and kernel yield. The data were analyzed using analysis of variance (ANOVA), and the averages were compared using Tukey's test at a 5% probability of error. Regression analysis was also carried out during the study. The experiment results demonstrated that increased nitrogen levels positively affected the measured characteristics, fitting a quadratic model, except for cob insertion height. Inoculation with *A. brasilense* significantly increased corn growth and productivity. The interaction of both factors produced a significant increase in cob length. Based on the experiment results, applying 104.30 kg ha^{-1} of N in combination with *A. brasilense* inoculation is recommended for improved maize production.

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1 Introduction

Maize, known as corn (*Zea mays* L.), is the second most important crop worldwide, and in 2022, global maize harvesting reached a record 1137 million tons (Erenstein et al. 2022). In Paraguay, maize is typically planted during the summer-fall season. As per estimates for the 2023 harvest, maize production in Paraguay is projected to be around 5 million tons, with an average yield of 5.8 tons per hectare (CAPECO 2023). Maize is an essential crop that contributes to food security. It is a staple food in many parts of the world and is also used as feed for animal production (Wang and Hu 2021). However, maize cultivation and production can be affected by various biotic and abiotic factors (Nigussie et al. 2021; Széles et al. 2023). In Paraguay, the most significant abiotic factor is the low fertility of subtropical soils. This, coupled with a scarcity of nitrogen-fixing microorganisms, gradually reduces the productivity of crops like maize (Teklewold et al. 2013).

Nitrogen is a macronutrient that is crucial in plant growth and development. It is essential for the production of organic plant compounds and chlorophyll. However, excessive nitrogen can lead to increased production costs and unnecessary loss of crop inputs through leaching (Picazevicz et al. 2017; Larramendi et al. 2023). Therefore, it is important to ensure that nitrogen is available in the required quantities for optimal plant growth without causing harm to the environment.

To achieve high yields, maize crops require nitrogen to be available throughout the entire vegetative phase. To ensure the provision of this element, management practices can be integrated into the farming of cereal crops (Zainab et al. 2021), such as the use of mineral fertilizers (Davies et al. 2020) and the incorporation of nitrogen-fixing microorganisms, especially the bacterium *A. brasilense*. This bacteria fulfils numerous functions, including producing phytohormones promoting root growth. In turn, this bacterium raises the capacity of plants to absorb nutrients and water (Cassán and Díaz-Zorita 2016; Niranjan et al. 2024). Furthermore, using this bacterium makes nitrogen available through biological fixation (Pedrinho et al. 2010; Dartora et al. 2013) and biocontrol of plant pathogens. These factors directly enhance root growth, thus increasing crop productivity (Milléo and Cristófoli 2016; Cadore et al. 2016). A study by Dartora et al. (2016) and Di Salvo et al. (2018) found that combining nitrogen fertilizer and seed inoculation with microorganisms increased maize growth and yield. Ferreira et al. (2013) found that maize grain yield increased by 29% when using *A. brasilense* and nitrogen compared to using nitrogen fertilization alone. In contrast, Lana et al. (2012) reported that *A. brasilense* inoculation without applying nitrogen significantly increased maize yields by 15%. In view of the above, this study aimed to evaluate the effect of different doses of nitrogen, with and without bacterial inoculation of *A. brasilense*, on maize crops.

2 Materials and Methods

The experiment occurred in Horqueta, Department of Concepción, Paraguay, during the 2020/2021 harvest season at coordinates 23°14'31.7"S and 56°53'05.9"W.

The climate in the region has an average temperature of 26°C, with maximum highs of 45°C in summer and minimum lows of 4°C in winter, with low levels of frost. The average annual rainfall during the study time is 1,400 mm. Figure 1 (DMH 2021) shows the average rainfall and temperature levels recorded throughout the experiment. The soil in the experimental area belongs to the Alfisol classification with the subgroup Mollic Paleudalf (López et

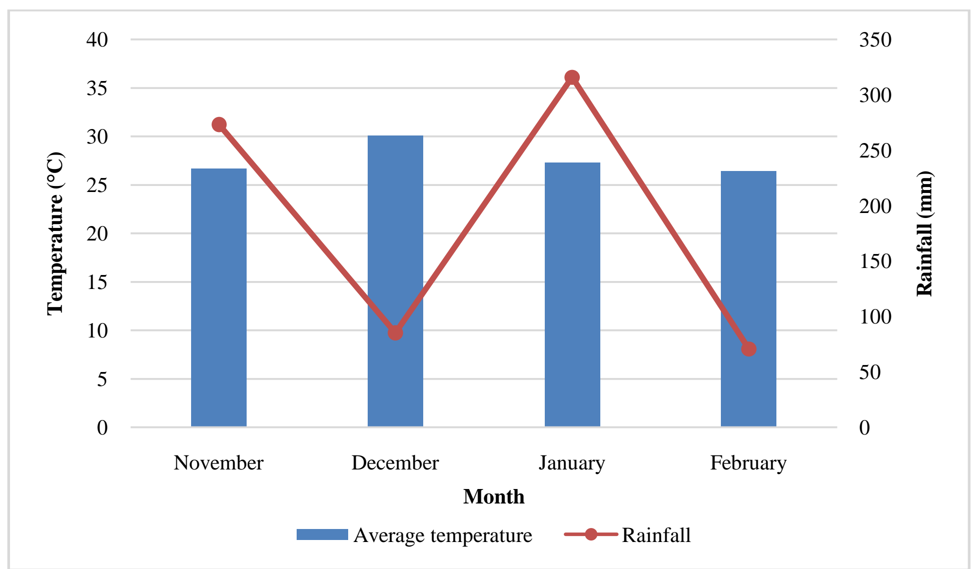


Figure 1 Average rainfall and temperature data for November to February 2020/2021

Table 1 Description of the formulated Treatments

Factor A (Nitrogen dose)	Factor B (Bacterial inoculation)	Combination
0 kg ha^{-1}	With *A. brasilense*	0 kg ha^{-1} + With *A. brasilense*
40 kg ha^{-1}		40 kg ha^{-1} + With *A. brasilense*
80 kg ha^{-1}		80 kg ha^{-1} + With *A. brasilense*
120 kg ha^{-1}		120 kg ha^{-1} + With *A. brasilense*
0 kg ha^{-1}	Without *A. brasilense*	0 kg ha^{-1} + Without *A. brasilense*
40 kg ha^{-1}		40 kg ha^{-1} + Without *A. brasilense*
80 kg ha^{-1}		80 kg ha^{-1} + Without *A. brasilense*
120 kg ha^{-1}		120 kg ha^{-1} + Without *A. brasilense*

al. 1995). Before the experiment, the soil's chemical attributes were evaluated using the methodology presented by Cardoso et al. (2009). The soil analysis showed the following results for the 0 – 20 cm soil layer: Phosphorus (4.50 mg dm^{-3}), Organic matter (11.16 g dm^{-3}), Potassium (0.16 cmol dm^{-3}), Ca (4.78 cmol dm^{-3}), Mg (0.97 cmol dm^{-3}), H+Al (2.36 cmol dm^{-3}), base saturation (5.91 cmol dm^{-3}), Cation exchange capacity (5.93 cmol dm^{-3}), pH (5.55), and volume (71.53%). The experimental design was a randomized complete block design (RCBD) arranged in a factorial design (4 X 2). Factor A corresponds to nitrogen doses (0, 40, 80, and 120 kg ha^{-1}), and factor B is bacterial inoculation (with and without *A. brasilense*) (Table 1). Each treatment had three replications. Each experimental unit (EU) measured 22.5 m² (5 m length by 4.5 m width).

A traditional sowing system was used for the experiment. In November 2020, the hybrid maize cultivar DKB 360 was planted manually with a spacing of 0.45 m between rows and 2.5 plants per linear meter, resulting in a population density of 55,500 plants per hectare. Before planting atrazine herbicide (2.0 kg ha^{-1}) with adjuvant (0.5 l ha^{-1}) and triflumuron insecticide (0.10 l ha^{-1}) were applied to the experimental field. Seed treatment was performed before sowing using a commercial liquid inoculant containing strains of *A. brasilense* bacteria at a concentration of 1.108 cfu.ml. Nitrogen fertilizer was applied twice, during sowing (30%) and at the V5 stage (70%). Urea was used as a nitrogen source (45% N) for all treatments. Phosphorus and potassium (57 kg ha^{-1} and 62 kg ha^{-1}, respectively) were applied during sowing using Triple Superphosphate and potassium chloride as the source of these nutrients. Weed control was carried out manually, while pest control was done chemically using insecticides such as thiodicarb (0.24 kg ha^{-1}) and triflumuron (0.10 l ha^{-1}). Disease was controlled using the fungicides tebucone + trifloxis (0.5 l ha^{-1}), which were applied using a 20 L manual sprayer.

The crop was harvested in February 2021, 115 days after sowing. A sampling area of 14.4 m^2 containing 80 plants was marked out to evaluate each experimental unit. The following variables were measured: (a) Plant height (m), measured when the plants reached physiological maturity. For this, ten plants were selected at random from the sampling area of each experimental unit and measured using a measuring tape from the base of the stem to the height of insertion of the flag leaf, (b) Cob insertion height (m), which was measured from the base at soil level to the insertion of the highest cob. This measurement was taken using a measuring tape from 10 plants from the sampling area of each experimental unit just before harvesting. (c) Cob length and diameter (cm) were measured by randomly selecting 10 cobs from each experimental unit. The length was measured using a measuring tape, and the diameter was measured at the center of the cobs using a vernier scale, (d) Weight of 1000 kernels (g), which was estimated using a scale with a precision of 0.01 g, at 13% moisture content (wet basis), and (e) Kernel yield (kg ha^{-1}), which was measured by harvesting the sampling area of each experimental unit and weighing the kernels on a digital scale.

A variance analysis (ANOVA) was carried out on the results obtained using Agrostat@ statistical software. Averages that showed significant differences were compared using the Tukey test at 5% probability. A regression analysis was conducted to identify doses providing maximum technical efficiency. The maximum fertilizer dosage was calculated using the formula X_{max} = -b/2a. The point of maximum productivity was calculated using the formula Y_{max} = D/ (4a), where D = b2 - 4ac.

3 Results and Discussion

Table 2 shows the calculated F values and their significance. The study found that Nitrogen doses significantly affected all variables except for the height of cob insertion. Bacterial inoculation also significantly affected various variables, including plant height, height of cob insertion, weight of 1000 kernels, and maize crop yield. Additionally, the interaction between Nitrogen doses and bacterial inoculation significantly affected cob length.

Table 2 Calculated F values and significance established by the effects of the factors under study on the variables of PH, HCI, CD, CL, WK, and YIEL

Factors	PH	HCI	CD	CL	WK	YIEL
	F Test					
ND	7.22**	0.30ns	5.13*	8.32**	41.36**	85.15**
BI	29.60**	11.64**	1.91ns	1.81ns	8.45*	6.45*
Interaction NDxBI	2.09ns	0.15ns	0.41ns	4.46*	0.74ns	0.32ns

ns: not significant; (**) (*) significant at 1 and 5 % probability, PH: Plant height, HCI: Height of cob insertion, CD: Cob diameter, CL: Cob length, WK: Weight of 1000 kernels, YIEL: Yield, ND: Nitrogen dose, BI: Bacterial inoculation

Table 3 Effect of *A. brasilense* inoculation and N dose on average plant height, cob insertion height, weight of 1000 grains, and maize yield

Factor	PH (m)	HCI (m)	WK (g)	YIEL (kg ha^{-1})
Bacterial Inoculation				
WI	2.08±0.011^{a}	1.17±0.010^{a}	229.94±4.23^{a}	4774.89±254.64^{a}
WOI	1.97±0.023^{b}	1.030±026 ^{b}	222.94+5.01 ^{b}	4523.02±240.94 ^{b}
N Dose (kg ha^{-1})				
0	1.96±0.045^{b}	1.09±0.041^{ns}	205.15±3.48^{c}	3492.25±56.47^{c}
40	2.000±030^{b}	1.08±0.043	220.91±2.70^{b}	4356.78±55.51^{b}
80	2.090±026^{a}	1.13±0.050	242.54±3.13^{a}	5516.42±81.92^{a}
120	2.050±020 ^{ab}	1.11±0.031	236.07±2.22^{a}	5230.38±194.11^{a}
CV %	2.54	8.66	2.80	5.22
MSD	0.45	0.08	5.56	212.77
OA	2.03	1.10	226.17	4648.96

Letters differ from each other statistically by Tukey's test at 5%, PH: Plant height, HCI: Height of cob insertion, WK: Weight of 1000 kernels, YIEL: Yield, CV: Coefficient of variation, MSD: Minimum significant difference, OA: Overall average, WI: With inoculation, WOI: Without inoculation

During the study, the application of *A. brasilense* on maize seeds before planting resulted in greater plant height (2.08 m) and cob insertion height (1.17 m) (Table 3). This can be attributed to the bacteria's synergistic effect on the seeds, which is fundamental to the plant's growth and development (Coelho et al. 2021). However, the data obtained in this study does not coincide with the findings of Marini et al. (2015), who did not observe significant effects on plant height to combined application. Similarly, the study does not support the results of Maestrelo et al. (2014), who also did not record a positive increase in the height of cob insertion. Regarding the application of N to the maize crop, it produced a positive response concerning plant height (Figure 2) and fitted with a quadratic equation. The study observed a reduction in average height when high doses of N were applied. A maximum height of 2.09 m was achieved using 90 kg ha^{-1} of N, similar to the results observed by Marini et al. (2015). Furthermore, Morais et al. (2015) found that applying nitrogen and bacteria led to greater plant development. Gutiérrez-Peña et al. (2022) stated that nitrogen plays a vital role in several metabolite production processes that influence the growth and maintenance of leaves' photosynthetic and reproductive capacity, promoting crop growth in terms of height and development.

The application of different doses of nitrogen significantly impacted the diameter of the cobs, as shown in Figure 3. A quadratic regression model was used to analyze the data. The maximum diameter of the cobs, measuring 6.75 cm, was observed at a dose of 83.33 kg ha-1. This highlights the importance of nitrogen for plant nutrition, as it facilitates the transformation of biomass in the plant's metabolic processes. Similar results were reported by Galindo et al. (2019), who found that the combination of nitrogen fertilizer and bioinoculant resulted in a significant increase in cob diameter.

According to Figure 4, the cob length was affected by a combination of bacterial inoculation and nitrogen (N) dose. The cob length was greater when *A. brasilense* bacteria was applied with doses of 0, 80, and 120 kg ha^{-1} N. However, when a 40 kg ha^{-1} N dose was used without *A. brasilense*, the cob length was the greatest. The quadratic regression model for N doses indicated

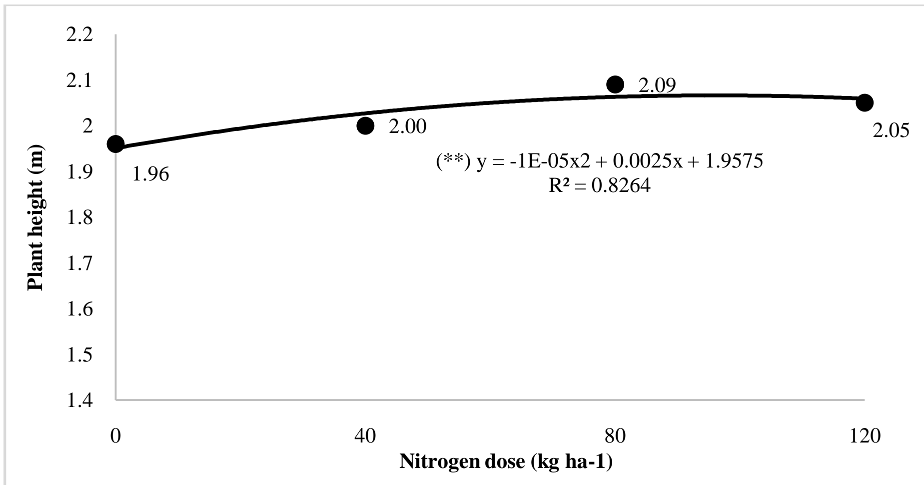


Figure 2 Regression analysis between nitrogen dose and maize plant height

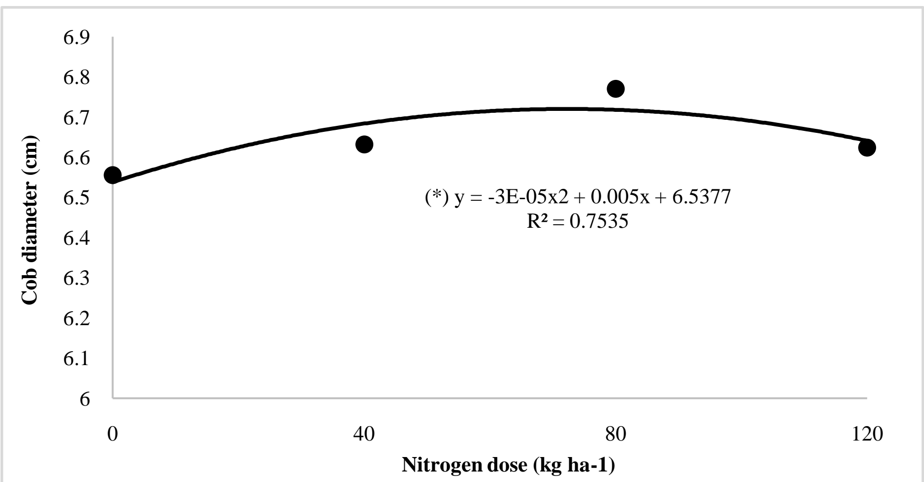


Figure 3 Regression analysis between nitrogen dose and diameter of maize cob

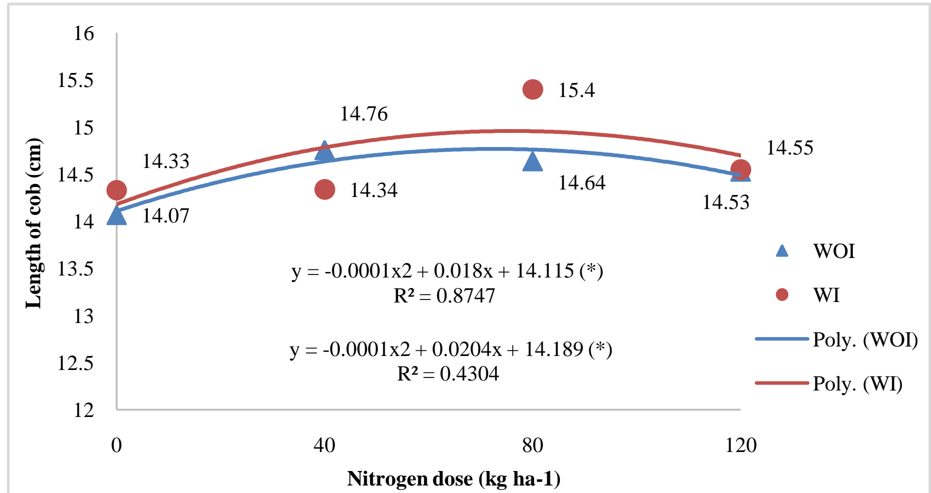
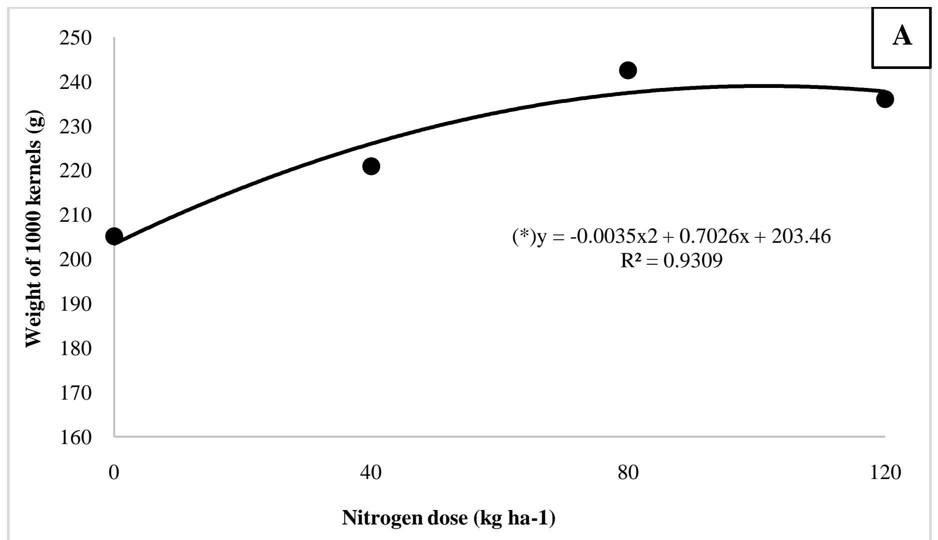


Figure 4 Regression analysis carried out on average cob length concerning inoculation and nitrogen dose, WI: With inoculation, WOI: Without inoculation, Y: cob length response as a function of X: nitrogen dose, *significant by Fisher's test at 5% probability of error.

that excessive N decreased average cob length. When *A. brasilense* bacteria was not used, the highest cob length of 15 cm was achieved with an N dose of 100 kg ha^{-1}. On the other hand, when *A. brasilense* bacteria was applied, the maximum cob length was 14.93 cm with a 90 kg ha^{-1} N dose.

In a study on the impact of nitrogen application and *A. brasilense* inoculation on maize crops, Souza et al. (2019) found no interaction between the two factors regarding cob length. Similarly, Cadore (2014) did not observe any significant effect on cob length when using *A. brasilense* and only detected differences with varying doses of nitrogen. Reis Junior et al. (2008) suggest that a lack of response to inoculant application can often be attributed to inconsistent root colonization, inoculum survival problems, or unfavorable environmental conditions.

In Table 3, the results for the weight of 1000 grains indicate that the application of bacterial inoculant led to a significant increase in weight (229.94 g). This finding contradicts the work of Galindo et al. (2019), who recorded a negative response to inoculation of *A. brasilense* to grain weight. The weight variable of 1000 grains responded positively to applying different N doses, fitting a quadratic equation, as shown in Figure 5a. The highest average weight of 238.72 g for 1000 grains was obtained using a dose of 100.37 kg ha-1 of nitrogen. According to Bulla and Balbinot Junior (2011), increasing the dose of N applied to the maize crop led to heavier grains, and a similar effect was observed in this experiment. Repke et al. (2013) recorded similar results for N doses, fitting a quadratic distribution. However, Mota et al. (2015) and Galindo et al. (2017) recorded a positive linear equation, displaying a tendency for an increase in the weight of the 1000 grains.



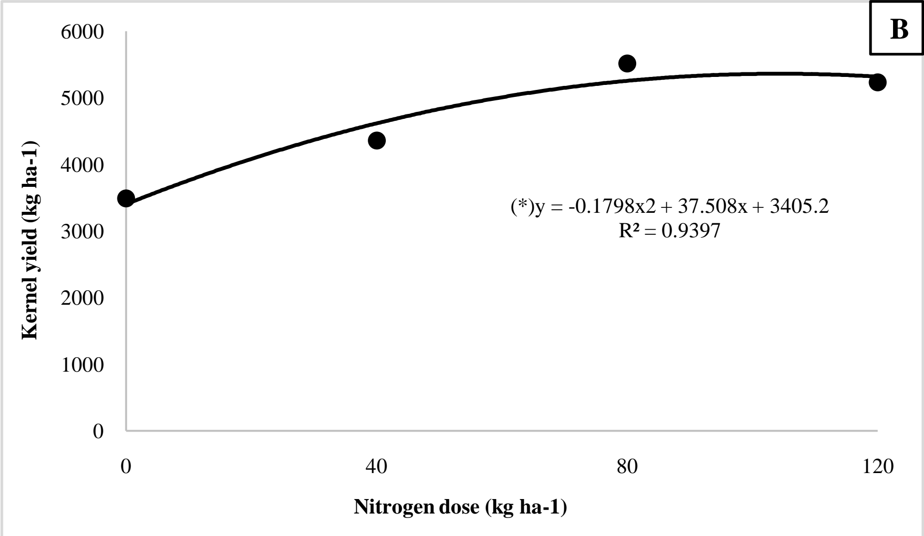


Figure 5 Regression analysis of nitrogen dose (A) weight of 1000 grains, (B) maize yield

In Table 3, the average grain yield showed a positive response to inoculation, with the highest result achieved by applying *A. brasilense* (4774.89 kg ha^{-1}). This result was more than 5.3% higher than the yield recorded without applying the bacteria. Similar results were obtained by Barbosa et al. (2022), who reported a 5.4% increase in maize grain yield when using bacterial inoculation compared to the control. The increase in yield could be attributed to greater plant development, more grains per cob, and higher grain mass, as previously reported by Oliveira et al. (2018). It is worth noting that according to Hungria (2011), the reaction of Gramineae and the plants' genetic characteristics can influence inoculation, the bacterial strains used, and environmental conditions.

A significant increase in grain yield was observed when applying nitrogen (N) to maize crops, as shown in Figure 5 B. Regression analysis revealed that the adjusted quadratic equation was substantial. The maximum yield efficiency was 5361.33 kg ha^{-1}, and the optimal dose of nitrogen fertilizer was 104.30 kg ha^{-1}. Several previous studies also support the positive effects of nitrogen fertilization. For instance, Mota et al. (2015) found that linear yield increases were obtained for nitrogen doses in maize crops, while Soratto et al. (2011) and Pereira et al. (2022) reported a quadratic increase in yield in response to increasing doses. Galindo et al. (2019) recommend using 100 kg ha^{-1} of N, in combination with the application of *A. brasilense*, to achieve the highest profitability in maize production, and similar results were obtained in this study.

Conclusion

According to the study, using nitrogen fertilizers on maize crops has a beneficial effect on factors associated with maize productivity. These were analyzed using a quadratic model. Moreover, most of these factors positively reacted to the introduction of *A. brasilense*. The research discovered that using *A. brasilense* led to a 5.3% average increase in maize yields.

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HPLC based Phytochemicals Analysis of *Phyllanthus emblica* (Indian Gooseberry/Amla): A mini Review

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ABSTRACT

High-Pressure Liquid Chromatography (HPLC) is an analytical tool extensively used for the scientific analysis of components in a mixture. Several reports attribute its high analytical potential to bioactive components from different medicinal plants. Hepatic disorders, which have been a major threat to public health for decades, affect cells, tissues, structure, or liver function. The damage caused by this can be triggered by biological causes, autoimmune diseases, excessive alcohol consumption, or the action of different compounds, for instance, few medicines. *Phyllanthus emblica* Linn. is a therapeutic plant that has been used to treat liver disorders in Asia for many decades now. It is mentioned in the Ayurvedic scriptures for its medicinal value. This review focuses on the intricacies of HPLC-based extraction and analysis of medicinally important phytochemicals, notably for hepatic disorders from the *P. emblica* plant. This will be useful for future phytochemical analysts working with medicinal plants.

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1 Introduction

Herbal products have long been used for medicinal purposes in the Asian subcontinent and their growing demand throughout the globe (Siddiqui 1993). The therapeutic benefits of plants are attributable to their less harmful nature, as well as the fact that they are inexpensive and effective (Payyappallimana 2009). Many medicinal or nutritional plants, as well as their compounds, are capable of delaying disease progression at various stages (Surh 2002). *Phyllanthus emblica* (Amla) is a prominent element in several herbal formulations, including patented pharmaceuticals (Gantait et al. 2021). It is a high-value plant in ancient systems of treatment. Fruits of Amla have generally been broadly investigated for their secondary metabolites, natural ingredients, and remedial use, and found to have a wide range of remedies like antidiabetic, antioxidative, hepatoprotective, immuno-modulatory, and other activities (Khan 2009; Kumar et al. 2012; Singh et al. 2015). To determine various physicochemical features of natural products, a number of analytical tools are applied. High Pressure Liquid Chromatography (HPLC) is an analytical technique that is used for the partition, purification, and quantitative analysis of components in a mixture. Several reports attribute its high value to the separation and purification of bioactive components from different parts of the *P. emblica* plant. Therefore, we have done a thorough review of the detection, separation, and purification of different phytochemicals from *P. emblica* having hepatoprotective activities.

2 HPLC based detection of medicinally important phytochemicals from amla plant

The constituents of distinct *Phyllanthus* species may be easily characterized by using HPLC with Mass Spectroscopy (MS), which is a potent analytical method. According to the time of retention (RT) and UV data, the majority of qualitative and quantitative assessments of phenolics in *Phyllanthus* species are reported by High Pressure Liquid Chromatography or High Performance Thin Layer Chromatography (Tripathi et al. 2006; Dhalwal et al. 2006; Dey et al. 2016). For the first time in the *Phyllanthus* species, thorough and original investigations of phenolics revealed the presence of nearly 10 different compounds derived from phenol, including Gallic acid (GA), five Gallic acid derivatives, Ellagic acid (EA), and three Ellagic acid derivatives as shown in Figure 1 (Rose et al. 2018). Using several separating solvents (both non-polar and polar), a recent prospective study

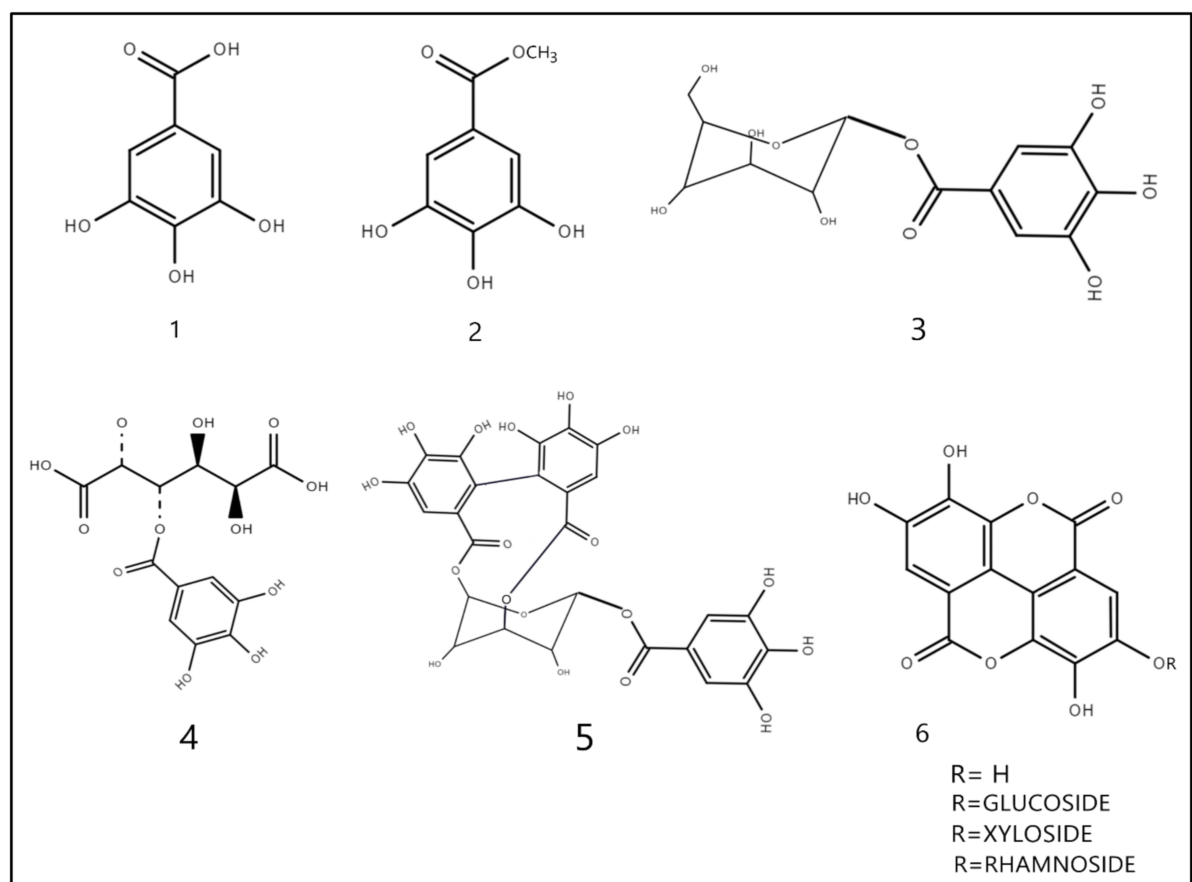

Figure 1 Structures of five GA derivatives (1-5), ellagic acid (6), and three ellagic acid derivatives obtained from Amla (Haddock et al. 1982; Shu et al. 2010; Yang et al. 2012; Rose et al. 2018)

revealed the presence of numerous phenols, flavonoids, and a plethora of other phytochemicals in dried fruits of Amla. The ethanolic extract of *P. emblica* fruits was screened for phytochemicals using conventional techniques. The presence of different primary metabolites like proteins, carbohydrates and many more secondary metabolites were discovered in the ethyl alcoholic extract of *P. emblica* fruits.

The whole phenolic and GA content of amla fruit, seed cover, and seed were measured using HPLC by Mishra and Mahanta (2014). With the comparison according to the standard, GA is found to be the major product among other phenolics in all of the 3 samples investigated; this conclusion accorded with Kumar et al. (2012), who stated that tannic acids and GAs are the crucial phenolics in Amla. Different polyphenols and other beneficial substances are observed in the fruit of *P. emblica* (Habib-ur-Rehman et al. 2007). The tannins emblicanin A, emblicanin B, phyllaemblicin B, pedunculagin, and punigluconin are also found in *P. emblica* fruit extract (Kapoor 2000). According to Liu et al. (2008) phytochemicals isolated from *P. emblica* fruit powder in methanolic extractions have various antioxidant properties.

Recently, β-sitosterol and Quercetin were discovered in alcoholic extracts of *P. emblica* leaves by Gupta et al. (2014). Balasubramanian (2014) found five main phytoconstituents in the methyl alcoholic extract of amla leaves, including Pyrogallol, 5-methyl-2-furylmethyl ketone, benzene carboxylic acid, and GA, Embrirol A, and B, two novel chalconoid analogs with the chemical formula $C_{19}H_{24}O_6$ were recently isolated from the lower parts of Gooseberry tree (Yan et al. 2017). It contains several important flavonoids as well as GA. Ellagi Tannins have a variety of functions and are found in many herbal medications (Krishnaveni and Mirunalini 2010; Variya et al. 2016). Besides, many other investigations have shown its importance in the mammalian body, particularly in liver detoxification and hepatotoxicity (Anilakumar et al. 2007; Reddy et al. 2009a) and lipid metabolism (Balusamy et al. 2020; Akhtar et al. 2011). Yugarani et al. (1993) and Kaleem et al. (2014) used the calorimetric method to extract tannins from freshly made fruit juice in powdered form, and the presence of tannic acid was recognized and validated using the HPLC technique. Starting from mucic acid, GA, and several other compounds in the crude methanolic fruit extracts, according to the author, who also reported 144 peaks identifying other compounds (Pramyothin et al. 2006; Khan 2009; Sharma et al. 2009). Preclinical investigations have demonstrated that amla extract and the phytochemicals ellagic acid, other polyphenols along with kaempferol derivatives are efficient as hepatoprotective agents against chronic liver damage caused by ethanol, mycotoxin, arsenic, and paracetamol (Kinoshita et al. 2007; Girish et al. 2009; Gaire and Subedi 2014; Yang and Liu 2014) (Figure 2).

3 Hepatoprotective behavior of the extracted compounds

Liver diseases remain a threat to humanity even though good advances occurred in the field of assessment and management of patients (Asrani et al. 2019). Compounds isolated from *P. emblica* exhibit hepatoprotective capabilities in several ways, including reducing inflammation, eliminating reactive oxygen species (ROS), inhibiting tumour growth, and so on (Figure 3). *P. emblica* fruits include various ethnomedical extracts rich in vitamin C, which have long been used in Unani and Ayurveda medicines in addition to traditional medicine (Poltanov et al. 2009)

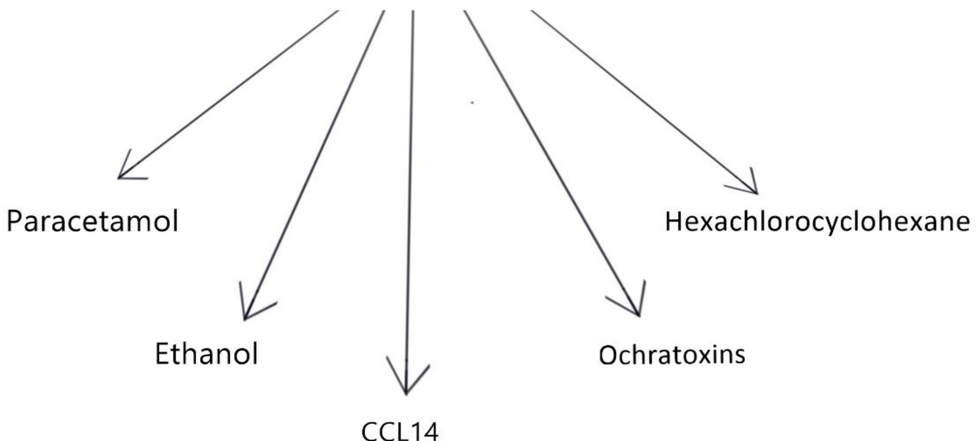

Figure 2 Hepatoprotective nature of Amla

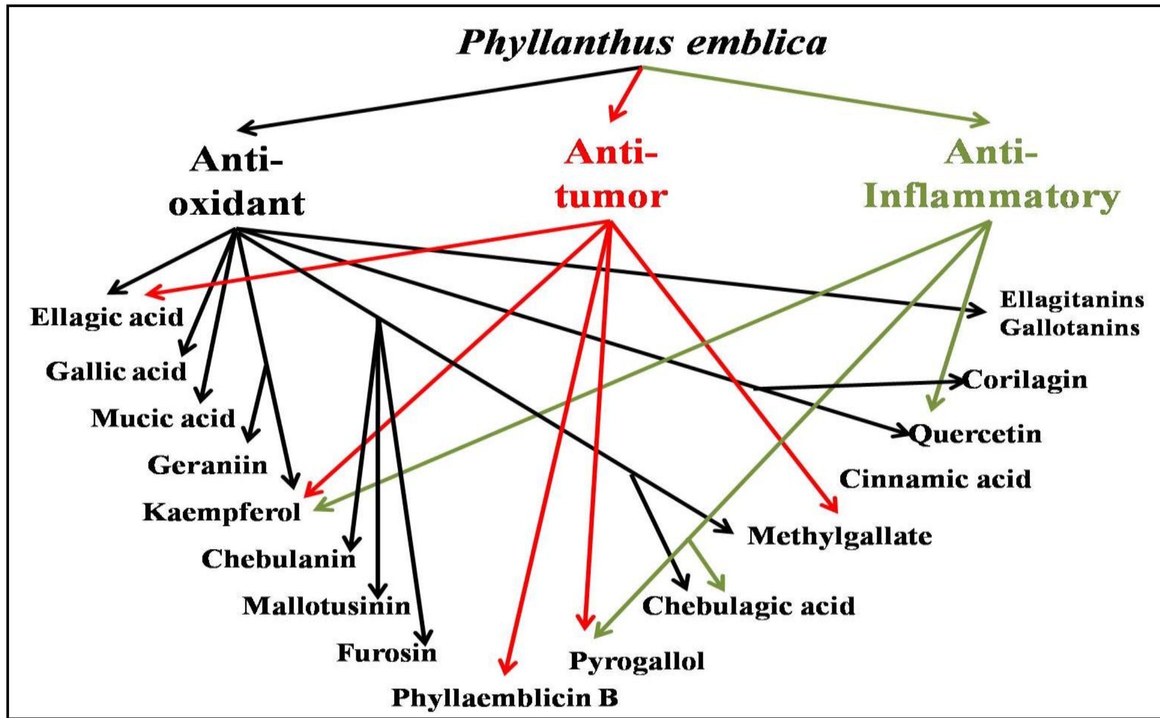

Figure 3 Pharmacological effects of isolated compounds from *P. emblica* showing hepatoprotective activities.

The fruit extracts have a mixture of different phytochemicals and compounds (Muthuraman et al. 2011; Dey et al. 2016). Phenolic extracts from gooseberry fruits are good Antiinflammatory agents when applied in combination with diclofenac (Middha et al. 2015). Butanol extract of *P. emblica's* fruit fraction also exhibits this property in indomethacin-induced gastric cancer (Zhu et al. 2013). The water and polyphenolic extracts of the fruit also showed anti-tumour effects against the ovarian and cervical cancer cells (Kang et al. 2006; Karimi et al. 2020).

Ellagic acid (EA) is a vital constitutes having antioxidant activity along with antiinflammatory responses (Singla et al. 2010). EA suppresses Hepatitis B-e antigen (HBeAg), thus plays a significant role in the reduction of hepatitis virus infection in HBV-infected as well as HBeAg transgenic mice (Fabbrini et al. 2010). EA is also effective against fatty liver disease as it decreases blood triglyceride concentration by increasing serum cholesterol HDL level (Bashar et al. 2021; Sanjay et al. 2021).

The phenolic compound Gallic acid (GA) is a well-established biochemical with therapeutic and pharmacological properties (Esmaeilzadeh et al. 2020). When GA is combined with anti-tuberculosis therapy, a better result is obtained via the reduction of liver function enzymes by maintaining homeostasis through Nrf2 activation and NFκB signalling pathway inhibition with respect to anti-tuberculosis therapy alone (Huang et al. 2021). It also has a very good antioxidant property for which administration of GA results in a significant increase in different enzymes like GSH, CAT, etc., and a remarkable decrease in protein carbonyl, Aspartate transaminase, Alkaline Phosphatase, etc. (Reyes-Farias and Carrasco-Pozo 2019).

Methyl gallate was reported to function as an anti-cell proliferator compound in both hepatocarcinoma cell lines and in the Zebrafish model (Ren et al. 2019). Flavonoid compound Quercetin showed descent pro-apoptotic activity by regulating several pathways like the PI3K / Akt / mTOR, etc. Further, Lin et al. (2011) and Yang et al. (2012) reported the antioxidant and antiinflammatory effects of Quercetin in the db/db mice model. Another flavonoid phytochemical constituent of *P. emblica* extract Kaempferol or kaempferol-3, was studied for the antiinflammatory and radical scavenging effects (Lin et al. 2011). Moreover, it has anti-tumour potential by inducing TNFα mediated tumour necrosis and increasing interleukin-1 (IL-1) by macrophage activation (Reddy et al. 2009b; Yang and Liu 2014).

The extraction of the different parts of the *P. emblica* containing ellagitannins and gallotannins (Reddy et al. 2009b) showed antioxidant effects in mice models (Zhang et al. 2001; Reddy et al. 2010a, b; Koo et al. 2016). Further, Zhang et al. (2001) reported significant radical scavenging activity of mucic acid and its derivatives like mucic acid 2-O-gallate and other lactone compounds, etc. The study by Lee et al. (2007) mentioned that chebulic acid inhibits hepatic fibrosis by regulating the Nrf2 pathway along with oxidative stress mitigation (Kanter 2010).

Conclusion and Future Prospects

The phytochemical Quercetin, which can be found in Amla, has been shown to help prevent liver injury caused by biliary hindrance. Healing with Quercetin before general bile duct constriction reduced changes in liver histology in rats, implying that it protects against liver damage, bile duct increase, and thickening or scarring of the tissue. Amla has been found to protect the liver from a number of toxins, including ethyl alcohol, analgesics, carbon tetra chloride, heavy metals, antitubercular medicines, and many more.

Amla treatment has been proven to reduce hyperlipidemia, metabolic syndrome, liver carcinogenesis, and liver toxicity caused by iron overload. In *P. emblica*, Quercetin, GA, corilagin, and ellagic acid were found to have liver protective effects against known analgesics, microcystins, galactosamine, and lipopolysaccharide toxicity. *P. emblica's* antioxidant, antiinflammatory, and hypolipidemic properties, as well as modulation of detoxifying enzymes, appear to have hepatoprotective properties. In light of these findings, it is reasonable to conclude that Amla needs further research, particularly in the high-risk category. The antioxidant capabilities of *P. emblica* are particularly important because oxidative changes of LDL cholesterol cause the hardening of arteries. More study, particularly on the effects of long-term use patterns, is required. With a growing interest in NSAIDs as a treatment for chronic swelling, research on the use of foodstuff extracts such as *P. emblica* is gaining attraction. In the future, additional scientific evidence depicting the advantages of *P. emblica* in overall health preservation and disease prevention should become available.

Conflict of Interest

There is no conflict of interest.

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Antibiotic-Induced Changes in Efflux Transporter Expression: A Key Factor in *Pseudomonas aeruginosa* Biofilm Resistance

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ABSTRACT

Listed by WHO as an antibiotic-resistant priority pathogen, *Pseudomonas aeruginosa* (P.A.) is a serious threat in nosocomial infections. Its high antibiotic resistance is attributed to major mechanisms that can be categorized into intrinsic, acquired, and adaptive resistance. This study tests the ability of three commonly used antibiotics to inhibit new biofilm formation and eradicate mature biofilm growth, as well as investigate changes in the expression levels of selected genes coding for multidrug efflux pumps in P.A. planktonic cells and biofilms before and after treatment with antibiotics to provide a conceptual estimate of the activity of the efflux transporters that work to extrude antibiotics leading to a reduction in their effectiveness. Antimicrobial susceptibility testing was conducted with Ofloxacin (OFLX), Tobramycin (TOB), and Ceftazidime (CAZ) to determine Mean Inhibitory Concentration (MIC) and Mean Bactericidal Concentration (MBC) using microtiter plate-based biofilm assay and spectrophotometric quantification. Extraction of total RNA was performed from planktonic cultures, inhibition phase, and eradication phase P.A. biofilms. Real-time quantitative reverse transcriptase PCR was utilized to analyze the changes in expression of the *mexAB*, *mexXY*, and *oprM* genes. Three (3) antibiotics that have proven to show less resistance are OFLX, TOB, and CAZ when tested against overnight cultures of P.A. strain PA01. Results showed that OFLX is best for bactericidal properties, which is also supported by the viability assay data obtained from Propidium Iodide staining. Our study showed that the PAO1 strain is susceptible to OFLX for both inhibition and eradication of mature biofilms. TOB was most effective at higher concentrations in the eradication phase.

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1 Introduction

Pseudomonas aeruginosa is a gram-negative, rod-shaped opportunistic bacterium and one of the most common nosocomial pathogens with high mortality rates (Sousa and Pereira 2014). *Pseudomonas* can easily grow in hospital water systems, humidifiers, and other types of medical equipment such as catheters, ventilators, and pacemakers (Walker and Moore 2014). Further, it can cause both acute and chronic infections as the course of the different infections can vary greatly. Acute infections often involve planktonic bacteria, which are usually treatable with antibiotics, while chronic infections are often difficult to treat, where the bacteria forms into a biofilm. The presence of this organism in biofilm communities exhibits a high degree of Multidrug Resistance (MDR), which makes biofilm-based bacterial infections such as Chronic Obstructive Pulmonary diseases (COPD) which is very challenging to treat. Prevention and treatment strategies are limited by the lack of sensitive detection methods and by the narrow availability of effective antibiotics (Lund-Palau et al. 2016). Thus, in addition to other health complications of *P. aeruginosa*, it contributes to a heavy cost burden on the hospital system.

P.aeruginosa's major resistance mechanisms to antibiotics can be classified as either intrinsic, acquired, or adaptive resistance. Intrinsic resistance may be associated with lower outer membrane permeability, expression of efflux pumps, and production of antibiotic-inactivating enzymes. Acquired resistance involves the horizontal transfer of resistance genes or mutational changes, and adaptive resistance may be characterized by the formation of biofilm or multidrug-tolerant cells in the biofilm. The active efflux pumps of antibiotics contribute to the bacterial multidrug-resistant phenotype; therefore, the development of efflux pump inhibitors is a promising adjuvant therapy (Li et al. 2015).

Several studies have shown efflux pumps may play a significant role in the multidrug-resistant phenotype and involvement in the efflux of bacterial factors essential for virulence (Alav et al. 2018). Efflux pumps are membrane proteins that are involved in the export of foreign substances within the bacterial cell. They can pump out a wide range of substrates, including antibiotics, dyes, toxins, waste metabolites, and detergents. The *P.aeruginosa* genome is predicted to encode multiple RNA efflux pumps, four of which are major role players, i.e. MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (Marquez 2005; Poole 2011). MexAB-OprM and MexXY-OprM, the RND-type efflux pumps, account for the major cause of intrinsic resistance to most antimicrobial agents in *P.aeruginosa* (Goli et al. 2018). Both MexXY-OprM and MexAB-OprMare are known to be some of the largest multidrug-resistant efflux pumps within the resistance nodulation division (RND) family in *P.* aeruginosa.

The pumps extrude antimicrobials across the outer membrane, which explains their ability to confer resistance to beta-lactams that target the cell wall synthesis. The pump is comprised of three different peptides, i.e. a MexB translocase belonging to the RND family of solute/proton antiporters, an outer membrane porin-OprM, and a "membrane fusion protein" MexA that docks MexB to OprM. Moreover, the active efflux system MexXY-OprM contributes to aminoglycoside resistance. MexXY-OprM is a multidrug efflux transporter whose specificity is extraordinarily broad but different compared with MexAB-OprM, MexCD-OprJ, MexEF-OprN, and other RND efflux transporters in *P.aeruginosa* (Masuda et al. 2000; Morita et al. 2012). Additionally, the overproduction of the major efflux pumps contributes to the carbapenem resistance in *P. aeruginosa* (Lee and Zhang 2014; Hassuna et al. 2020).

The broad specificity of MDRs seems to match the resistance of biofilms to antimicrobials qualitatively. Biofilms can show very high levels of resistance, and it is unclear whether mechanisms operating in planktonic cells that confer lower levels of resistance play a key role in biofilms. This study utilizes the hypothesis that the deletion of individual pumps or pairs of pumps would make *P.aeruginosa* more susceptible to antibiotic treatment and observe increased activity or mutations (if any) in other pumps quantified by reverse transcription polymerase chain reaction. The objectives of this study were to determine the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of *P.aeruginosa* PA01 biofilm and to elucidate the role of the efflux pumps in antibiotic resistance in biofilms by comparing the expression levels of *mexA*, *mexB*, *mexX*, *mexY*, and *oprM* genes in *P.aeruginosa* planktonic cells and biofilms before and after treatment with antibiotics.

2 Materials and Methods

2.1 Bacterial strains, growth media, and conditions

The *P. aeruginosa* strain PA01 utilized in this study was preserved by our laboratory at −80°C. Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) media were employed to culture the planktonic form of *P. aeruginosa* PA01 bacteria, whereas M63 media supplemented with arginine, glucose, and casamino acid were used for the formation of PA01 biofilm.

2.2 Literature Search

A literature search was conducted from August 2020 to December 2020. Studies on current antibiotic treatment regimens for *P.aeruginosa* and various efflux transporters were collected. Tobramycin (TOB), Ofloxacin (OFLX), and Ceftazidime (CAZ) are promising antibiotics for current *P. aeruginosa* treatment. In addition to a literature search, a Basic Local Alignment Search Tool (BLAST) was utilized, which allowed the finding of regions

with similarity between biological sequences of *mexA, mexB, mexX*, and *mexY* genes of the PA01 strain by comparing it to select genes of various strains that have been previously studied extensively and are well documented in literature with regards to how they function under different stressors. This is an essential tool for biologists since its efficiency and sensitivity allow scientists to compare nucleotide and protein sequences to both single sequences and large databases to design a primer.

2.3 Planktonic cell growth

The planktonic cell growth was measured using a microtiter plate-based assay (Qu et al., 2016). Overnight-grown PA01 cells were diluted 1:100 in L.B. broth. These samples were then grown at 37°C with agitation (160 rpm). The planktonic culture turbidity was read using an Eppendorf spectrophotometer by measuring absorbance at 550nm every hour.

2.4 Determination of Minimal Inhibition Concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by the broth microdilution method described by the CLSI 2015. Using McFarlan 0.5 solution as a standard for turbidity of the overnight broth culture, 10 µl of *P. aeruginosa* was dispensed per well in a 96-well plate. The various concentrations of antibiotics were added, and the volume was adjusted with TSB to 100 µl in each well. Each antibiotic (TOB, OFLX, and CAZ) was diluted to a final concentration of 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, and control. The plates were incubated overnight at 37°C. MIC was established as the lowest concentration with the absence of any visible bacterial growth.

2.5 Determination of Minimal Bacterial Concentration (MBC)

The MBC of planktonic *P. aeruginosa* was determined for the four antibiotics according to the Manual of Antimicrobial Susceptibility Testing published by the American Society of Microbiology (2005). An overnight PAO1 plate was incubated with 3 loops full of cells in 150 ml of TSB on a shaker at 37°C. Ten-milliliter (10 mL) of culture were taken during the logarithmic phase into 36 sterile tubes (triplicated) and standardized to OD600 before treating with the various concentrations of antibiotics (Ofloxacin: 0.5 µg/ml, 1µg/ml, and 32 µg/ml, Ceftazidime: 4 µg/ml, 8 µg/ml, 16 µg/ml, Tobramycin: 0.5 µg/ml, 1µg/ml, 2 µg/ml). The antibiotic-treated culture was serial diluted 10^{-1} to 10^{-7} and plated. The plates were incubated at 37°C overnight. CFUs and MBC were established for each plate.

2.6 Biofilm Eradication and Inhibition Assay

Newly formed biofilm growth inhibition was analyzed by exposing the culture to antibiotics at the time of inoculation. In contrast, the eradication of mature biofilms was measured by adding antibiotics 24 hours after inoculation, allowing the formation of mature biofilm first, followed by spectrophotometric quantification of the residual biofilm using a modified Microtiter Dish Biofilm Formation Assay (O'Toole 2011).

The biofilm inhibitory assay was carried out in microplates (Qu et al. 2016). 4 µl bacterial suspension from 1: 100 overnight culture and 196 µl TSB was dispensed per well in a 96-well microplate and exposed to different concentrations of the 3 antibiotics as described before, followed by incubation for 24 hours at 37°C. After incubation with antibiotics, the plates were washed with deionized water three times and dried for 4-6 hours. The dried biofilms were stained with a 0.1% solution of crystal violet and incubated at room temperature for 10-15 minutes. Excess stain was removed by rinsing the plate wells three times with deionized water and drying overnight. The residual biofilm biomass in the plate wells was quantified by dissolving the crystal violet attached to the cells in the biofilm in 30% acetic acid and reading the absorbance (O.D. at 550nm) plate reader spectrophotometer.

For biofilm eradication, 100 µl bacterial suspension from 1:100 overnight culture was dispensed per well in microplates. After static incubation at 37°C for 24 hours to allow the formation of a mature biofilm, the supernatants were removed, and the wells were treated with various concentrations of antibiotics. The plates were incubated for 24 hours at 37°C, and biofilm biomass was determined as previously described.

2.7 Gene expression analysis

Upon determining the MIC/MBC, concentrations of 8 µg/ml and 32µg/ml of TOB, OFLX, and CAZ were utilized for MIC and MBC, respectively. These concentrations were selected because this is where inhibition (at 8 µg/ml) and eradication (at 32µg/ml) were best observed. Total RNA from the selected concentrations was extracted using RNeasy Mini kit (Qiagen) instructions with RNA protect®Bacteria Reagent added at the required step as indicated in the RNeasy kit instructions. RNA purity and concentration were determined by measuring the absorbance of the sample at 260 and 280 nm (260/280 nm), and 1µL of RNA was used for cDNA synthesis, which was done using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix (Transgene, China).

Expression of the efflux transporter genes was measured by qRT PCR amplification and quantification using the synthesized cDNA. qRTPCR was performed using TransStart™ Green qPCR SuperMix UDG kit (Transgene, China). Conditions for qRT-PCR were the following: 50°C for 2 minutes, initial denaturation at 94°C for 10 minutes, 40 cycles of 5 seconds at 94°C, and 30 seconds at 60°C. The data obtained were normalized to the

endogenous reference gene *pslA* of the *P. aeruginosa* PA01 strain. The threshold cycle method ($2^{-\Delta\Delta CT}$) was used to analyze changes in gene expression in each sample relative to the control, qRT-PCR studies were performed in triplicate using the ProtoScript® First Strand cDNA Synthesis Kit, and the entire experiment was repeated twice with RNA samples extracted from independent cultures.

3 Results and Discussion

3.1 Selection of primers for gene expression studies

The BLAST tool available on the National Library of Medicine website was utilized to align and compare the DNA sequences of the genes *mexA*, *mexB*, *mexX* and *mexY* (Figure 1), which were selected for further studies on effect of antibiotics on planktonic cells and biofilms formed by the *P. aeruginosa* PA01 strain based on literature search to design the primers needed to facilitate the amplification and quantification of gene expression levels by quantitative real-time polymerase chain reaction (qRTPCR).

The BLAST alignment tool was used to find regions of similarities between the biological sequences of the four efflux transporter genes *mexA*, *mexB*, *mexX* and *mexY*, from *P. aeruginosa* PA01 and that of similar genes from other strains that are documented in the literature. The sequences of the primers used for the qRT PCR-based amplification and quantification of cDNA obtained from the transcribed RNA isolated from the tested samples for analysis of relative expression of these genes, which code for proteins functioning as efflux transporter pumps known to have a potential role in active extrusion of antibiotics from bacterial cells (Bhandari et al. 2022; Lorusso et al. 2022), are listed in Table 1.

The functions of MexA, MexB, MexX, and MexY are primarily transport to structurally varied molecules, including antibiotics out of the bacterial cell. It also functions to lower the intracellular concentration of antibiotics, allowing *P.aeruginosa* to survive at higher antibiotic concentrations, thereby leading to antibiotic resistance (Table 1). In addition, the ribosomal subunit (RpsL) responsible for rRNA and tRNA binding and is a structural constituent of the ribosome is expressed constitutively and therefore

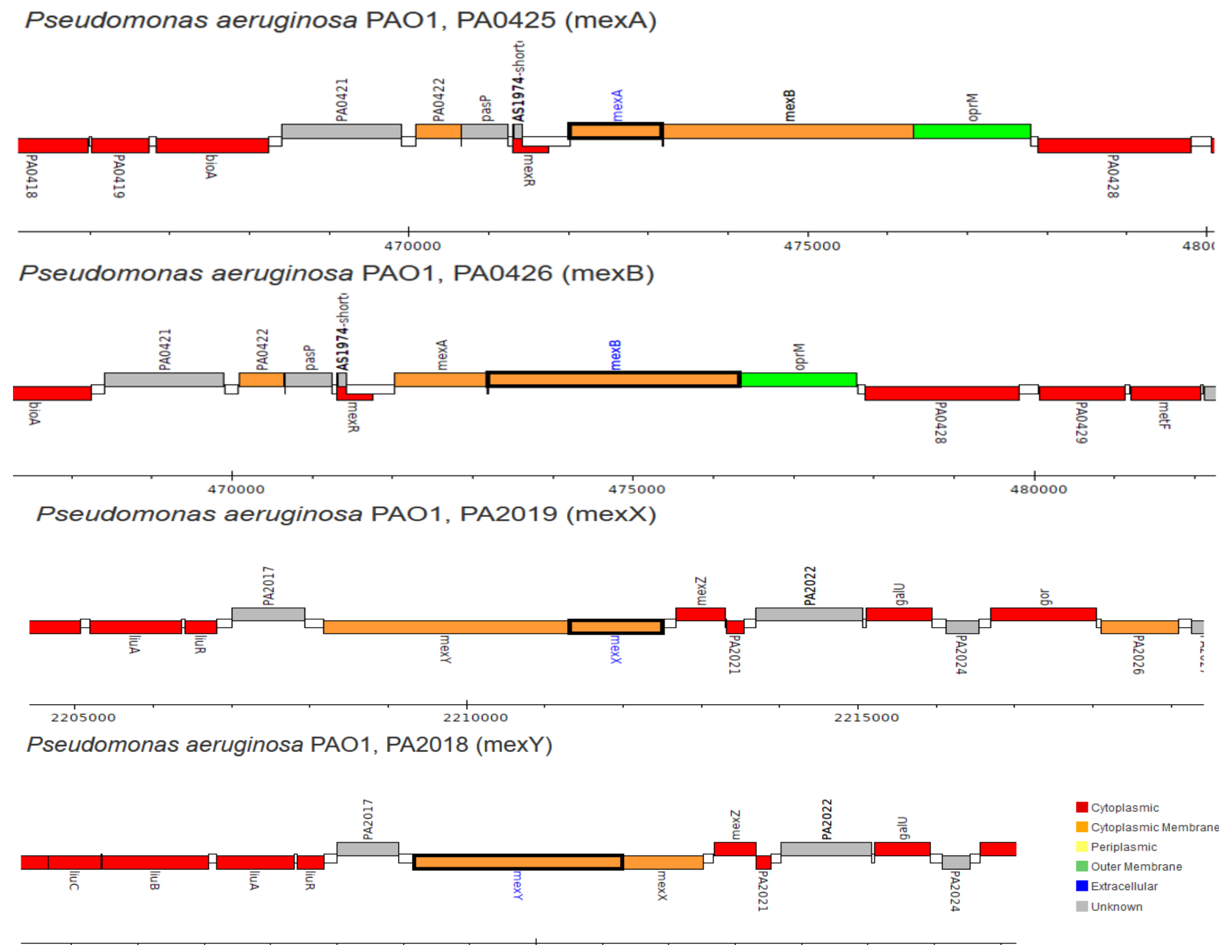


Figure 1 BLAST results for the four main genes responsible for MexA, MexB, MexX, and MexY efflux transporters.

Table 1 Summary of the Functions of Efflux Transporters MexA, MexB, MexX, and MexYin Bacteria.

Protein	Function	Role in Antibiotic Resistance	Primer Sequence
Multidrug-Resistant Efflux Pump *Mex*A	Resistance Nodulation Cell Division (RND) multidrug efflux Periplasmic membrane fusion protein precursor	Transports structurally varied molecules, including antibiotics, out of the bacterial cell	F: 5'-acctacgaggccgactaccaga-3' R: 5'-gttggtcaccagggcgcctc-3'
Multidrug Resistant Efflux Pump *Mex*B	Resistance Nodulation Cell Division (RND) Inner membrane multidrug efflux Transporter protein	Transports structurally varied molecules, including antibiotics, out of the bacterial cell	F: 5'-gtgttcggctcgcagtactc-3' R: 5'-aaccgtcgggattgaccttg-3'
Outer Membrane Protein *Opr*M	Major intrinsic multiple antibiotic resistance efflux outer membrane protein OprM precursor	Channel-forming outer membrane protein	F: 5'- ccatgagccgccaactgtc-3' R: 5'-cctggaacgccgtctggat-3'
Multidrug Resistant Efflux Pump *Mex*X	Resistance Nodulation Cell Division (RND) multidrug efflux membrane fusion protein Mex X precursor	Transports structurally varied molecules, including antibiotics, out of the bacterial cell	F: 5'-tgtacgcgtattcggaacaaggcgtctgc-3' R: 5'-ttctgctagcgatgtgcatgggtgtccctc-3'
Multidrug Resistant Efflux Pump *MexY*	Resistance Nodulation Cell Division (RND) multidrug efflux transporter *MexY*	Transports structurally varied molecules, including antibiotics, out of the bacterial cell	F: 5'-tgtactagttgatgcccctagcgaaactctc-3' R: 5'-tttaagcttgacctacaggacgctgctg-3'
Ribosomal Subunit *RpsL*	Ribosomal subunit binding rRNA and tRNA expressed constitutively	Structural constituent of ribosome, which serves as Internal control	F: 5'-gctgcaaaactgcccgcaacg-3' R: 5'-acccgaggtggtccagcgaacc-3'

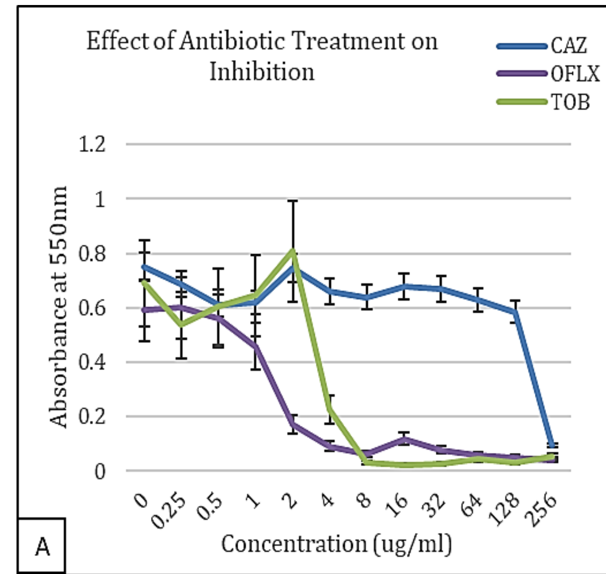
utilized as the internal control for evaluating the changes in level of expression of the efflux transporters in response to biofilm formation and presence of various antibiotics (Smith and Iglewski 2003; Rutherford and Bassler 2012; Kishk et al. 2020; Lorusso et al. 2022).

3.2 Effectiveness of different antibiotics for inhibition and eradication of *P. aeruginosa* biofilm

The ability of the 3 antibiotics ceftazidime [CAZ], ofloxacin [OFLX] and tobramycin [TOB] to inhibit biofilm formation and eradicate mature biofilms in the *P. aeruginosa* PA01 strain were compared by determination of their Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of the antibiotics. The eradication data compares the effectiveness of these antibiotics on a mature biofilm, which represents clinical biofilm infections. In contrast, the inhibition data compares the effects of the antibiotics mentioned above on a biofilm starting to form from free-living or planktonic cells.

Results presented in Figure 2 revealed the effects of antibiotic application time and concentration on the inhibition of biofilm formation. The antibiotics are applied at the time of inoculation (a) and biofilm eradication (n = 6), where the antibiotic is applied after mature biofilm formation (b) in various concentrations. The x-axis represents the various concentrations of the three antibiotics used, and the y-axis represents the inhibition or eradication of *P. aeruginosa* biofilms obtained because of treatment with the antibiotics as measured by spectrophotometric absorbance at 550 nm to quantify the residual biofilms.

For comparing the effectiveness of the three tested antibiotics for the inhibition of *P. aeruginosa* biofilms, the effect of various concentrations of each antibiotic on the biofilm-forming capacity was assessed using the micro broth format in order to determine the Minimum Inhibitory Concentration (MIC) values which showed dose-dependent sensitivity of PA01 biofilms to both ofloxacin and tobramycin at 8μg/ml concentrations and high resistance to ceftazidime in inhibition phase (Figure 2A). On the other hand, the effectiveness of the three tested antibiotics for eradication of *P. aeruginosa* biofilms were compared by measuring the residual biofilm after incubation of a mature biofilm with various concentrations of each antibiotic to determine the corresponding Minimum Bactericidal Concentration (MBC) values, where a significant resistance to all antibiotics except for ofloxacin was exhibited. In the eradication phase, *P. aeruginosa* biofilms displayed sensitivity to ofloxacin but at much higher concentrations (32 μg/ml) compared to that seen in the inhibition phase, in addition to sensitivity at only the highest concentration of tobramycin (256 μg/ml). The highest level of resistance was observed for ceftazidime, which showed a lack of sensitivity even at the highest concentration, and also displayed significant resistance to tobramycin at lower concentrations, respectively, during the eradication phase (Figure 2B). Antibiotics were applied at the time of inoculation in the 96-well microtiter plate for inhibition, while antibiotics were applied 24 hours after 37°C inoculation for eradication. The optical density of each sample at 550 nm was utilized for collection of data on the inhibition of young biofilms and eradication of mature PA01 biofilms after incubation with increasing concentrations of tobramycin,



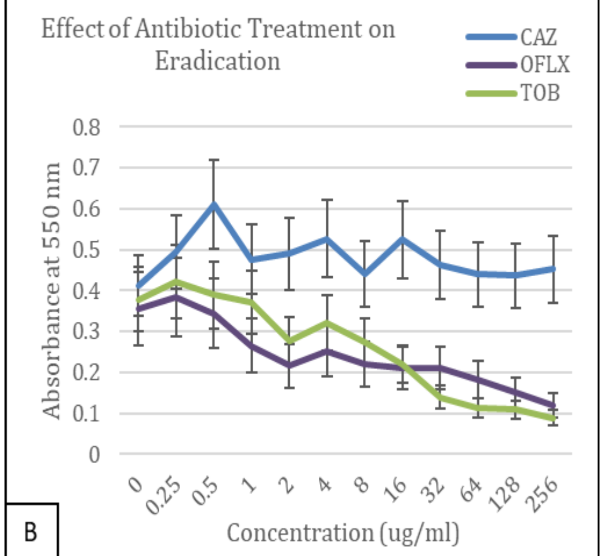


Figure 2 Effect of increasing concentration of the antibiotics ceftazidime[CAZ], ofloxacin [OFLX]and tobramycin [TOB] on (A) inhibition and (B) eradication *of P. aeruginosa* biofilm

ceftazidime, and ofloxacin. Overall, ofloxacin showed the greatest inhibitory and eradicative effect on *P.aeruginosa,* while ceftazidime proved the least effective (Figures 2A and 2B) in both conditions. These observations are corroborated by the data reported by recent studies, which indicated that *P. aeruginosa* biofilms would not be eradicated with low-dose tobramycin (Mangiaterra et al. 2020) and exhibit resistance to beta-lactams, aminoglycosides, fluoroquinones and carbapenems (Zakhour et al. 2022) through processes such as production of carbapenemase, and upregulation of efflux pumps (Hassuna et al. 2020).

3.3 Changes in expression of efflux transporter genes in *P. aeruginosa* during biofilm formation

The expression levels of the genes coding for efflux transporters systems MexAB-OprM and Mex XY-OprM were compared in PA01 planktonic cells and biofilms to elucidate changes in their expression in response to factors that trigger the formation of biofilms, which are known to be more resistant than planktonic cells, to inhibition as well as eradication by treatment with antibiotics (Patel et al. 2021).

The expression of the MexAB-OprM and MexXY efflux transporter genes was measured using qRT PCR to compare their expression in planktonic cells and biofilms. All 5 genes showed some degree of upregulation in the biofilm stage as opposed to the planktonic stage (Figure 3), which is also supported by recently published reports showing that a significant majority of antibiotic-resistant clinical isolates of *P. aeruginosa* possessed *mexA* and *mexB* genes which indicates the presence of active efflux-pump system (Bhandari et al. 2022) and their contribution to the higher antibiotic resistance shown by biofilms formed by clinical isolates of *P.aeruginosa* (Hassuna et al. 2020). The isolates possessed mexA and mexB genes, indicating the presence of an active efflux pump system. The *rpsl* gene, which is considered the housekeeping gene, had the same RNA expression levels in both planktonic and biofilm stages.

3.4 Effect of various antibiotics on expression of efflux transporter genes in inhibition and eradication phases

The inhibitory and eradicative effects of ceftazidime, ofloxacin and tobramycin on relative expression levels of the MexAB-OprM and Mex XY-OprM transporters system genes were studied in newly formed and mature PA01 biofilms, respectively, by comparing the relative expression level of *mexA, mexB, mexX, mexY* and *oprM* after exposure to the antibiotics at their corresponding MIC values under inhibitory conditions for newly formed biofilms from planktonic cells and MBC values under eradication conditions for mature biofilms to evaluate the possibility that these efflux pumps are involved in the observed decrease in sensitivity to the tested antibiotics during and after biofilm formation.

Figure 4 represents the relative gene expression levels during (A) inhibition of biofilms on treatment with the antibiotics at their determined MIC and (B) eradication of mature biofilms for tobramycin, ceftazidime, and ofloxacin at their determined MBC values. On comparing the effect of the tested antibiotics on the level of expression of *mexA*, *mexB*, *mexX*, *mexY* and *oprM* genes after 24-hour treatment of newly forming and mature PA01 biofilms with each of them at their determined MIC and MBC values, all of the five efflux transporter genes showed significantly increased expression relative to the internal control during both inhibition and eradication phases (Figures 4A and 4B) when

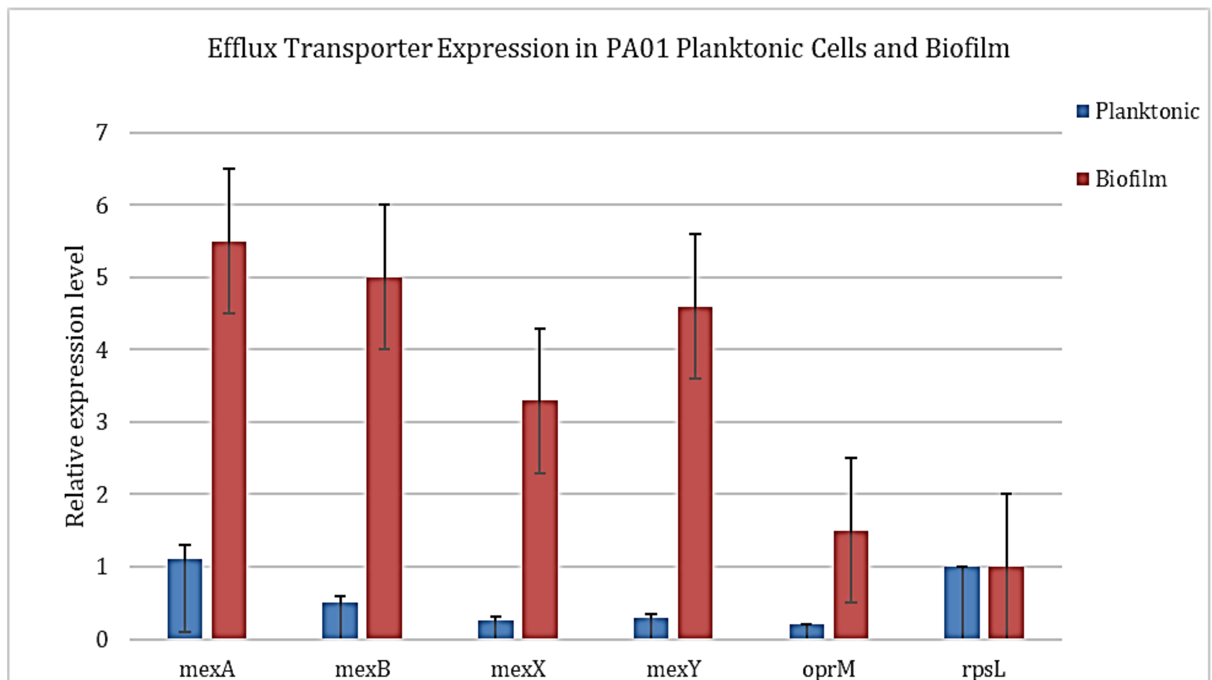


Figure 3 Normalized Expression of Efflux Transporters in PA01 Planktonic Cells and Biofilm; Values represent fold change (mean of triplicate samples) in comparison with the transcription level of the internal control rpsL.

treated with ceftazidime. Earlier studies have reported overexpression of MexAB-OprM systems associated with increased resistance towards cephalosporins (Pourakbari et al. 2016) in *P. aeruginosa* biofilms. On the other hand, treatment with ofloxacin and tobramycin shows a much lesser increase in the expression of *mexA*, *mexB*, *mexX* and *mexY* compared to the expression of *rpsL* during inhibition (Figure 4A) as well as eradication (Figure 4B). This data supports the results obtained on studying the effectiveness of these antibiotics for inhibition and eradication of new and mature *P. aeruginosa* biofilms, which showed that ceftazidime was least effective having the highest MIC and MBC values (Figures 2A and 2B), which could probably be due to the higher expression of the MexAB-OprM and MexXY-OprM efflux pumps leading to increased extrusion of this antibiotic, thus rendering it less useful. Also, both ofloxacin and tobramycin showed comparable effectiveness with similar MIC and MBC values (Figures 2A and 2B), which is backed by the similar level of expression of the efflux pump genes shown after treatment of the PA01 biofilms with ofloxacin and tobramycin under both inhibition and eradication conditions (Figures 4A and 4B).

Empirical antibiotic therapy for suspected cases of *P.aeruginosa* includes monotherapy and combination therapy, such as a β-lactam antibiotic with aminoglycosides. At the same time, the current treatment uses a combination of an antipseudomonal β-lactam (penicillin or cephalosporin) and an aminoglycoside or carbapenem (imipenem or meropenem) with antipseudomonal quinolones with an aminoglycoside. The World Health Organization (WHO) has listed carbapenem-resistant *P.aeruginosa* as one of three bacterial species in critical need of the development of new antibiotics (World Health Organization 2017).

The results reported in this study also show that the efficacy of antibiotics against biofilm formation is time-dependent; the results shown in Figure 2 indicate that less concentration was required to treat an early *P.aeruginosa* infection compared to 24 hours later when a mature biofilm has formed. This study demonstrated that PA01 biofilms are more susceptible to antibiotic treatment in the inhibition phase, as shown by their sensitivity to lower antibiotic concentrations when they are newly forming, compared to the eradication phase when they have matured and only show sensitivity to much higher concentrations. This supports the treatment regimen in monotherapy empirical antibiotic therapy as opposed to combination treatment once the infection is confirmed. Additionally, shifting to a newer combination of antibiotic therapy to treat resistant strains of *P.aeruginosa* may improve outcomes. As *P.aeruginosa* becomes a growing concern, the discovery of antibiotics that the bacteria are not resistant to is significant.

Further genomic analysis was conducted to compare the expression levels of genes coding for proteins that function as efflux pumps, identify those that are overexpressed or suppressed during the antibiotics treatment, and understand the mechanism of PA01 biofilm resistance to the selected antibiotics. With regards to the normalized RNA expression of the efflux transporter genes, it was

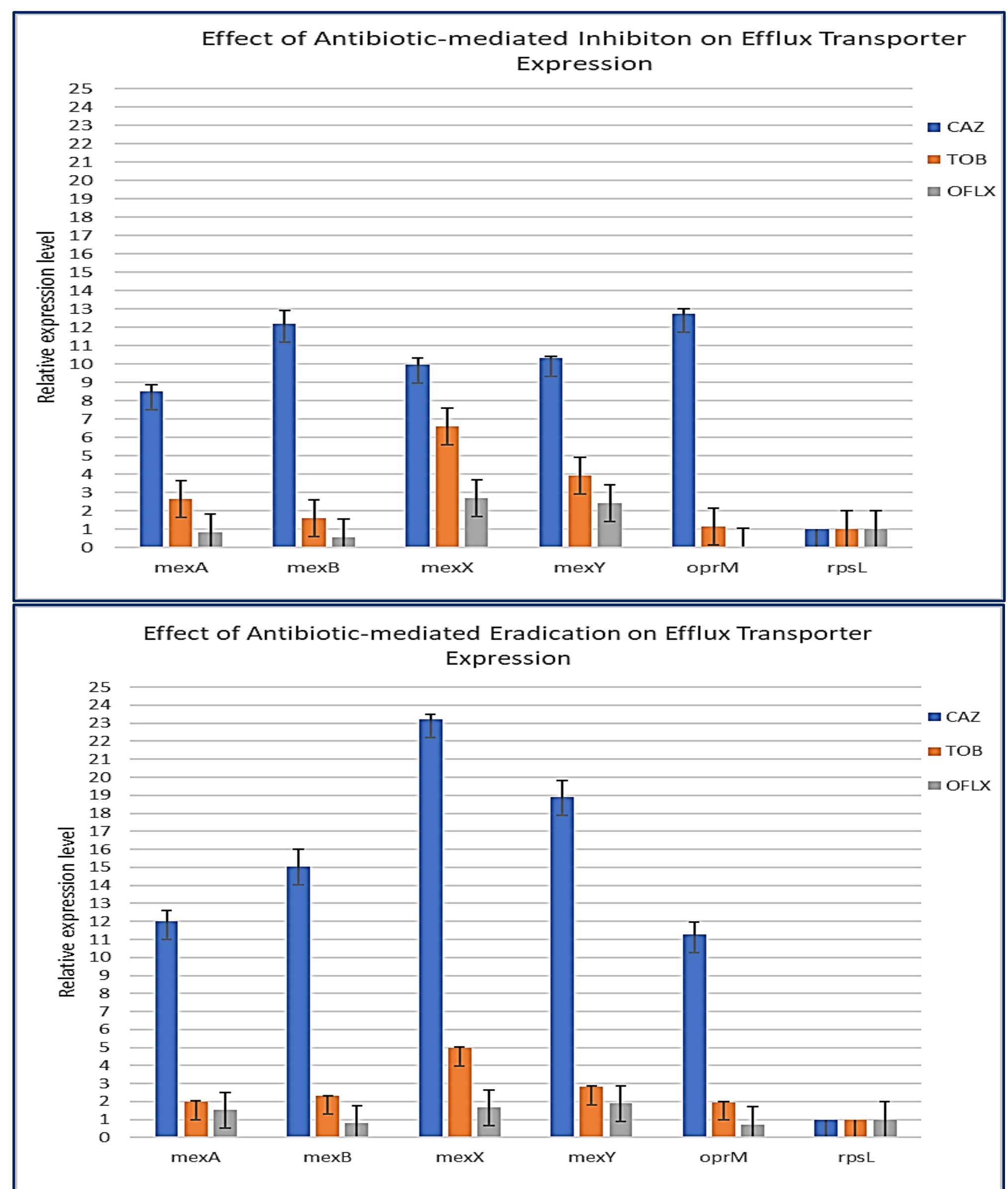


Figure 4 Relative Expression of five Efflux Pump Genes in (*mexA*, *mexB*, *mexX*, *mexY* and *oprM*) PA01 during (A) Inhibition of new biofilm formation and also during (B) Eradication of mature biofilms in the presence of three antibiotics, ceftazidime [CAZ], tobramycin [TOB] and ofloxacin [OFLX] at their Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) respectively as determined previously; Values represent fold change (mean of triplicate samples) in comparison with the transcription level of the internal control *rpsL*

observed that the genes were overexpressed in the biofilm phase as opposed to the planktonic phase (Figure 3). Higher expression of the *mexAB-oprM* and *mexXY-oprM* genes were also observed when PA01 biofilms were treated with ceftazidime compared to ofloxacin and tobramycin as presented in Figure 4. These genes that were upregulated could play a role in lowering the sensitivity to antibiotics by pumping the antibiotics out of the bacterial cells in the biofilm phase. Consequently, they have the potential to act as drug targets for overcoming antibiotic resistance in the future.

4 Conclusions

Ofloxacin, tobramycin, and ceftazidime were the most used antibiotics, and they are reported to be effective for the treatment of *P. aeruginosa* infections in this study as well as in the current literature. Out of the three antibiotics tested in both inhibition and eradication phases, ofloxacin [OFLX] was observed to be the most effective since it showed the most inhibitory effect against *P.aeruginosa* strain PA01compared to tobramycin [TOB] and

ceftazidime [CAZ], which showed the least effectiveness. Additionally, both tobramycin and ofloxacin were effective in eradicating biofilm growth. Based on all these results, ofloxacin was the most effective antibiotic for both inhibition and eradication of *P.aeruginosa* biofilms at lower concentrations as opposed to ceftazidime, which was seen to be less effective since the PAO1 strain selected in this study was the most resistant in both MIC and MBC phases.

Furthermore, a comparative analysis of gene expression levels was also done to determine the probable role of the MexAB and MexXY efflux transporters in the observed antibiotic resistance occurring with biofilm formation, which showed higher expression of these genes in PA01 biofilms compared to planktonic cells. Significantly higher expression levels of all the selected efflux pump genes were detected for the treatment of *P. aeruginosa* biofilms with ceftazidime treatment, which was proven to be the least effective, compared to ofloxacin and tobramycin in both inhibition and eradication phases, which may indicate a probable contribution of these efflux pumps in the mechanism of antibiotic resistance associated with these PA01 biofilms.

Deletion studies of both MexA-MexB-OprM and MexX-MexY-OprM efflux pumps also need to be conducted to determine whether they play a significant role in reducing the susceptibility of PAO1 biofilms to antibiotics. This would reduce intrinsic resistance, making *P.aeruginosa* biofilms more sensitive to antibiotics, and reverse acquired resistance could be a promising target for developing new strategies for the treatment of *P. aeruginosa* infections.

Although the efflux pumps play important roles in increasing the resistance towards different antibiotics, the role of other agents and mechanisms in the evolution of resistance should not be ignored. Since the concomitant overproduction of other Mex efflux systems might have additive effects on antibiotic resistance, the co-expressing of a multicomponent efflux pump is recommended. On the other hand, the concomitant overproduction of two Mex pumps might have additive effects on resistance to antibiotics, which would necessitate the co-expression of Mex efflux systems to study their effects. The development of novel antibiotics that can bypass the effects of efflux pumps is still a challenging task. Therefore, further studies on involved mechanisms and structure-function association of bacterial efflux systems, as well as the interactions between the pumps and other resistance mechanisms, are highly recommended.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Assess the antioxidant and antimicrobial activity of herbal popsicles prepared by *Hibiscus sabdariffa L.* and *Clitorea ternatea* floral waste

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KEYWORDS

Crude floral waste extract

Formulated product

Antioxidant

Antibacterial

ABSTRACT

In this study, we extracted bio-colour from two commonly available flowers, Rosella (*Hibiscus sabdariffa* L.) and Butterfly pea flower (*Clitoria ternatea*), and evaluated their potential therapeutic benefits by examining their antioxidant and antibacterial activity. To assess the suitability and quality of the extracted bio-colour as a food additive, we formulated ice popsicles using bio-colour derived from *H. sabdariffa* and *C. ternatea*. The crude floral waste extract of *H. sabdariffa* showed the highest reducing capacity (FRAP assay), antioxidant activity (DPPH, ABTS assay), and antibacterial potential. This may be attributed to polyphenols, flavonoids, anthocyanins, ascorbic acids, organic acids, hibiscus acid, and other compounds in *H. sabdariffa* flower parts. The ice popsicles formulated with these two bio-colours contained significant polyphenol and flavonoid content, contributing to their antioxidant potential comparable to ice popsicles available in the local market. The formulated ice popsicles also retained better physical properties (texture, melting, smoothness/hardness) and sensory qualities (as per hedonic scale rating) than market-derived ice popsicles. Therefore, these two crude floral wastes can be utilized as functional food bio-colourants in the food industry.

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1 Introduction

Food colour is a critical factor directly linked to consumers' acceptance of food items (Solymosi et al. 2015; Dey and Nagababu 2022). However, a significant amount of colour is lost while processing various food items. As a result, various synthetic or natural food colouring agents are used to restore the colour intensity, texture, and taste of food items (Xing et al. 2012; Dey and Nagababu 2022). In recent decades, synthetic food colourants have been predominantly used in food processing. It has been reported that these synthetic food colourants, when present in food industrial discharges, can cause water pollution by increasing the biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of water bodies (Ardila-Leal et al. 2021; Al-Tohamy et al. 2022; Patil et al. 2022). They can interfere with the process of photosynthesis, inhibit the growth of aquatic plants (Yang et al. 2011; Hussain et al. 2020; Slama et al. 2021), and also cause biomagnification after entering the food chain (Shivani et al. 2020; Alsukaibi 2022). Additionally, numerous reports have highlighted the hazardous effects of these synthetic food colourants, such as hyperactivity, depression, hives, asthma, and brain tumours (Bora et al. 2019; Dey and Nagababu 2022; John et al. 2022). Therefore, scientists face the challenge of developing new, cost-effective, nutritious food colourants that enhance food flavours and are safe to use.

Bio-colourants refer to natural colouring agents from various living organisms such as plants, insects, and animals. The main food bio-colourants include carotenoids, flavonoids, anthocyanidins, and chlorophyll, which are extracted from different parts of plants (Rymbai et al. 2011; Singh et al. 2023; Vega et al. 2023). In India, permitted bio-colours include beta-carotene, beetroot concentrates, grape extract, annatto, lutein, cochineal extract, paprika oleoresin, turmeric oleoresin, phycocyanin, and saffron (Bora et al. 2019). The orange-yellow pigment beta-carotene, isolated from carrot (*Daucus carota*), algae (*Dunaliella salina*), oranges, pumpkins, apricots, mangoes, papayas, and red bell peppers, can act as an antioxidant and antiproliferative agent (Mortensen 2006; Rymbai et al. 2011; Sowmya Shree et al. 2017; Young and Lowe 2018; Renita et al. 2023). Betanin found in beetroot (Beta *vulgaris*) possesses potent radical scavenging, anti-inflammatory, hepato-protective, cardioprotective, antiproliferative, and antimicrobial activity and is widely used as a food colourant in yoghurt, candy, and ice cream (Bora et al. 2019; Silva et al. 2021; Luzardo-Ocampo et al. 2021; Novais et al. 2022).

Annatto is a yellow-orange coloured bio-colourant used in dairy products extracted from *Bixa orellana* tree seeds (Novais et al. 2022). Lutein, a yellow-coloured carotenoid with prominent antioxidant potential, is extracted from the petals of the marigold (*Tagetes erecta*) flower and is widely used in beverages, chewing gums, and oils, as a food colourant (Rymbai et al. 2011; Manzoor et al. 2022; Saini et al. 2023). The yellow pigment Crocin is isolated from the dried stigma of the saffron plant (*Crocus sativa*) and exerts efficient radical scavenging activity, thereby preventing metabolic syndromes (Farrell 1998; Naidu and Sowbhagya 2012; Bora et al. 2019). Similarly, Cochineal Extract, also known as Carmine (Carminic acid), is extracted from *Dactylopius coccus* (AlAshkar and Hassabo 2021; Li et al. 2021). Paprika red colour oleoresin is obtained from *Capsicum annuum* (Pérez-Gálvez et al. 2003; Rymbai et al. 2011; Kostrzewa et al. 2023). Curcumin, a bright yellow-coloured pigment, is isolated from the rhizome of *Curcuma longa* (Rajendran et al. 2022) and is used as a food colouring agent in various food items, having significant antioxidant and antimicrobial activities (Bora et al. 2019). Most non-food plant parts (flowers, leaves, fruit peels, etc.) are discarded as agro waste from the food processing or agricultural industry (Helkar et al. 2016; Torres et al. 2018).

The accumulation of agro-waste poses disposal issues and can lead to environmental pollution. However, these problems can be mitigated by using agro-waste to extract bio-colourants. These bio-colourants not only add colour to food but also contain various bioactive compounds, resulting in greater antioxidant and antimicrobial activity, as well as enhanced therapeutic potential (Rymbai et al. 2011; Bora et al. 2019; Singh et al. 2023; Pasdaran et al. 2023). As a result, natural bio-colourants are becoming preferred over conventional synthetic colourants due to their easy availability, cost-effectiveness, and lack of side effects (Rymbai et al. 2011; Ghosh et al. 2022; Nabi et al. 2023). In this study, we have endeavoured to extract bio-colourants from the natural crude floral waste of Rosella flowers (*Hibiscus sabdariffa*) and Butterfly pea flowers (*Clitoria ternatea*) and evaluate their antioxidant and antibacterial activity. These flowers are known for their beneficial health effects, including anti-inflammatory, radical scavenging, and antiproliferative and anti-carcinogenic potential (Goh et al. 2021; Jeyaraj et al. 2021). We investigated food products' antioxidant properties using these bio-colourants and compared their antioxidant potential with crude floral waste extract. Additionally, this study assessed the radical scavenging and antimicrobial activity of herbal popsicles prepared from the floral waste of *H. sabdariffa* and *C. ternatea*.

2 Materials and Methods

2.1 Preparation of floral waste extracts

The fallen floral parts of the Rosella flower (*H. sabdariffa*) were obtained from Adamas University Campus located in Barasat, West Bengal, India, and the flower parts of the Butterfly pea (*Clitoria ternatea*) were collected from a nearby temple as floral waste. Ten grams of Rosella and Butterfly pea flower petals were dried and blended with a mortar and pestle until the petals became a paste while being extracted with 100 ml of 1% citric acid

solution at a ratio of 1:10. The prepared extracts were filtered through Whatman filter paper no. 1 and transferred into a centrifuge tube, and centrifugation was carried out at 12000 RPM. As a result of the centrifugation, only the supernatant was collected in a new centrifuge tube, dried at 50°C on a hot plate, and stored at 4˚C for further study.

2.2 Estimation of the phenol content

The phenol content of crude floral waste extracts and their formulated products was evaluated using Folin-Ciocalteu's reagent, as Mathur and Vijayvergia (2017) described. In this method, 100 µL of the sample solution was mixed with 1 mL of Folin-Ciocalteu reagent (diluted in a 1:20 ratio, W/V) and 1 mL of 7% Sodium Carbonate was added, followed by 90 minutes of dark incubation. Finally, the absorbance reading was taken at 760 nm using a spectrophotometer (Hitachi, U-2910) (Shirazi et al. 2014), and the values were represented in terms of standard gallic acid.

2.3 Total flavonoid content Estimation

The flavonoid content of crude floral waste samples and their formulated products was determined using a spectrophotometric method (Shirazi et al. 2014). 1 ml of the sample solution and 4 ml of dH_2O were combined in a 10 ml volumetric flask. After incubating for 5 minutes, 0.3 mL of 5% (w/v) Sodium nitrite ($NaNO_2$) and 0.3 mL of 10% (w/v) Aluminium chloride ($AlCl_3$) were added. Then, 1 ml of 1M Sodium hydroxide was added to each tube, and the final volume was adjusted to 10 mL. The absorbance was measured at 510 nm using a spectrophotometer. Quercetin was used as a control, and the data was expressed as equivalent to quercetin in mg QE/g Dry Weight (DW) (Mathur and Vijayvergia 2017).

2.4 DPPH scavenging method

The antioxidant potential of crude floral waste samples and their formulated products was estimated using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging method. 0.5 mL of the stock sample solution was mixed with 2 mL of 1 mM DPPH solution in a tube. After 5 minutes of mixing, the solution was incubated in the dark for 1 hour. The absorbance was then measured in a spectrophotometer at 517nm. Ascorbic acid was used as a standard (Kouassi et al. 2016; Fanta Yadang et al. 2019), and the radical scavenging rate was calculated using the following formula:

$$\text{Radical scavenging rate (\%)} = [(A_{Control} - A_{Sample}) / A_{Control}] \times 100$$

2.5 ABTS radical scavenging method

The ABTS method offers an alternative approach to measure the antioxidant capacity of crude floral waste extracts and their formulated products by reducing the ABTS cation radical. The reaction mixture consists of equal proportions of 7 mM ABTS solution and 2.45 mM $K_2S_2O_8$ (potassium persulfate) solution, which is kept in the dark for 1-2 days. The ABTS solution is diluted in aqueous methanol at a 1:25 ratio. An aliquot of 20 µL of ten times diluted crude floral waste extract or formulated product and 2 mL of ABTS solution is added to a tube and kept at 30°C for a specific duration. The absorbance reading is then recorded at 734 nm using a spectrophotometer at 0, 10, and 20 minutes (Proestos et al. 2013; Kouassi et al. 2016; Fanta Yadang et al. 2019).

2.6 FRAP assay

The FRAP assay is a promising method for determining the reducing potential of crude floral waste extracts and their formulated products. The reaction mixture consists of 1 ml of sample solution, 2.5 ml of 0.2M PBS (pH 6.6), and 2.5 ml of 1% $K_3Fe(CN)_6$ solution. This mixture is well mixed and then heated in a water bath for 20 minutes at 50°C. After heating, 2.5 ml of 10% trichloroacetic acid (TCA) is added. The solution is centrifuged at 3000 rpm for 10 minutes, and 2.5 ml of the supernatant is collected. An equal volume of distilled water is added to the collected supernatant, followed by adding 0.5 ml of 0.1% $FeCl_3$ solution. Finally, the absorbance is measured at 700 nm using a spectrophotometer after 10 minutes of incubation. Ascorbic acid is the standard for comparison (Vijayalakshmi and Ruckmani 2016).

2.7 Estimation of TAC

The total antioxidant capacity (TAC) of the crude floral waste extracts and their formulated products was determined using the phosphomolybdenum method. A mixture was prepared by combining $(NH_4)_6Mo_7O_{24}$ (4mM), H_2SO_4 (0.6M), and Na_3PO_4 (28mM). Next, 0.1 mL of the sample solution was mixed with 1 mL of the abovementioned mixture, which was then placed in a water bath at 95°C for 90 minutes, then cooling to room temperature. The absorbance was measured at 695 nm using a spectrophotometer, and ascorbic acid was used as the standard (Re et al. 1999).

2.8 Agar well diffusion method

The antibacterial activity of bio-colours extracted from crude floral waste was assessed using the well diffusion method against two Gram-positive bacteria, *Staphylococcus aureus* (ATCC25923-0360P) and *Bacillus subtilis* (ATCC11774-0269P), as well as two Gram-negative bacteria, *Escherichia coli* (ATCC35218-0495P) and *Salmonella typhi* (ATCC14028-0363P). Ampicillin and chloramphenicol were used as positive controls. Each bacterial strain was cultured in 20mL of nutrient broth medium and then incubated for 24 hours to optimize growth. After incubation, 100 µL of the bacterial inoculum was spread over the agar surface. Wells were created in the agar media plates using a cork borer, and

the antibacterial agents and test extract solutions were added to these wells. The plates were then incubated at 37˚C for 24 hours. The antibacterial activity of the test crude floral waste extracts was evaluated by measuring the inhibition zone diameter (Mathivanan and Suseem 2016; Naqvi et al. 2020).

2.9 Product formulation

Initially, 50ml of water and 5 grams of sugar syrup were combined in a falcon. Next, 100 microliters of bio-colourant, extracted from crude floral wastes, was added to the mixture and thoroughly mixed using a mixer grinder. The solution was then poured into an ice cream maker, covered with a lid, and left to incubate overnight at 4˚C until the product formed. In this process, four different types of ice popsicles were produced: (1) Untreated ice popsicle A (Control, without any bio-colour), (2) Ice popsicle B (formulated with Rosella-derived bio-colour), (3) Ice popsicle C (formulated with Butterfly pea-derived bio-colour), and (4) Ice Popsicle D (a store-bought ice cream named SLICE)

2.10 Estimation of the Melting rate

The sample's melting rate was assessed by placing the ice popsicle at room temperature on the laboratory balance to measure the mass of the melting ice popsicles. The amount of melting was observed at 5-minute intervals until the structure completely melted. Ice popsicles with minimal overruns often melt faster than those with large overflows. The percentage of melted weight was plotted against time in minutes (Yeon et al. 2017; Martins et al. 2018).

2.11 Analysis of Organoleptic Properties

The prepared ice popsicles were assessed for taste, texture, appearance, colour, and overall preference and then stored overnight at 4°C. One hundred regular ice popsicle consumers, with an average age range of 17-55 years, including 50 males and 50 females, participated in the study. They were requested to evaluate the ice popsicles using a 9-point structured hedonic scale (Martins et al. 2018). The ice popsicles were presented to the participants randomly on coded opaque plastic plates. All participants received prior instructions about the test.

2.12 Statistical evaluations

Each experiment was repeated three times. Data was presented as Mean ± SD and analyzed statistically using one-way and two-way Analysis of Variance with the GraphPad Prism5 software V5.03 (San Diego, USA).

3 Results

3.1 Total phenolic content (TPC)

The total phenolic content (TPC) was significantly higher in the crude floral waste extract of *H. sabdariffa* derived bio-colour (47.16 mg GAE/g DW) compared to its formulated product, which had a TPC value of 25.53 mg GAE/g DW (Figure 1). Similarly, the crude floral waste extract of Butterfly pea (*C. ternatea*) derived bio-colour exhibited a higher TPC value than its formulated product, as depicted

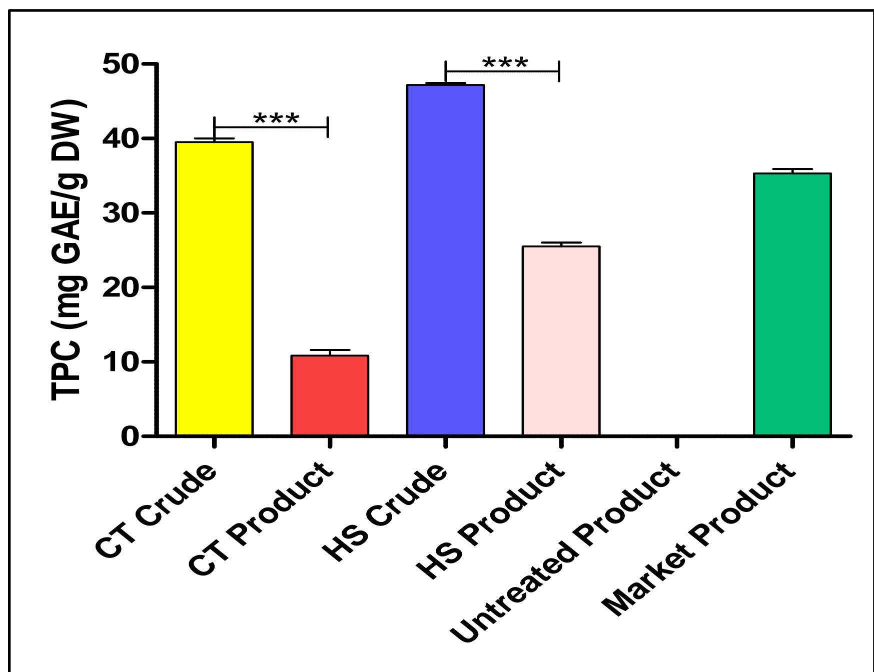


Figure 1 Comparing TPC crude floral waste samples and the formulated products (ice popsicles) expressed as GAE/g DW, the graph represents data in Mean ± SD (n=3) with ***P ≤ 0.001, **P≤0.01, *P≤0.05, CT – *C. ternatea;* HS – *H. sabdariffa*

in Figure 1. In the formulated products, the TPC content was lower than that of their crude samples, but the highest TPC content was found in *H. sabdariffa* derived bio-colour and its formulated product.

3.2 Total flavonoid content (TFC)

Rosella's crude floral waste extract shows almost six times higher total flavonoid content (TFC) than its formulated product, similar to TPC. Similarly, the crude floral waste extract of Butterfly pea exhibits a higher TFC value than its formulated product, as shown in Figure 2.

3.3 FRAP assay

The ferric ion reduction potential (FRAP) of a crude floral waste extract of *H. sabdariffa* was 9.31 µg/ml, significantly higher than its formulated product's FRAP value (4.45 µg/ml). Similarly, the crude floral waste extract of Butterfly pea showed a FRAP value of 8.02 µg/ml, higher than its formulated product (3.12 µg/ml), as depicted in Figure 3. This suggests that the ferric ion reduction potential of the crude sample of Rosella and its formulated product was slightly higher than that of the Butterfly pea derived bio-colour and its formulated product.

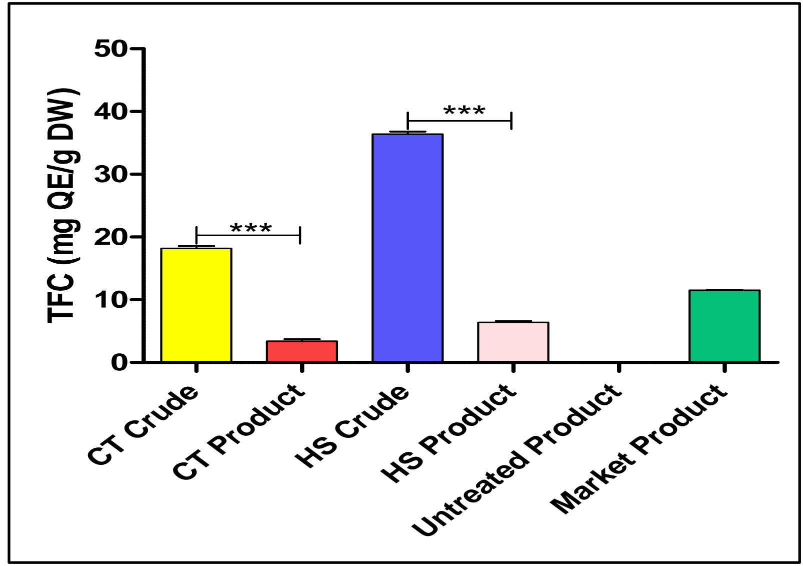


Figure 2 Comparison of TFC of crude floral waste samples and the formulated products (ice popsicles) expressed as QE/g DW, the graph represents data in Mean ± SD (n=3) with ***P ≤ 0.001, **P≤0.01, *P≤0.05, CT – *C. ternatea; HS – H. sabdariffa*

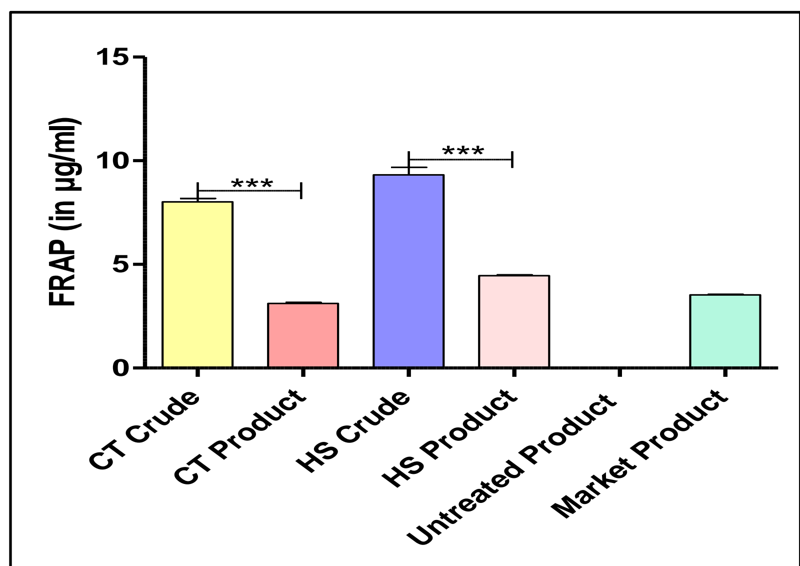


Figure 3 Result of the FRAP assay of crude floral waste samples and the formulated products (ice popsicles), the graph represents data in Mean ± SD (n=3) with ***P ≤ 0.001, **P≤0.01, *P≤0.05; CT – *C. ternatea; HS – H. sabdariffa*

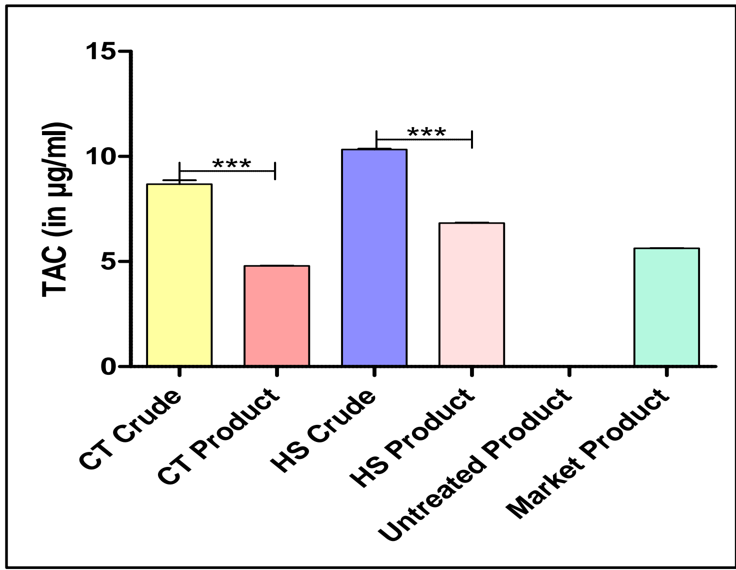


Figure 4 Result of the total antioxidant capacity of the crude floral waste extracts and the formulated products (ice popsicles); the graph represents data in Mean ± SD (n=3) with ***P ≤ 0.001, **P≤0.01, *P≤0.05; CT – *C. ternatea; HS – H. sabdariffa*

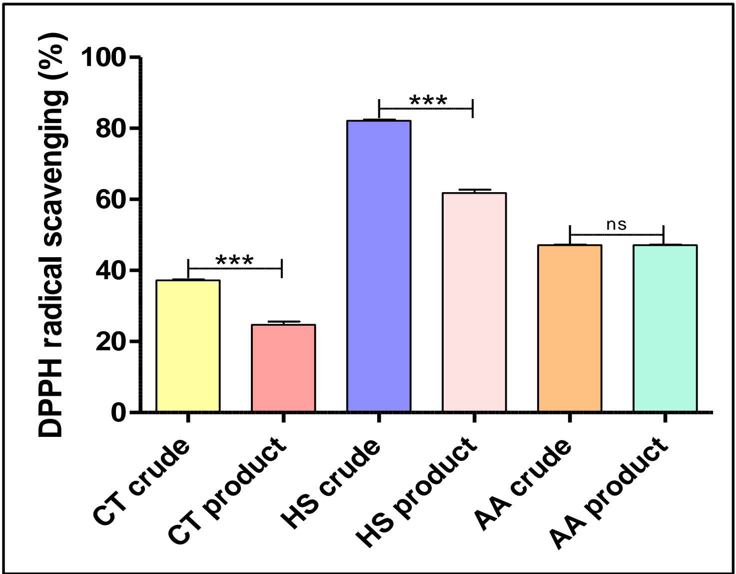


Figure 5 Comparison of the DPPH method of crude floral waste samples and the formulated products (ice popsicles) of *C. ternatea* and *H. sabdariffa*, the graph represents data in Mean ± SD (n=3) with ***P ≤ 0.001, **P≤0.01, *P≤0.05, CT – *C. ternatea; HS – H. sabdariffa,* AA – Ascorbic Acid.

3.4 TAC assay

The results of the total antioxidant capacity (TAC) of the floral wastes and their formulated products are shown in Figure 4. Similar to other parameters, the TAC value of the crude floral waste extract of *H. sabdariffa* was higher than that of its formulated product. The crude floral waste extract of *C. ternatea* exhibited almost double the total antioxidant capacity of its formulated product. The TAC value was significantly lower in the formulated product compared to the crude samples.

3.5 DPPH assay

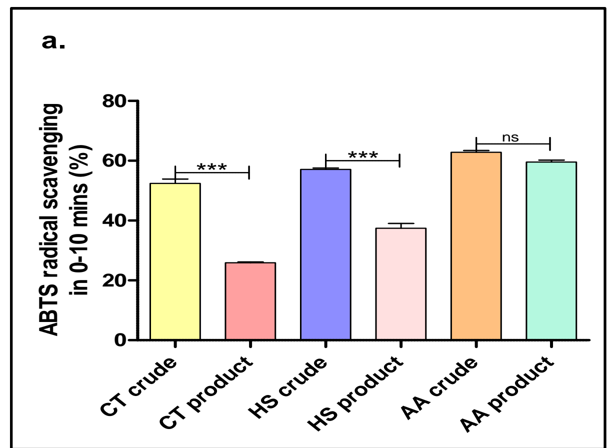
The antioxidant content of the crude floral waste extracts and their formulated products was assessed based on their DPPH radical scavenging capacity (Figure 5). Rosella's crude floral waste extract exhibited a higher DPPH radical scavenging potential at 82% compared to its derived formulated product, which showed 61% scavenging potential. Similarly, the DPPH inhibition potential of the crude floral waste extract was 37%, exceeding the DPPH inhibition potential of its derived formulated product (24%).

3.6 ABTS assay

The results of percent ABTS inhibition are presented in Figure 6. In the ABTS radical scavenging assay, Rosella's crude floral waste extract and its formulated product showed the highest percent inhibition of ABTS radical after 10 and 20 minutes. For the 0-10 minute interval of the assay, the percent inhibition potential of the crude floral waste extract of Rosella was 57%, which was significantly greater than its derived formulated product, which had a rate of 37% (Figure 6a). In the case of the Butterfly pea crude floral waste, the ABTS percent inhibition value was 52% for 0-10 minutes, which was higher than its derived formulated product (25%), as shown in Figure 6a. As time passed, the percent inhibition decreased because the antioxidants of the plant-derived bio-colourants scavenge the cation radical $ABTS^{\bullet+}$. For 10-20 minutes, the floral waste of *H. sabdariffa* and its derived formulated product exhibited ABTS percent inhibition values of 51% and 31%, respectively (Figure 6b). The crude floral waste of Butterfly pea exhibited 49% ABTS inhibition for 10-20 minutes, which was higher than its derived product (20%), as shown in Figure 6b.

3.7 Antibacterial activity

The results of the antibacterial activity of the two crude floral waste extracts of Butterfly pea and Rosella are represented in Figure 7. Ampicillin and chloramphenicol were used as positive controls, showing maximum inhibition zone. The Butterfly pea



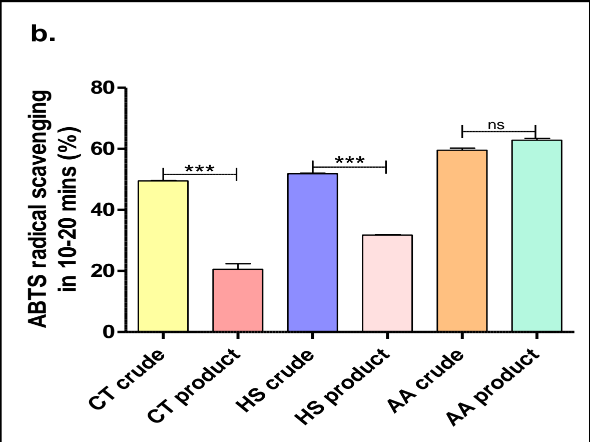


Figure 6 Comparison of the ABTS radical scavenging potential of crude floral waste samples and formulated products (ice popsicles) after (a.) 10 minutes & (b.) 20 minutes of *C. ternatea* and *H. sabdariffa*, the graph represents data in Mean ± SD (n=3) with ***P ≤ 0.001, **P≤0.01, *P≤0.05, CT – *C. ternatea; HS – H. sabdariffa*, AA – Ascorbic Acid

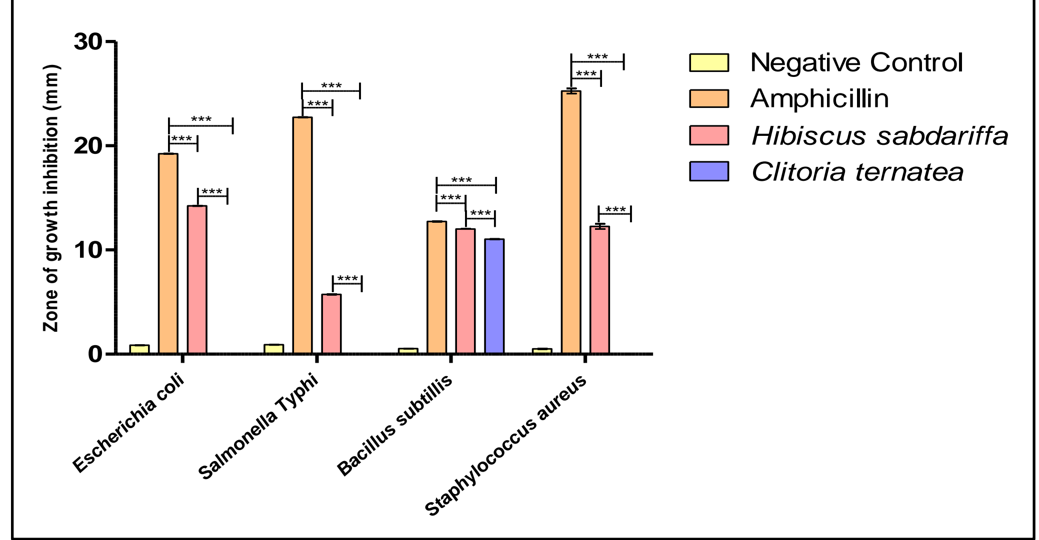


Figure 7 Bar graph showing the zone of growth inhibition (in mm) by the crude floral waste extracts against different Bacterial Strains, the values are represented as Mean ± SD (n=3) with *P≤0.05, **P≤0.01, ***P≤0.001, here, the inhibition of bacterial strains was compared with that of Ampicillin.

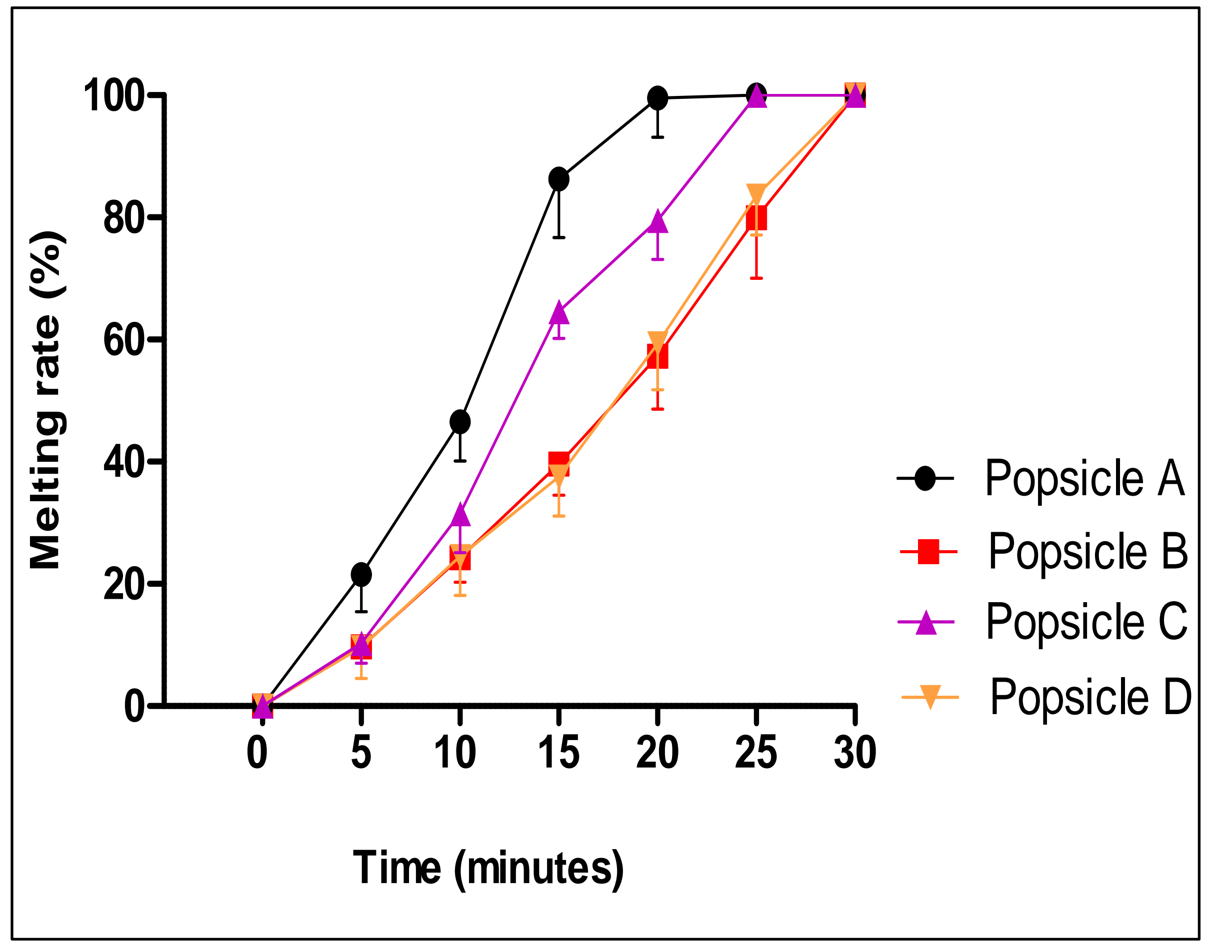


Figure 8 Melting quality of formulated Ice popsicles by observation after 5 minutes intervals, here, Ice popsicle A is Control i.e., without added bio-colour, Ice popsicle B is formulated with Rosella derived bio-colour, Ice popsicle C is formulated with Butterfly pea derived bio-colour added in it, Ice Popsicle D is popsicle ice-cream of brand name SLICE brought from Local market, The graph represents data in Mean± SD (n=3) with ***P ≤ 0.001, **P≤0.01, *P≤0.05.

flower extract exhibits antimicrobial activity only for *B. aureus* (11.035 mm). However, Rosella showed bacterial growth inhibitory effect against four different bacterial strains with diameter ranges from 5.735 mm for *S. typhi*, 12 mm for *B. subtilis*, 12 mm for *S. aureus* and 14 mm for *Escherichia coli*.

3.8 Melting quality

Ice popsicles of good quality are less likely to melt when exposed to room temperature for 10-15 minutes. Ice popsicle A (Control) melted after 20 minutes, ice popsicle C entirely melted after 25 minutes, and ice popsicle B thoroughly melted after 30 minutes. Ice popsicle B maintained its appearance longer than ice popsicles A and C. Ice popsicle D, purchased from the local market, also melted after 30 minutes, indicating a similarity in melting time between the formulated and commercial ice popsicles (Figure 8). The dishes' melted substance should conge into a uniform, homogeneous, smooth liquid.

3.9 Analysis of Organoleptic properties

The results shown in Figure 9 demonstrate the sensory characteristics of the three different ice popsicles created. The sensory properties were rated on a 9-point structured hedonic scale (Martins et al. 2018) and scored between 6.07 and 8.25. There were significant differences (≤0.05) among the three ice popsicles in colour, taste, texture, appearance, and overall rating. The ice popsicle made with Rosella-derived bio-colour had an attractive red colour, while the one made with Butterfly pea derived bio-colour had an appealing blue colour. The mean scores for colour ranged from 6.07 (Popsicle D) to 8.25 (Popsicle B). For taste, the mean score ranged from 6.92 (Popsicle C) to 7.72 (Popsicle B), and for texture, the scores ranged from 6.81 (Popsicle D) to 8.21 (Popsicle B). Regarding appearance, the mean scores ranged from 7.18 (Popsicle C) to 7.22 (Popsicle D). Consequently, the mean overall rating ranged from 7.38 (Popsicle C) to 8.02 (Popsicle B). All 100 consumers reported no health issues within 24 hours after consumption.

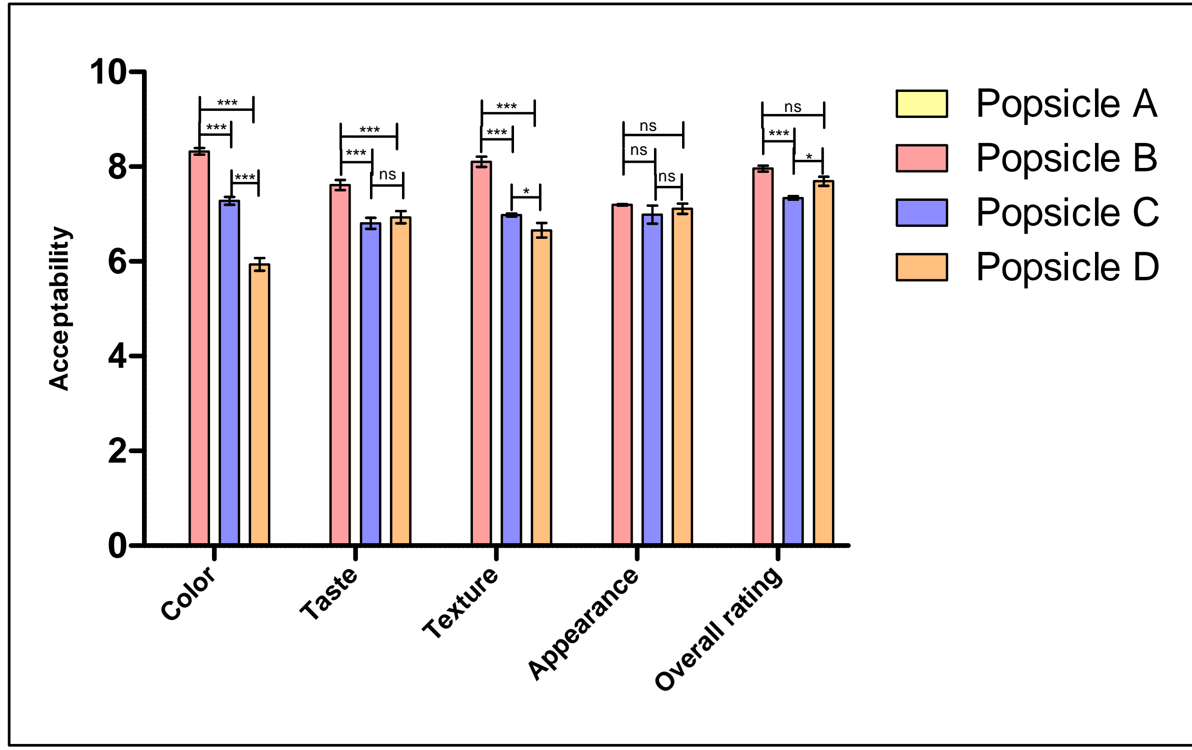


Figure 9 The graph showed consumers' acceptance of untreated ice popsicles and ice popsicles formulated with *H. sabdariffa* and *C. ternatea* derived bio-colour, the values were shown as Mean ± Standard deviation (n=3) with ***P ≤ 0.001, **P≤0.01, *P≤0.05 and compared with sensory attributes of the untreated sample. Mean data from 100 consumers based on a 9-point structured hedonic scale, Formulations of Popsicle A, i.e. Control is without added bio-colour, Popsicle B - Rosella derived bio-colour, Popsicle C - Butterfly pea derived bio-colour, Popsicle D - Popsicle ice cream brought from Local market.

4 Discussion

Food colour is a crucial factor affecting consumers' acceptance of food items. For decades, synthetic colourants have been widely used in food processing. However, recent reports have highlighted the harmful effects of these synthetic food colourants, such as hyperactivity, depression, hives, asthma, Attention Deficit Hyperactivity Disorder (ADHD), and brain tumours (Khanavi et al. 2012; Dey and Nagababu 2022; John et al. 2022). As a result, scientists have been developing new cost-effective and nutritious food colourants that enhance food flavours and are safe to use. Bio-colourants are increasingly preferred over synthetic colourants due to their easy availability, cost-effectiveness, and lack of side effects (Rymbai et al. 2011; Ghosh et al. 2022; Nabi et al. 2023).

A recent study evaluated various bio-colourant properties extracted from natural crude floral waste of H. sabdariffa and C. ternatea. The high total antioxidant capacity (TAC) and total flavonoid content (TFC) values of Rosella's crude floral waste extract can be attributed to higher phenolic and flavonoid content. Results from the ferric ion reduction potential (FRAP) and TAC assay showed that Rosella's crude floral waste extract exhibited maximum reducing capacity and antioxidant potential. The DPPH and ABTS assay results also confirmed the high antioxidant capacity of the Rosella flower's crude floral waste extract. The superior antioxidant properties of the Rosella flower extracts may be associated with the higher presence of polyphenols, flavonoids, anthocyanins, ascorbic acids, organic acids, and hibiscus acid, in the extract (Prenesti et al. 2007; Aurelio et al. 2008; Cisse et al. 2009; Abou-Arab et al. 2011). Additionally, the crude floral waste extract of Rosella demonstrates good antibacterial potential against different bacterial strains due to its high phenolic content.

The red colour of the petals of *H. sabdariffa* and the deep blue colour of *C. ternatea* petals are governed by anthocyanin (Cisse et al. 2009; Pasukamonset et al. 2016; Goh et al. 2021). These floral pigments are commonly used as food colourants and are FDA-approved (Aurelio et al. 2008; Abou-Arab et al. 2011; Goh et al. 2021, Jeyaraj et al. 2021). Rosella juice, derived from water extract of fresh or dried Rosella flowers, is a popular soft drink in various countries such as Nigeria and Thailand (Abou-Arab et al. 2011). Additionally, previous reports have suggested that rosella calyces can serve as food colourants and emulsifiers in the food industry (Duangmal et al. 2004). Surmani et al. (2022) found that administering rosella flower petal ethanolic extract capsules can stimulate erythropoiesis in female anaemic

adolescents without side effects. Furthermore, Srichaikul (2018) and Jeyaraj et al. (2021) reported that oral administration of aqueous ethanol extract of *C. ternatea* flower (2000 mg/kg body weight) in mice did not cause acute toxicity, suggesting its safety for consumption.

In this study, to investigate the acceptability and quality of bio-colour extracted as a food additive, ice popsicles were prepared using bio-colourants derived from *H. sabdariffa* and *C. ternatea*. A locally bought ice popsicle served as the quality control. Ice popsicles are popular globally regardless of age, culture, and economic status (Balthazar et al. 2017) and can be made from dairy or non-dairy ingredients. Although dairy-based ice popsicles are considered nutritious, their quality is sometimes compromised (Bahram-Parvar 2015). Therefore, this study also tested the antioxidant and antibacterial potential of the formulated ice popsicles, as these parameters are directly associated with their quality. The study also assessed the physical and sensory properties of the formulated ice popsicles. The results revealed that ice popsicles formulated with these two bio-colours possess significant polyphenol and flavonoid content, contributing to their antioxidant potential, almost similar to locally bought ice popsicles. Furthermore, the formulated ice popsicle maintained physical and sensory properties better than the locally bought one, indicating that these two crude floral wastes can be used as a functional food bio-colourant in the food industry. Although natural bio-colourants are environmentally friendly, less toxic, non-carcinogenic, facilitate incorporation into aqueous food systems, and promote protection against diverse chronic diseases (Clinton 1998; Siva 2007), there are some limitations to using these bio-colours in the food industry, such as lack of proper knowledge of extracting bio-colour, difficulty in sample collection, decolourization, sensitivity to light, temperature, oxygen, pH, and associated allergic reactions (Francis and Markakis 1989; Hallagan et al. 1995; Duangmal et al. 2004).

Conclusion

The crude floral waste extract of Rosella (*H. sabdariffa*) exhibited higher total phenolic and total flavonoid content, greater reducing capacity, more substantial radical scavenging potential, and better antibacterial activity compared to the Butterfly pea (*C. ternatea*). This trend was also observed in their respective formulated food products. Ice popsicles made with Rosella-derived bio-colour maintained physical and sensory properties better than those bought from the local market, indicating that Rosella-derived bio-colour can be used as a functional food colouring in the food industry. This discovery paves the way for utilizing floral agro-waste to produce valuable food additives, thereby addressing agro-waste-related environmental pollution. Further research is needed to investigate the therapeutic potential of this bio-colour in disease prevention.

Abbreviations

TPC - Total Phenolic Content, TFC - Total Flavonoid Content, DPPH – 2,2-Diphenyl-1-picrylhydrazyl, ABTS - 2,2′-azinobis-(3-ethylbenzothiaziline-6- sulfonic acids), FRAP - Ferric Reducing Antioxidant Power Assay, CT - *Clitoria ternatea*, HS - *Hibiscus sabdariffa L*,PBS- Phosphate Buffer Solution**.**

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