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Significance of Incorporating Biotechnology in Vaccine Development

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Timely vaccination helps people to live a healthy life with no infectious diseases. Agents in vaccines are derived by inactivation of microbes, which may also contain subunits, e.g. parts of surface antigens, or toxins. In the past years, a number of new biotechnological methods have been added to the development of vaccines that have revolutionized the conventional practices. Biotechnology and its development and use have led to immense diversification of the clinical outcomes and strategies of healthcare. The occurrence of contagious diseases has decreased in the contemporary world; non infectious diseases on the other hand are on the increase and they present a significant burden to the healthcare systems of the world at large. Biotechnology is providing solutions that are promising to the prevention of the infectious diseases and to the features of non-infectious diseases [1].

It is critical to comprehend the importance of vaccines before discussing the importance of biotechnology in vaccine development. Although there is an improvement in medicine, some of the deadliest pathogens in the world such as malaria and HIV are yet to have vaccines in place [2]. Vaccines are biological agents that are used to boost the immune system against bacterial or viral infections, and as a result, prevent diseases proactively. They usually include inactivated pathogens or certain antigenic constituents, usually those of surface-binding proteins of the pathogens. These antigens, when introduced into the body, induce the immune system to react to the respective pathogen efficiently equipping the body with defence against possible infections in future [1,2].

Biotechnological approaches such as genetic engineering and cell culture are contemporary technologies that have transformed vaccine production. Such techniques can be used to create vaccines which are easier to manufacture, less expensive and can produce more robust and sustained immunity. Prevention of infectious diseases and promotion of patient outcomes is the main aim of applying biotechnology in the development of vaccines [1]. They involve the insertion of desired genes into plants or body cells and the production of the encoded proteins. These genetically engineered vaccines make the body more immune even in situations when conventional vaccines and treatment methods have failed, and this leads to hope of recovery of serious and persistent illnesses. Biotechnology has played out in three main ways per majorly in generating specific monoclonal antibodies, application of cloned genes to produce antigens and synthesis of peptides which may be used as vaccines.

Reverse vaccinology is one of the most important innovations in the field in which biotechnology holds a key position in changing the research on vaccines. Reverse vaccinology refers to the process of cloning and genome analysis of entire pathogenic genomes by applying bioinformatics tools in a proactive fashion with the intent of identifying targets that would be the basis of vaccines. DNA microarrays, proteomics and comparative genome analysis are functional genomic techniques used to discover virulence factors and promising vaccine targets. Although reverse vaccinology was originally developed to make MenB vaccines, this technology has now been applied to other bacterial vaccines, including Staphylococcus aureus and Streptococcus pneumoniae [3]. The antigens can be predicted using modern computational techniques without paying attention to their abundance or immunogenicity, which offers a more accurate and effective approach to developing vaccines.

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Biotechnology has further enhanced the quicker velocity in creating vaccines to emerging pathogens. As an example, the COVID-19 pandemic emphasized the urgency to make vaccines as quickly as possible. Biotechnological tools helped develop effective vaccines in a several months, compared to traditional vaccine development which would have taken years to develop. Such technologies like mRNA vaccines, viral vectors vaccines and recombinant protein vaccines demonstrate how biotechnology can be used directly to offer new and effective responses during a crisis in the life of the people. In addition to infectious diseases, biotechnology has the prospects of employing non-infectious diseases like some types of cancers by developing therapeutic vaccines which induce immune responses against cancerous cells [2,3].

Biotechnology is a necessity of contemporary vaccinology. Its use in vaccine development can integrate the process of quick, accurate, and effective vaccine manufacturing and improve the health outcomes of the population at a global level. Biotechnology deployment in strategic innovation of vaccines is not only crucial in tackling the current infectious hazards, but also providing a remedy of the diseases that could not be treated before or controlled effectively. With an ever-growing development of scientific skills, biotechnology in vaccinology will have an increased role to play to make the global health issues safer, efficient, and comprehensive in prevention efforts.

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Review Article

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Keratinophilic Fungi as Eco-Friendly Agents for Poultry Waste Biodegradation: Mechanisms, Applications, and Sustainable Management Strategies

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ABSTRACT

The rapid expansion of the poultry industry has resulted in the accumulation of large quantities of keratin-rich waste such as feathers, nails, and skin, which pose significant environmental challenges due to their recalcitrant nature. Conventional disposal methods, including incineration and chemical treatment, are energy-intensive and potentially harmful to ecosystems. Keratinophilic fungi offer an eco-friendly alternative through their ability to produce keratinase enzymes that degrade insoluble keratin into valuable amino acids and peptides. This study investigates the biodegradation potential of keratinophilic fungi isolated from poultry waste, focusing on their keratinase production, activity, and potential applications in sustainable waste management. The findings highlight promising fungal strains capable of efficient keratin degradation, underscoring their potential role in circular bioeconomy strategies and industrial applications such as animal feed production, fertilizer formulation, and leather processing.

INTRODUCTION

According to the United Nations, the global human population is projected to reach 10.1 billion by 2060 [1]. Such rapid growth poses serious challenges for humanity, including the persistent issue of hunger. In 2020, approximately 690 million people, around 9% of the global population, were undernourished and lacked access to sufficient and nutritious food [2]. Achieving food security, improving nutrition, and ending hunger are among the core Sustainable Development Goals (SDGs) [3]. However, factors such as climate variability, pest outbreaks, and, more recently, the COVID-19 pandemic have hindered the establishment of resilient and efficient food systems. One promising approach to addressing these challenges is the promotion of sustainable agricultural practices [4]. As the human population expands, the demand for agricultural products continues to grow, leading to a corresponding increase in agricultural waste. Among these wastes, keratin-rich materials such as poultry feathers are particularly problematic due to their resistance to natural degradation. Presently, most protein-based agro-

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industrial by-products are disposed of through incineration, landfilling, or chemical hydrolysis. While these methods are effective for waste reduction, they conflict with sustainable economy principles, often causing environmental damage and missing opportunities for resource recovery. In contrast, biodegradation, the use of living organisms and their enzymes to decompose waste, offers an eco-friendly alternative. This method not only minimizes environmental impact but also yields valuable hydrolysis products, such as amino acids and peptides, which can be used in fertilizers, animal feed, and other biotechnological applications. Such an approach supports a circular economy model, making waste management both sustainable and economically viable [5]. Although both bacteria and fungi produce keratinases, bacterial strains are often favored in industrial applications due to their faster growth rates and relatively simpler enzyme purification processes. In contrast, fungal keratinases generally exhibit broader substrate specificity and greater resilience under harsh conditions [6]. This review aims to investigate the biodegradation potential of keratinophilic fungi isolated from poultry waste, focusing on their keratinase production, activity, and potential applications in sustainable waste management.

Despite the abundance of keratin-rich poultry waste, its effective disposal remains a major environmental challenge due to the recalcitrant nature of keratin and limitations of conventional methods like incineration or chemical hydrolysis. While microbial keratin degradation offers an eco-friendly alternative, most studies have focused on bacterial keratinases, leaving fungal keratinases particularly from indigenous poultry waste isolates—underexplored. Furthermore, there is limited information on optimizing fungal strains for industrial-scale biodegradation and sustainable valorization of keratin into value-added products.

Keratinophilic Fungi: Biology, Ecology, and Pathogenicity

Keratinophilic fungi are saprotrophic microorganisms capable of degrading highly recalcitrant keratinous structures, including feathers, hair, nails, and skin. Their keratinolytic capacity allows them to utilize keratin as the sole source of carbon and nitrogen. These fungi are classified ecologically into three groups: geophilic, zoophilic, and anthropophilic. Geophilic species inhabit soils rich in keratin debris and are mostly saprophytic. Zoophilic species are associated with animals and can cause infections in keratinized tissues, while anthropophilic species primarily infect humans. Among the notable keratinophilic fungi are members of the genera *Trichophyton*, *Microsporum*, and Epidermophyton. Although most species are harmless in their natural habitats, some act as opportunistic pathogens capable of causing dermatophytosis in humans and animals. To ensure safe application in industrial and environmental processes, several mitigation strategies are recommended. Non-pathogenic or low-risk strains such as *Chrysosporium keratinophilum* should be prioritized. Fungi should be cultivated under controlled conditions, including regulated temperature, pH, and containment, to prevent accidental release. Laboratory and industrial personnel must use appropriate personal protective equipment (PPE) such as gloves, masks, and protective clothing. Residual fungal biomass should be inactivated post-application via autoclaving or chemical sterilization, and adherence to biosafety protocols with routine culture monitoring under BSL-1/2 guidelines is essential. Implementing these measures allows the safe integration of keratinophilic fungi in waste management, enzyme production, and other biotechnological applications without compromising human or animal health [7]. Beyond their biotechnological potential, keratinophilic fungi play a significant ecological role in keratin turnover, contributing to nutrient cycling and the biodegradation of otherwise persistent organic matter [8]. Their ecological versatility has encouraged exploration of diverse environments, including poultry waste, to identify strains capable of efficient keratin degradation.

Keratinolytic Fungi from Different Environments

Chicken-associated microbes, particularly fungi, actinomycetes, and certain bacteria, are effective keratin-degrading organisms due to their production of diverse proteolytic and keratinolytic enzymes [9]. Poultry feathers are rich in nitrogen (15–18% dry weight) and sulfur (2–5% dry weight) and are highly resistant to degradation because of strong disulfide bonds. Only specialized organisms, including dermatophytes, Chrysosporium species, and some molds like Fusarium spp., can biologically break down keratin [10]. Keratin-degrading dermatophytes include anthropophilic species (*Trichophyton rubrum*), zoophilic species (*Trichophyton verrucosum*), and geophilic species (*Trichophyton terrestre, Trichophyton georgie, Trichophyton ajelloi, Microsporum gypseum, M. fulvum*). Geophilic dermatophytes and saprophytic Chrysosporium species are widespread in soils enriched with keratinous materials and are also found in bird nests and pellets. Among them, *Chrysosporium keratinophilum* (*Aphanoascus keratinophilus*) is particularly effective, producing keratinolytic and proteolytic enzymes that release nutrients such as ammonia and sulfates [11]. The ecological significance of fungal keratinases lies in their ability to degrade otherwise persistent keratin, contributing to nutrient cycling. Often, this degradation involves a complex interplay of enzymes produced by multiple cooperating microorganisms [12].

Keratin Structure and Degradation Mechanism

There is a huge quantity of keratin waste produced by the poultry processing industry in the form of chicken feathers,

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consisting of up to 90 percent keratin protein [13, 14]. Keratin is a very stable, fibrous protein, usually rich in a dense lattice of disulfide-bonded structures and the hydrogen bonds, and hydrophobic interactions altogether, of which it is insoluble and resistant against degradation caused by ordinary proteolytic enzymes [15, 16]. Accumulation of such waste material continuously leads to environmental pollution and requires sustainable strategies of management. Structurally, keratin can be classified into α-keratin, β-keratin, and γ-keratin. α-keratins, found in all vertebrates, possess α-helical secondary structures; β-keratins, predominant in reptiles and birds, form β-sheet structures stabilized by non-covalent interactions, making them especially resistant to hydrolysis [17-19]. The high cysteine content in keratin contributes to its recalcitrance, as cysteine residues form strong disulfide bridges that maintain the rigid protein structure. Certain microorganisms, particularly keratinolytic fungi, have evolved mechanisms to degrade keratin through the secretion of keratinases and associated proteases [11]. Notably, members of the phylum Ascomycota, such as *Onygena corvina,* are recognized for their potent keratin-degrading capabilities [20]. Indigenous fungal isolates from poultry waste, therefore, represent a valuable, yet underexplored, biotechnological resource. Keratin degradation generally proceeds via two key stages: sulfitolysis and proteolysis. In the sulfitolysis phase, disulfide bonds are cleaved either enzymatically (e.g., by sulfide reductases) or chemically (e.g., by reducing agents), converting cysteine residues into more accessible forms and loosening the keratin matrix [21-23]. This destabilization enables the next stage of proteolysis, whereby keratinases cleave the exposed peptide bonds to give rise to soluble peptides and free amino acids [24]. Keratinases may also act synergistically with other accessory enzymes, and crude enzyme preparations may prove to be more efficient in keratin degradation as compared to pure keratinase. The realization of the structural complexity of keratin and the enzyme reactions provoking its degradation is essential in the realization of effective, eco-friendly techniques of converting poultry wastes into useful products like biofertilizers, livestock feed, and bioactive molecules [25]. Subsequently, proteolysis takes place after the staining sulfitolysis whereby the exposed peptide bonds are broken and soluble peptides and free amino acids are released by keratin conductivity. There are frequently greater rates of Keratin degradation by crude enzyme preparations as compared with purified Keratinase alone, indicating that there is an interaction between Keratinase and other accessory enzymes. This two-step enzymatic process is essential for the complete biodegradation of keratin-rich materials [26]. Understanding these mechanisms also informs the strategies for isolating and screening keratinophilic fungi capable of producing potent keratinases, thereby bridging laboratory studies with practical applications.

Isolation and Screening of Keratinophilic Fungi

Keratinophilic fungi are specialized microorganisms capable of degrading keratin, the highly stable structural protein found in feathers, hair, nails, and other keratinized tissues. Their enzymatic keratinolytic activity enables the breakdown of keratin into amino acids and peptides, making them valuable for biotechnological applications, including poultry waste management [27, 28]. These fungi are commonly isolated from soils, poultry litter, and keratin-rich environments using keratin baiting techniques, where materials such as hair, feathers, or nails serve as selective substrates. Dominant genera include *Chrysosporium, Trichophyton, Microsporum, Aspergillus,* and *Penicillium* [29, 30]. Strains are screened for keratinase activity using keratin agar or quantitative feather degradation assays, and potent isolates are selected for further studies under controlled fermentation conditions. Several studies have demonstrated the potential of keratinophilic fungi in poultry waste biodegradation. For instance, Shestakova *et al.* [5] highlighted the environmental advantages of microbial keratin degradation over conventional disposal methods like incineration or landfilling, emphasizing its role in sustainable waste management and circular economy practices.

Biodegradation of Waste: Microbial Approaches

Building upon laboratory studies of microbial keratin degradation, these fungi can be further utilized in industrial and agricultural settings to transform poultry feather waste into value-added products such as animal feed, fertilizers, and bioactive compounds. Poultry feathers are a significant by-product of the poultry industry, comprising about 5–7% of chicken weight and accounting for several million tons of waste generated annually [32]. These wastes are composed of approximately 90% keratin, a structural protein characterized by high stability due to disulfide bonds [33]. While feathers possess desirable properties such as warmth retention, sound insulation, flexibility, and low density, their accumulation in landfills poses environmental concerns due to their resistance to natural degradation [34, 35]. Microbial degradation of feathers offers an eco-friendly solution for waste management. Various microorganisms, particularly keratinolytic fungi, can hydrolyze keratin into soluble proteins and amino acids by producing keratinase and protease enzymes [11]. These fungi play a crucial role in converting recalcitrant keratin into value-added products, with potential applications in agriculture, biotechnology, and other industries that utilize keratin-containing raw materials [27]. The Ascomycetes group, including species

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such as *Onygena corvina*, is well known for its keratin-degrading abilities [21]. Despite promising results, the practical application of fungal biodegradation faces several hurdles. The efficiency of degradation can be influenced by environmental factors such as temperature, pH, and moisture content. Additionally, the degradation products may be toxic or accumulate in the environment, posing further ecological risks. The economic viability of large-scale fungal biodegradation processes also remains a significant challenge [36] (Table 1).

Table 1: Common Keratinophilic Fungi Isolated from Poultry Waste and their Keratinolytic Properties

Fungal Genus / Species	Sources	Keratinase Activity	Optimum pH	Optimum Temp (°C)	References
Scopulariopsis brevicaulis	Poultry farm soil, feathers	~3.2 KU/mL; ~79% feather degradation	alkaline (≈ 8)	~35°C*	[37]
Trichophyton mentagrophytes	Poultry soil, feather waste	~2.7 KU/mL; ~72% feather degradation	~7.0–8.0	~30–40°C	[38]
Aspergillus flavus (strain K-03)	Feather meal compost/medium	Moderate to high; purified keratinase active up to 2.39× purification, broad activity	~8.0	~45°C	[39]
Chrysosporium keratinophilum	Poultry litter–enriched soil	Potent keratinase producer under submerged culture†	~7–9	mesophilic (28–37°C)	[40]

Applications of Keratinolytic Biodegradation in Poultry Waste Management

Wastes generated from livestock industries such as poultry, swine, and cattle farming are rich in keratin. Among these, chicken feathers represent the largest source, accounting for nearly 9% of a bird's total body weight, with global production estimated at approximately 11.85 million tonnes annually. Despite this abundance, most feathers are discarded, and only a small proportion is reprocessed into products such as feather meal or fertilizers. Improper disposal can make feather residues reservoirs of pathogenic microorganisms, including *Salmonella* spp., *Staphylococcus* spp., and *Clostridium* spp., as well as carriers of veterinary drugs, antibiotics, and chemical contaminants, all of which contribute to soil and water pollution [41]. Current feather disposal practices, such as incineration, eliminate waste but release greenhouse gases and prevent resource recovery [42]. Other non-biodegradation methods similarly pose environmental drawbacks and fail to align with sustainable waste management goals. In contrast, biodegradation using microbial keratinases presents a green alternative, transforming feather waste into value-added products such as fertilizers, animal feed additives, bioelectricity, and biofuels [43]. Both bacteria and fungi produce Keratinases (EC 3.4.21/24/99); while initially linked mainly to pathogenic dermatophytes, non-pathogenic keratinolytic strains have since been identified [44]. Using poultry feathers as a growth substrate for keratinase-producing microorganisms not only lowers enzyme production costs but also supports circular economy practices and reduces the environmental footprint of poultry farming [45].

Limitations and Future prospects

Although keratinophilic fungi show promising biodegradation potential, challenges remain in scaling up processes, ensuring consistent enzyme activity under variable environmental conditions, and managing potential pathogenicity of certain strains. Future research should focus on developing genetically safe or non-pathogenic fungal strains, optimizing fermentation and enzymatic conditions, and integrating fungal keratin degradation with other biotechnological approaches to enhance efficiency and expand applications in sustainable animal feed, biofertilizers, and circular bioeconomy practices.

CONCLUSION

Keratinophilic fungi isolated from poultry waste represent a promising and eco-friendly solution for the biodegradation of highly recalcitrant keratinous materials. Through their keratinase activity, these fungi can efficiently convert feathers, nails, and other keratin-rich waste into valuable by-products such as amino acids, peptides, and biofertilizers, supporting both environmental sustainability and circular bioeconomy strategies. Beyond waste valorization, their application can reduce reliance on energy-intensive or chemically harsh disposal methods, mitigating environmental pollution and greenhouse gas emissions. However, the presence of opportunistic pathogenic strains highlights the need for careful strain selection, controlled cultivation, and strict adherence to biosafety protocols to prevent human or animal health risks. Future research should focus on optimizing fermentation and degradation conditions, developing non-pathogenic or genetically safe strains, and integrating keratinophilic fungi into large-scale industrial processes. Moreover, combining fungal biodegradation with other biotechnological approaches such as microbial consortia or enzyme engineering may enhance efficiency and expand the range of feasible applications, including sustainable animal feed, biofertilizers, and novel bioproducts. Ultimately, strategic deployment of keratinophilic fungi offers a scalable, environmentally responsible pathway to transform poultry waste into high-value resources.

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Methodology: TB, A, AAK, HI
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All authors approved the final manuscript and take responsibility for the integrity of the work.

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Review Article

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Extremophilic α-Amylases: Structural Adaptations, Discovery Strategies, and Industrial Applications (2020-2025)

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ABSTRACT

The use of the enzyme α-amylases is a large-scale industrial enzyme used in the manufacture of food and beverages, textiles, detergents, paper, pharmaceuticals, and biofuels. Conventional microbial α-amylases, primarily Bacillus and Aspergillus-based ones, have been in use for many years, but their effectiveness is often limited by the harsh conditions of industrial processes. Extremophilic enzymes such as thermophiles, halophiles, acidophiles, alkaliphiles, and psychrophiles are an attractive alternative to resilient α-amylases with exceptional thermostability, pH tolerance, salt resistance, and, in some cases, cold activity. This review sums up recent developments (2020-2025) in the discovery, biochemical characterization, as well as industrial application of extremophilic α-amylases. New culture-independent technologies, such as metagenomics, high-throughput functional screening, and machine learning-guided enzyme mining, are highlighted because they help to increase the number of genes in α-amylases of previously unculturable microorganisms. The discussion is centered on structural and mechanistic understanding concerning enzyme stability with reference to comparison to conventional counterparts. Although considerable advances have been made, there are still several gaps in the exploration of unexplored habitats, structural explanation of identified new enzymes, and cost-effectiveness of industrial applications. A combination of extremophilic scaffolds with protein engineering, synthetic biology, and sustainable fermentation has great potential for the realization of tailored α-amylases to serve advanced bioprocesses. The advances make extremophilic α-amylases an important source of industrial biotechnology innovation.

INTRODUCTION

It is a metalloenzyme (EC 3.2.1.1) that catalyzes the hydrolysis of internal 1, 4 glycosidic bonds in starch, glycogen, and related polysaccharides in the presence of calcium to produce maltose, glucose, and limit dextrins [1]. Thanks to using starch in plants as the main source of carbohydrates, as well as in many industries as one of the main raw materials, 2-amylases are the crucial biocatalysts in starch processing. Today, they form some of the most manufactured commercial enzymes, with a presence of about 25-30% of the world's enzyme market, just being surpassed by proteases [2]. The worldwide market of α-amylase is expected to grow at a compound annual growth rate (CAGR) of 5.9 percent between the year 2023 and 2033. In 2023, the market is estimated to be valued at over USD 1,840.8 million, and this is expected to reach approximately USD 2,692.5 million in 2033 [3]. They have extensive applications in the food and beverage sector, brewing, baking, making sweeteners, in the textile industry to desize fabrics, in the detergent industry to remove stains made by starch, in the pulp and paper industry to reduce their viscosity, the pharmaceutical industry and as a source of biofuels among other things, indicating their widespread

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relevance in economics [4]. The major use of α-amylase was demonstrated (Figure 1).

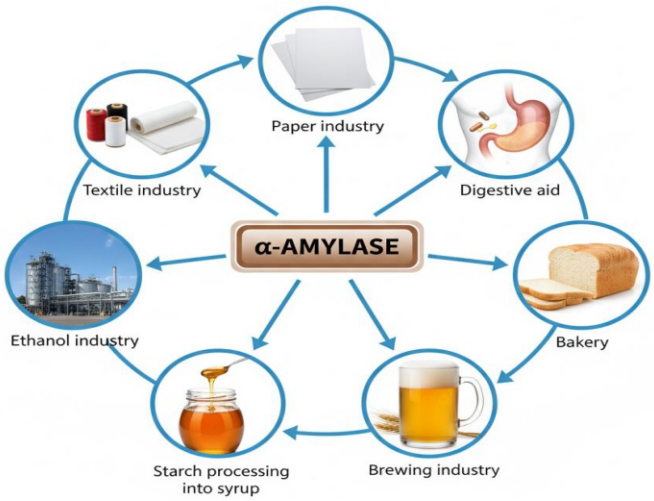

Figure 1: Major Industrial Sectors Utilizing α-Amylase

Its applications are important in the food and beverage sector (bakery, brewing, starch processing into syrups), the textile and paper industry, and in the production of ethanol and as a digestive aid in pharmaceuticals and nutraceuticals. In the past, Bacillus species (e.g., B. licheniformis, B. amyloliquefaciens) and filamentous Aspergillus species have been used as the major microbiological sources of commercial alpha-amylase production because of their high secretion capacity, established fermentation techniques, and regulatory approvals [5]. The enzymes are often found to show poor functioning in high-pressure industries characterized by high temperatures, harsh pH, or heavy salt. This can lead to denaturation of enzymes, loss of activity, and increased production costs due to the need to have stabilizers or process adjustments [6]. The paper gives a comparative overview of traditional microbial α-amylases and extremophilic α-amylases, their optimal temperature, pH, halotolerance, and applications. Stability is better at high temperature, strong pH, and high salinity than conventional Bacillus and Aspergillus enzymes, and extremophilic α-amylases are therefore better adapted to harsh industrial environments (Figure 2).

Comparative Chart

Feature	Conventional α-Amylase	Extremophilic α-Amylase
Optimal Temperature	Typically mesophilic, ranging from 37°C (human) to 70°C (bacterial).	Often highly thermophilic, with optimal temperatures above 70°C and some functional up to 100°C or more.
Optimal pH	Generally neutral to slightly acidic, typically pH 6.0 to 7.5.	Highly variable, with optimal activity in a broad range, from acidic (pH 4.0) to alkaline (pH 10.5).
Halotolerance	Low tolerance; activity is often inhibited by high salt concentrations.	High tolerance; many are active and stable in high salt environments, tolerating several molar concentrations of NaCl.
Sensitivity to Solvents	Generally low tolerance; can be denatured by organic solvents.	High tolerance; often stable and functional in the presence of organic solvents.

Figure 2: Comparison of Conventional and Extremophilic Microbial α-Amylases

Microorganisms known as extremophiles, which flourish in conditions previously deemed inhospitable to life, present a significant and largely unexplored source of resilient biocatalysts [7]. Organisms such as thermophiles found in geothermal springs, compost heaps, and hydrothermal vents generate enzymes that maintain stability at elevated temperatures. Halophiles, which inhabit salt flats and saline lakes, produce enzymes that can withstand high salt concentrations [8]. Acidophiles, sourced from acidic mines or hot pools, along with alkaliphiles from soda lakes, demonstrate stability across varying pH levels [9]. Meanwhile, psychrophiles from polar and alpine regions develop cold-active enzymes that function effectively at low temperatures [10]. α-Amylases derived from these organisms frequently exhibit a range of beneficial characteristics, including thermostability, halotolerance, and pH stability. These traits can contribute to minimizing contamination risks, decreasing energy consumption, and enhancing process efficiency in industrial applications [11]. The representative global habitats of microorganisms that thrive in extreme conditions, including thermophilic, halophilic, acidophilic/alkaliphilic, and psychrophilic environments, all of which are capable of producing α-amylases, were illustrated. Thermophiles (red) are associated with geothermal springs and hydrothermal zones; halophiles (blue) inhabit saline lakes and salt flats; acidophiles/alkaliphiles (orange) occur in acidic mines and soda lakes; psychrophiles (light blue) are found in polar and alpine environments. Callouts indicate representative locations (Figure 3).

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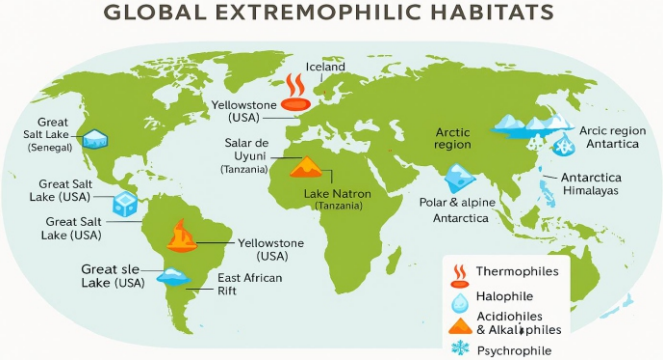

Figure 3: Global Distribution of Representative Extremophilic Habitats

The culture-independent methodologies of the recent past, especially metagenomics, amplicon sequencing, and high-throughput functional screening, have given us a wide knowledge of microbial diversity beyond the small number of culturable species [12, 13]. These approaches have been used to identify a large number of new α-amylase genes whose properties may be improved using protein structure modeling, homology-based mining, and machine learning techniques [14]. However, few comprehensive reviews have been conducted to capture such improvements. The present review provides a revised synthesis of microbial α-amylases in extreme environments with their structural and biochemical adaptations, their discovery methods, and their use in the food processing sector, textile sector, detergents, paper production, pharmaceutical sectors, as well as biofuel production. Traditional alpha-amylases, predominantly found with Bacillus and Aspergillus strains, control the market but usually exhibit low stability in high temperatures, extreme pH, or high salt content. These shortcomings add to production expenses and limit them in severe industrial processes. Thermophiles, halophiles, acidophiles, alkaliphiles, and psychrophiles are examples of extremophilic microorganisms that provide enzymes with impressive thermostability, halotolerance, and pH adaptability that are more suited to the needs of industry. New technologies (within the last five years, 2020-2025), including metagenomics, functional screening, structural biology, and machine learning controlled enzyme mining, have increased the finding of novel α-amylase genes by previously unculturable microbes. Synthetic biology and protein engineering are also making the optimization of extremophilic scaffolds for particular uses feasible. Nevertheless, there are still significant knowledge gaps in terms of studying unexplored habitats, resolution structures, enhancing heterologous expression, and creating cost-efficient scale-up techniques. This review aims to fill this gap between environmental enzyme diversity and commercial application by combining ecological, biochemical, and technological views on the research in extremophilic 2-surrendered Aylase, presenting both the present and possible future developments of this research. The left track shows culture-dependent screening (isolation, fermentation, and activity assays), while the right track shows culture-independent approaches (DNA extraction, metagenomic sequencing, bioinformatic mining, and functional screening). Both approaches converge at the characterization of new α-amylase candidates for industrial application (Figure 4).

DISCOVERY OF NOVEL α-AMYLASES

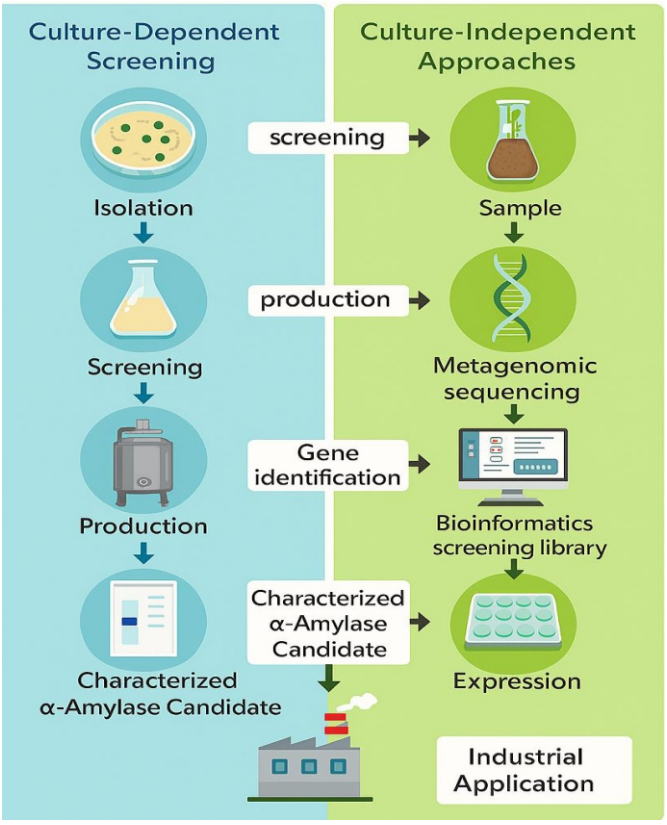

Figure 4: Workflow for Discovery of Novel α-Amylases

Despite the widespread industrial use of α-amylases, conventional enzymes derived from Bacillus and Aspergillus often fail under extreme conditions such as high temperature, extreme pH, and high salinity, limiting their efficiency and increasing production costs. Although extremophilic microorganisms offer a promising source of robust α-amylases, many habitats remain underexplored, and the structural-functional relationships of newly discovered enzymes are not fully understood. Moreover, challenges in heterologous expression and cost-effective industrial-scale application persist. Addressing these gaps is crucial to develop tailored enzymes for enhanced industrial processes.

Classification

α-Amylases (EC 3.2.1.1) are found in the glycoside hydrolase (GH) family and are mostly defined by their action on the

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polysaccharide chains [15]. Two main types of amylolytic enzymes are possible. Endo-amylases break internal α-1,4 bonds of starch and other related polysaccharides, producing oligosaccharides of variable length. Exo-amylases, such as α-amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3), sequentially cleave α-1,4 or α-1,6 bonds at the non-reducing terminus of the polypeptide to yield maltose or glucose [16]. The most outstanding endo-hydrolase in such categories is α-amylase, with its high catalytic ability and extensive substrate selectivity. It finds widespread application in the food industry, detergent industry, and biofuel industry because of its flexibility and cost-effectiveness [17]. A schematic categorization of the amylolytic enzymes into endo-type and exo-type is distinguishing between endo-type enzymes, which cut internal α-1,4 bonds, and exo-type enzymes, which cleave one or two terminals of the chains.

Mechanism of Action

The hydrolysis of starch is catalyzed by alpha-amylase using the retaining, double-displacement reaction that involves a covalent glucosyl-enzyme intermediate. A conserved catalytic triad of a nucleophilic aspartate, an acid/base glutamate, and a stabilizing aspartate residue at the catalytic domain facilitates this reaction [18, 19]. Active-site water molecules coordinate and play a key role in the glycosylation phase and determine the reaction rate [20]. Cofactors such as bound Ca 2 ions have an effect on domain interfaces, but neighbouring Cl ions alter acid-base arrangements and further enhance the activity of the catalyst, particularly in chloride-dependent subfamilies. The combination of these dynamics, along with the help of metal/anion-induced modifications, can be considered the significant determinant of the activity, stability, and commercial performance of alpha-amylase. The catalytic action of 2010 kb of alpha-amylase with its preserved active-site residues and necessary metal/anion cofactors. Left: internal 14-alpha-1,4 bond is cleaved by endo-amylases (e.g., 2-amylase, EC 3.2.1.1) into oligosaccharides. Right: exo-amylases (e.g., α-amylase, EC 3.2.1.2; glucoamylase, EC 3.2.1.3; pullulanase, EC 3.2.1.41) remove glucose/maltose units from chain termini. Examples indicate common sources and representative industrial applications (Figure 5).

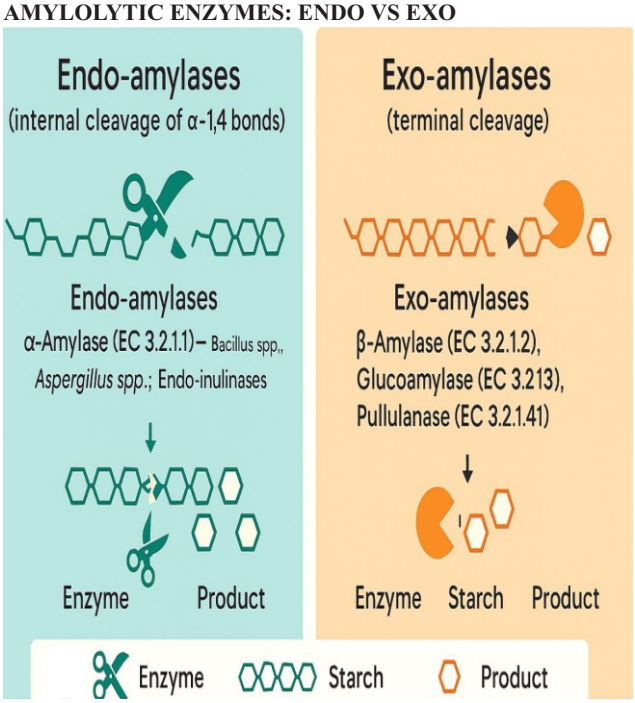

Figure 5: Classification of Amylolytic Enzymes into Endo and Exo-Acting Types

The mechanism employed by the enzyme is a retaining double-displacement reaction that includes an Asp nucleophile, an acid /base Glu, and a stabilizing Asp. The structural stability of the ion is supplied by calcium ions (Ca 2 +), whereas the allosteric modulators are chloride ions (Cl -). Schematically, the two-step formation of the covalent glucosyl enzyme intermediate and hydrolysis to give maltose/glucose is depicted.

The Structural and Functional Features

The alpha-amylases are usually monomeric proteins with a molecular weight ranging between 45 and 60 kDa [21]. They exhibit three domain folds: Domain A (the β/α barrel catalytic domain), Domain B (a loop area needed in binding Ca 2), and Domain C (a β-sheet domain commonly involved in binding raw starch). This modular design enables the α-amylases to interact with a large number of substrates and maintain their functions in different working conditions [22]. The variations of loop-regions, surface residues, and metal-binding sites between extremophilic and α-amylases are considered to be the causes of their higher stability at high temperature levels, salinity, or extreme pH levels [23]. Extremophilic Micro-organisms as Novel Suppliers of 2-Amylases [24]. The α-amylases that are secreted by these microorganisms are physically and functionally adapted to survive high temperatures, high PH, high salinity, or low temperatures. These features make them attractive replacements for conventional microbial α-amylases in the industry [25, 26]. Findings: The species of microbes and their location and optimum temperature and pH. (Table 1).

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Table 1: Representative Extremophilic Microorganisms Producing α-Amylases and Their Key Biochemical Properties

Microorganism (Species)	Environment/ Habitat	Optimal Temp (°C)	Optimal pH	Unique Traits/ Notes
Geobacillus stearothermophilus	Hot springs (thermophile)	70-80	5.5-6.5	High thermostability, rapid starch liquefaction
Anoxybacillus flavithermus	Compost/ thermal soils	65-75	6.0-7.0	Calcium-independent α-amylase (REF)
Haloferax mediterranei	Salt flats (halophile)	40-50	6.5-7.5	Active at 3-4 M NaCl
Halomonas meridiana	Saline lakes (halophile)	35-45	7.0-8.0	Broad salt tolerance
Alicyclobacillus acidocaldarius	Acidic hot springs (acidophile)	55-65	3.0-4.0	pH stability at low pH
Bacillus alcalophilus	Soda lakes (alkaliphile)	40-50	9.0-10.5	High alkaline stability
Pseudoalteromonas haloplanktis	Polar sea (psychrophile)	0-15	6.0-7.0	Cold-active α-amylase
Colwellia psychrerythraea	Arctic sediments (psychrophile)	0-10	6.0-7.0	Catalysis at low temperature

This table summarizes major extremophilic microbial species reported between 2020 and 2025, highlighting their native habitats, optimal temperature and pH for α-amylase activity, and unique adaptive traits such as thermostability, halotolerance, acid/alkali stability, and cold activity.

Thermophilic Organisms

Thermophilic archaea and bacteria thrive in geothermal springs, compost heaps and hydrothermal vents [27]. Their amylases have 2, 3-alpha2 stabilities and activities at high temperatures above 70° C and they are capable of rapid liquefaction of starch as well as reduced viscosity without the use of additional stabilizers [28, 29]. Some examples include Geobacillus stearothermophilus, Anoxybacillus flavithermus, and some archaeal species of thermal springs [30]. Hermophilic microorganisms of geothermal environments generate α-amylases that are stable and active at high temperatures, allowing efficient liquefaction of starch under industrial conditions.

Halophiles

Halophiles live in the salt lakes, solar salterns and saline soils, producing α-amylases that remain active in high salt levels [31]. Halotolerant enzymes reduce the risk of contamination and stabilise industrial reactions within a brine or high-ionic strength environment. Other notable producers include Haloferax spp., Halomonas meridiana and Natrialba magadii [32-34].

Acidophiles and Alkaliphiles

Alpha-amylases that are produced by acidophilic and alkaliphilic bacteria in the acidic mines or soda lakes have the ability to remain active at extreme levels of pH widening the scope of their application in food processing, detergents and in producing biofuels [35]. They include Alicyclobacillus acidocaldarius (acidophilic) and Bacillus alcalophilus(alkaliphilic)[36].

Psychrophilic Organisms

The cold-active alpha-amylases synthesized by psychrophilic polar region and alpine microorganisms enable low-temperature catalysis thus reducing energy costs in processes like cold-wash detergents as well as the fermentation of chilled food [37, 38]. Such species as Pseudoalteromonas haloplanktis and Colwellia psychrerythraea[39].

Other Potential Market Niches.

Other than well-studied thermophilic, halophilic, acidophilic/alkaliphilic, and psychrophilic environments, deserts, deep-sea hydrothermal vents, and cave microbiomes are ecosystems with novel α-amylases of unknown properties. Metagenomic studies of such environments have demonstrated numerous families of amylase genes. The continued research can reveal the existence of enzymes with unique substrate specificities or stability properties that can be used in new industrial applications.

Biochemical and Functional Properties of Extremophilic α-Amylases

Extremophilic 2-amylases are biochemically and structurally distinct to enable their stability and functioning in harsh conditions. These properties make them unlike the conventional microbial α-amylases which make them suitable to specific industrial use[28].

Thermal Stability

High temperature thermophilic α-amylases have increased optimum temperatures (typically over 70°C) and high temperature half-lives, which reduce the viscosity of starch slurries, speed up processing, and reduce the risk of contamination [40]. Improved thermostability is often due to increased hydrogen bonding, additional salt bridges, reduced hydrophobic cores of reduced size, and greater loop region rigidity [41].

pH Stability

The acidophilic and alkaliphilic 1/ 2 -amylases can be catalytically active in extreme pH and at the same time they can be used at pH 2-4 and also at pH 9-11 [42] The large pH stability would ensure that the processes of adjusting the pH would be significantly reduced in the industrial processes, which would lead to higher efficiency and lower costs [43]. Some of the structural adaptations include in the modification of surface charge distribution and the strengthening of Ion-pair networks[44].

Salt Tolerance

Halophilic alpha-amylases are active at high ionic strength (i.e. 3M NaCl) and thus, at that concentration, other

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enzymes would precipitate or denature. This feature works to the advantage of brine-based processes and improves the stability of enzymes to proteolysis [45]. To guarantee solubility, halophilic enzymes tend to have an increased concentration of acidic amino acids on their surfaces and reduce their hydrophobicity [46].

Cold Activity

Psychrophilic -amylases make efficient use of low temperature (0-15°C) to catalyze hydrolysis of starch which may be used in cold-wash laundry product and chilled food fermentation with low energy consumption [47]. Their high catalytic turnover and low thermostability are evidence of active-site loops that are flexible, as well as a small number of stabilizing interactions.

Substrate Specificity and Raw Starch Affinity

The substrate specificity of α-amylases extremophiles has a broad range of substrate specificity in comparison to traditional enzymes, which have a low affinity to raw starch. The features can reduce the number of pretreatment steps and increase yields of the starch-based processes [48]. This affinity is increased by aromatic residue-binding domains or surface loops. Industrial uses of extremophilic α-Amylases Applications The enzyme is useful in industries to substitute traditional enzymes in diverse functionalities. They have the advantage of being naturally stable in high temperature, extreme pH or high salinity conditions to promote shorter processing times, less risk of contamination, and less need to use stabilizing additives [49]. Alpha-amylases are thermophilic enzymes involved in the liquefaction of starch used in brewing, baking, as well as preparation of sweeteners like high fructose syrups and maltodextrins [50]. They have a high tolerance to high temperatures and can be directly incorporated into hot mash or dough processes and as a result, reduce cooling processes and increase efficiency [51].

Paper and Textile Industries

In the case of textiles, 3-amylases are used in the removal of starch-based finishes in fabrics. Enzymes that are thermostable promote short desizing times and water saving. In pulp and paper industry, alpha-amylases reduce the viscosity of the pulp, hence increasing drain and sheet forming [52].

Surfactants

Psychoactive 0-amylases are cold-active enzymes, which can be used at low washing temperatures to save energy during domestic and commercial laundry. They still remain active in highly salty and alkaline detergents formulations, outperforming conventional enzymes [53, 54].

Biofuels and Biorefineries

By eliminating the additional cooling stages and providing the possibility of saccharification and fermentation in one step, thermophilic α-amylases promote the effectiveness of the starch-to-ethanol transformation. This saves on energy use and enhances yield on production of bioethanol [55].

Pharmaceuticals and Special Applications.

Extremophilic 6-amylases have been studied in terms of their possible uses as digestive enzymes, diagnostic enzymes and in controlled-release formulations in which they must endure atypical conditions [56].

Identifying Research Gaps and Future Directions

Nevertheless, in spite of the major achievements of the isolation and characterization of a-amylases of extremophiles, there are some critical gaps in knowledge. Limited Exploration of Ecosystem Research has mainly focused on readily available geothermal springs, saline lakes and soda lakes. There are many untapped niches, such as deep-sea sediments, desert-dwelling crusts, cave microbiomes and extreme oligotrophic environments, that are not well characterized. The use of higher sampling and global cooperation can reveal new enzyme families with unanticipated properties. Clues to Structure and Mechanism despite many new α-amylase genes having been discovered, few have high-resolution 3D structures. To explore the atomic basis of thermostability, halotolerance, and pH stability through crystallography, cryo-EM, and computational modeling is necessary to engineer the rational enzyme. Several metagenomic studies have been performed based on functional metagenomics and high-throughput screening in which sequence-based mining is commonly applied, whereas functional screening has been constrained by throughput and expression issues. The use of novel microfluidic and cell free systems of expression can facilitate a greater number of active enzymes that are acquired directly out of environmental DNA, protein engineering and directed evolution. A combination of extremophilic scaffolds with site-directed mutagenesis, domain swapping, and directed evolution can be used to create tailored α-amylases that can be used in specific industrial settings. The inclusion of machine learning models in stability or substrate prediction could reduce the experimental effort (REF) that is needed. Regulation, scale up and sustainability. Regulatory approval, cost of production and impact to the environment are subject to commercial acceptance of a product. The future directions vital are optimization of the fermentation processes involving extremophilic enzymes, research on GRAS (generally regarded as safe) status and use of renewable substances during fermentation.

Limitations and Future prospects

While significant advances have been made in isolating and characterizing extremophilic α-amylases, the exploration of novel ecosystems such as deep-sea sediments, deserts, and caves remains limited. Structural elucidation at high resolution is lacking for many newly identified enzymes,

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hindering rational design for industrial optimization. Future prospects include integrating metagenomics, protein engineering, synthetic biology, and machine learning to design enzymes with enhanced stability and substrate specificity, as well as developing cost-effective, scalable, and sustainable fermentation processes to translate laboratory discoveries into practical industrial applications.

CONCLUSION

Because they are stable in harsh environments including high temperatures, salinity, and fluctuating pH, extremophilic α-amylases have a great deal of promise for industrial use. Enzyme discovery is now a systematic, predictive approach rather than a random screening method because to developments in metagenomics, bioinformatics, and machine learning. Continued integration of protein engineering, synthetic biology, and sustainable processing is anticipated to produce highly effective, personalized enzymes, despite obstacles in structural characterization, large-scale production, and regulatory approval. To fully realize the industrial and environmental benefits of extremophilic α-amylases, interdisciplinary collaboration will be essential.

Authors' Contribution

Conceptualization: IUH
Methodology: IUH, HW, MA, AK
Formal analysis: IUH
Writing and Drafting: IUH, HS, HW, AK, AIM
Review and Editing: IUH, HS, HW, AK, AIM

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Review Article

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Use of Artificial Intelligence in Breast Ultrasound Imaging: Diagnosis and Clinical Decision Support

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ABSTRACT

Breast ultrasound (US) is a critical non-invasive imaging modality for evaluating breast lesions, particularly in women with dense breast tissue. However, conventional interpretation suffers from inter-observer variability and high false-positive rates due to operator dependence and subjectivity. **Objectives:** To evaluate the role of Artificial Intelligence (AI), specifically deep learning models, in enhancing diagnostic accuracy, reducing unnecessary interventions, and supporting clinical decision-making in breast ultrasound imaging. **Methods:** A comprehensive review of recent literature (2000-2025) was conducted, focusing on AI applications in breast ultrasound for lesion detection, classification, segmentation, and clinical workflow integration. **Results:** AI systems, particularly convolutional neural networks, demonstrate diagnostic accuracy with area under the curve (AUC) values ranging from 0.92 to 0.98, often matching or exceeding expert radiologist performance. These systems achieve sensitivities and specificities typically exceeding 85%, with some studies reporting up to 98% sensitivity. AI integration reduces false-positive rates by up to 37% and unnecessary biopsies by approximately 28%. Beyond diagnosis, AI assists in lesion segmentation, BI-RADS classification consistency, and risk stratification. Portable AI-powered devices have shown promise in resource-limited settings, achieving 96-98% sensitivity. Integration of quantitative ultrasound parameters with AI enhances lesion differentiation and treatment planning. **Conclusions:** AI in breast ultrasound significantly improves diagnostic precision, workflow efficiency, and accessibility. Despite challenges, including dataset diversity, model interpretability, and clinical integration, ongoing developments support AI as a valuable adjunct tool for enhancing breast cancer detection and supporting personalized patient management.

INTRODUCTION

Breast cancer remains one of the most prevalent malignancies affecting women globally, accounting for approximately 2.3 million new cases and over 680,000 deaths in 2020 alone [1]. Early and accurate diagnosis is critical to improving patient outcomes and facilitating appropriate treatment planning. Among diagnostic imaging modalities, breast ultrasound (US) has become increasingly significant, particularly for women with dense breast tissue where mammography's sensitivity is compromised [2, 3]. Breast ultrasound is a non-invasive, radiation-free, and cost-effective imaging technique providing real-time visualization of breast tissue [4]. However, conventional US interpretation is highly operator-dependent and susceptible to inter-observer variability, leading to increased false-positive findings, unnecessary biopsies, and inconsistent clinical decisions [5]. Artificial Intelligence (AI), encompassing machine learning (ML) and deep learning (DL), has emerged as a transformative approach to address these challenges [6]. Deep learning models, especially convolutional neural networks (CNNs), have demonstrated high performance in identifying complex image patterns, often equating or exceeding expert-level diagnostic accuracy [7]. In breast ultrasound, AI applications have shown potential in improving diagnostic precision, reducing false positives, and assisting with early cancer detection [8, 9]. Despite

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significant advances, challenges persist. Many AI models are trained on limited datasets, restricting their applicability in diverse clinical settings. Additionally, integration into routine workflows remains nascent, with unresolved issues regarding model interpretability, regulatory oversight, and clinician trust [10]. While numerous studies have demonstrated AI's technical capabilities in breast ultrasound, there remains a need for a comprehensive synthesis of its clinical applications, performance across diverse settings, and practical implementation challenges. This systematic narrative review aims to: (1) evaluate the diagnostic performance of AI techniques in breast ultrasound imaging, (2) assess their impact on clinical decision-making and workflow efficiency, (3) identify persistent research gaps and implementation barriers, and (4) propose evidence-based directions for clinically integrated AI solutions in breast cancer diagnosis and management.

Despite the widespread use of breast ultrasound, conventional interpretation remains limited by operator dependence, inter-observer variability, and high false-positive rates, often leading to unnecessary biopsies and patient anxiety. While AI and deep learning have shown promise in improving diagnostic accuracy, most studies rely on limited datasets or single-institution cohorts, restricting generalizability. Furthermore, integration of AI into routine clinical workflows and evaluation across diverse populations remains insufficiently explored, highlighting a critical gap for evidence-based, clinically deployable AI solutions in breast ultrasound imaging.

The Role of Ultrasound in Breast Cancer Diagnosis

Breast ultrasound, also known as sonography, is a non-invasive imaging technique using high-frequency sound waves (typically 5-14 MHz) to produce detailed images of internal breast structures. Ultrasound (US) plays an essential role in breast cancer imaging and diagnosis, particularly in women with dense breast tissue [11]. While mammography remains the standard for screening, its sensitivity significantly decreases in dense breast composition. In such cases, breast US becomes more effective, detecting lesions that mammography may miss [12]. The US is also used to evaluate palpable lumps, guide needle biopsies, and assess abnormal findings from other imaging tests. The versatility of breast US includes grayscale B-mode imaging and Doppler modalities, allowing clinicians to observe lesion vascularity [13]. However, breast US has limitations. It is highly dependent on operator skill, patient anatomy, and equipment quality, resulting in interpretation variability and both false-negative and false-positive findings [14]. US diagnostic utility is further challenged by its subjective nature. Radiologists assess lesion features such as shape, margin, echogenicity, orientation, and posterior acoustic behavior. The Breast Imaging Reporting and Data System (BI-RADS) helps standardize interpretations but still relies on human input [15]. This has driven demand for computer-aided diagnosis (CAD) systems and AI applications that can support or augment human interpretation [16].

Limitations of Traditional Ultrasound Interpretation

Traditional interpretation of breast US involves manual evaluation by radiologists examining features such as lesion morphology, acoustic patterns, and margins. While experienced radiologists achieve high diagnostic accuracy, studies highlight significant inter-reader variability [17]. The subjective nature of feature assessment, along with variability in training and experience, leads to inconsistent conclusions. Interpretation of BI-RADS categories 3 and 4 remains particularly challenging [18]. Another limitation is the high false-positive rate. Studies show that adding US to mammography can increase recall rates by 5-15% and biopsy rates by 4-8%, but only 7-8% of these biopsies yield malignant results [4]. This means many patients undergo unnecessary invasive procedures, leading to anxiety, discomfort, and increased healthcare costs. Furthermore, the increasing volume of breast US exams places pressure on radiologists, raising the risk of fatigue-related errors [19]. Inconsistent interpretation due to cognitive load or lack of standardized reporting protocols further reduces reliability. These challenges create opportunities for AI and deep learning algorithms that can provide standardized, reproducible, and accurate interpretations [20].

Emergence of Artificial Intelligence in Medical Imaging

Artificial Intelligence (AI) has significantly transformed medical imaging. AI refers to the simulation of human intelligence by machines and encompasses subfields such as machine learning (ML) and deep learning (DL) [6]. In imaging applications, AI algorithms are trained on large datasets to detect patterns, classify anomalies, and provide diagnostic suggestions. Deep learning, particularly through convolutional neural networks (CNNs), has been especially impactful in medical image analysis [7]. CNNs can learn spatial hierarchies from image data, extracting increasingly complex features as the network deepens. Unlike traditional ML, which relies on handcrafted features, CNNs learn directly from raw image inputs, improving accuracy and reducing bias. These characteristics make CNNs particularly suited for the US, which is known for variability in image quality [21]. In breast imaging, AI is applied for classification (benign vs. malignant), lesion detection, segmentation, and disease prognosis prediction [22]. AI systems can analyze millions of images faster than humans and can be deployed to flag suspicious cases, aid in triage, or serve as second readers. Importantly, these systems are now being designed with explainability features like saliency maps, allowing

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clinicians to understand AI-generated decisions, fostering trust in clinical environments [17].

This review article evaluates the role of Artificial Intelligence (AI), specifically deep learning models, in enhancing diagnostic accuracy, reducing unnecessary interventions, and supporting clinical decision-making in breast ultrasound imaging.

RESULTS

The aggregated data comprises 123 cases from women (95% female) with a mean age of 52 ± 15 years, all evaluated for breast masses. Of the total lesions, 27 were malignant, primarily invasive ductal carcinoma (IDC) and ductal carcinoma in situ (DCIS), while 96 were benign. Lesion size distribution varied, with 7% measuring ≤10 mm and 28% falling within the 10-20 mm range. Most patients (85%) exhibited low breast density. Ultrasound features observed included non-circumscribed margins (44.7%), irregular lesion shapes (34.1%), and spiculation (25.2%). Additional findings included calcifications in 14.6% of cases and evidence of surrounding tissue alterations or increased vascularity in 19.5%. These varied morphological and textural characteristics provide representative examples of the diverse datasets used for training, validating, and optimizing AI-based diagnostic models in breast US imaging (Table 1).

Table 1: Representative Demographics and Imaging Features from AI-Assisted Ultrasound Studies (Illustrative Case Series, N=123)

Characteristics	Values
Number of patients	123
Female (%)	95.1%
Mean age (years)	52.0 ± 14.7
Lesions	123 breast masses
Malignant / Benign	27 malignant (22 IDC, 4 DCIS,1 mucinous) 96 benign
Tumor size ≤10 mm	9 (7.3%)
Tumor size 10-20 mm	34 (27.6%)
Breast density (low/med/high)	105/17/1
Non-circumscribed margins	55 (44.7%)
Irregular shape	42 (34.1%)
Spiculation	31 (25.2%)
Calcification	18 (14.6%)
Moderate-high blood flow	7 (5.7%)
Surrounding tissue changes	24 (19.5%)

Several high-quality studies have demonstrated the effectiveness of AI in interpreting breast US images. One of the most robust efforts involved an AI model trained on over 5.4 million US images from 288,767 breast exams [23]. This model achieved an area under the receiver operating characteristic curve (AUROC) of 0.976 on a test dataset of 44,755 exams. When compared to 10 board-certified radiologists, the AI system not only matched but outperformed them in diagnostic accuracy, reducing false-positive rates by 37.3% and unnecessary biopsy recommendations by 27.8%. Transfer learning, which allows models pre-trained on general image datasets to be fine-tuned for specific tasks, has been used with great success [8, 23]. Byra *et al.* employed a VGG19 model pre-trained on ImageNet and adapted it for breast US classification, achieving a high AUC of 0.936 for classifying malignant vs. benign lesions [12]. Another study by Xiao et al. compared different CNN architectures and found that transfer learning-based models achieved superior diagnostic accuracy over traditional ML and standard CNNs [13]. AI has also been shown to improve diagnostic consistency across diverse patient populations. The AI system developed by Shen *et al.* maintained high accuracy across all age groups, breast densities, and US machine types [8]. The model was further validated on an external dataset (BUSI) from Egypt, achieving a strong AUROC of 0.927, which suggests good generalizability [3]. Automated Breast Ultrasound; CAD = Computer-Aided Diagnosis; AUC = Area Under the Curve. Across numerous studies, AI systems consistently demonstrate high diagnostic performance, with mean sensitivities and specificities often ranging between 80% and 100% for breast cancer detection and classification. AI is also increasingly utilized to predict molecular subtypes, axillary lymph node involvement, and response to neoadjuvant chemotherapy, enabling more personalized treatment strategies. Some models, such as recurrent neural networks (RNNs), have achieved over 98% accuracy in experimental settings [23-26]. The integration of AI with automated breast US (ABUS) and radiomics has further improved diagnostic precision and enabled quantitative assessment for therapy monitoring. Additionally, smartphone-based AI applications have shown promise in delivering rapid and accurate diagnoses, particularly in resource-constrained settings [20, 24] (Table 2).

Table 2: Comparison of AI Model Efficacy in Breast Ultrasound Imaging (2000-2025)

References	AI Approach	Dataset Size/ Type	Diagnostic Task (s)	Sensitivity (%)	Specificity (%)	Accuracy (%)	AUC	Key Applications
[20]	ABUS radiomics + AI	Not specified	Diagnosis, therapeutic evaluation	Not stated	Not stated	Not stated	Not stated	Personalized treatment, therapy monitoring
[22]	ML/DL (77.6% DL)	58 studies (2017-2022)	Diagnosis, prognosis, subtyping, axillary status, response to therapy	Mean: 85-95	Mean: 80-95	Mean: 85-95	0.85-0.95	Treatment planning, response prediction

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[24]	YOLOv3 (DL)	316 images (benign/ malignant)	Lesion detection/ classification	100 (smart phone)	75 (AI server) 97.5 (smart phone)	Not stated	Not stated	Point-of-care diagnosis
[25]	Various AI	Not specified	Detection, diagnosis, subtyping, axillary status, response to therapy	Not stated	Not stated	Not stated	Not stated	Treatment response, molecular subtyping
[26]	RNN, GP, TL, ANN, CNN	30 datasets, 310 articles	Early diagnosis, precision treatment	>98 (RNN)	>96 (GP, TL, ANN)	>96 (DL)	Not stated	Precision treatment, automated triage
[27]	ML, DL	Not specified	Benign/malignant differentiation	Not stated	Not stated	Not stated	Not stated	Early screening, workflow improvement
[28]	ML, CAD	Not specified	Early diagnosis, detection	Improved vs. traditional ML	Improved	Improved	Not stated	Reducing misdiagnosis, workflow efficiency

ML = Machine Learning; DL = Deep Learning; RNN = Recurrent Neural Network; GP = Gaussian Process; TL = Transfer Learning; ANN = Artificial Neural Network; CNN = Convolutional Neural Network

The study summarizes the distribution of breast US cases across multiple AI research studies conducted between 2002 and 2025, reflecting variations in dataset composition, imaging platforms, and study designs (Table 3).

Table 3: Case Overview of AI Applications in Breast Ultrasound Imaging

Scanner Model	Malignant Cases	Benign Cases (Biopsy / Follow-up)	Total Cases
Canon Aplio 500 & GE LOGIQ E10	79	92 (77 via follow-up)	171
Siemens ACUSON Sequoia & Canon Aplio 500 (portable)	95	107 (unspecified method)	202
Samsung S Detect (multi-mode clinical analysis)	70 (27%)	190 (44 biopsy, 100 follow-up)	260
Handheld B-mode ultrasound (not specified)	450	601	1,051
Koios DS with US-guided biopsy	45	155	200

Accurate lesion segmentation is critical for measuring tumor size, planning treatment, and monitoring progression or response to therapy [13]. Traditionally, segmentation requires manual annotation, which is time-consuming and prone to variability. AI-powered segmentation tools can automate this process with high accuracy, designed to delineate lesion boundaries from surrounding tissue, even in cases of poor contrast or irregular shapes, which are common in US imaging [9]. Gu *et al.* developed a 3D segmentation method for breast US using morphological reconstruction and edge-detection techniques [14]. This approach achieved high accuracy in differentiating tissues and structures within 3D US volumes. Beyond segmentation, AI has been applied to assess tumor heterogeneity and predict biological behavior. Deep learning models have been trained to classify lesion stiffness, vascularity, and posterior acoustic features, attributes that help determine malignancy risk. In some cases, AI has outperformed radiologists in distinguishing between BI-RADS 3 and 4 lesions, aiding in biopsy decision-making and potentially reducing overtreatment [15, 18]. One of the most critical applications of AI in breast US is the reduction of false positives and unnecessary biopsies [3]. False positives not only burden healthcare systems but also cause significant psychological stress to patients. AI can mitigate this by accurately identifying lesions that do not require biopsy and flagging those that do with greater precision [29] (Table 4).

Table 4: AI in Breast Ultrasound for Low-Resource Settings: Key Studies (2000-2025)

References	Setting and Sample	AI Task	Key Performance
[30]	Rural Mexico, portable handheld US by minimally trained users (758 masses in 300 women)	CADx classification using Koios DS	Sensitivity 96-98%, specificity 38-67%, AUC ≥ 0.95
[31]	Mexico, low-cost handheld US by non-physicians (subset of Berg cohort)	CAD-assisted triage	Accuracy comparable to radiologists (100% sensitivity/specificity in small subset)
[32]	Brazil, 83 biopsy-proven breast masses	CAD system on elastography +BI-RADS lexicon	AUC improved from ~0.80 to 0.90-0.93 across readers; κ_i.c.c. improved
[33]	Dataset from clinical breast US images	Semi-supervised DL integrating BI-RADS features (BIRADS-SDL)	Classification accuracy ~83.9-92.0% on two datasets
[34]	Automation via 3D ABUS, 418 patients	3D detection + classification network	Sensitivity 97.7%, AUC ≈ 0.872
[35]	BUS images (multiple datasets)	ROI-free Transformer (HoVer-Trans)	Outperformed CNNs/sonographers; state-of-the-art accuracy

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Several quantitative ultrasound (QUS) parameters significantly differ between malignant and benign breast lesions, offering valuable diagnostic insights. Malignant lesions generally exhibited higher attenuation coefficients and speed of sound values, likely reflecting increased tissue density and stiffness [36]. In contrast, benign lesions showed greater effective scatterer diameter (ESD), indicating a more uniform internal microstructure [37]. Parameters such as mid-band fit, spectral slope, and spectral intercept also trended higher in malignant lesions, corresponding to increased tissue heterogeneity [38]. Although some features, like effective scatterer concentration, did not show significant variation, the overall combination of spectral and textural QUS features enabled high diagnostic accuracy, with reported AUCs nearing 0.97 [39]. These findings support the integration of QUS metrics into AI systems for more accurate lesion classification and early breast cancer detection (Table 5).

Table 5: Quantitative Ultrasound Parameters by Final Diagnosis and Pathology Outcome

QUS Parameter	Malignant (mean ± SD)	Benign (mean ± SD)	Significance
Attenuation co-efficient (AC)	Higher	Lower	$p < 0.05$
Speed of sound (SoS)	Higher	Lower	$p < 0.05$
Effective scatterer diameter (ESD)	Lower	Higher	$p < 0.05$
Effective scatterer concentration (ESC)	No significant difference	–	–
Mid-band fit (MBF)	↑	↓	–
Spectral slope (SS)	↑	↓	–
Spectral intercept (SI)	↑	↓	–
Textural QUS features	More heterogeneous	Less heterogeneous	AUC 0.97

BI-RADS 4 lesions displayed markedly higher elasticity values (e.g., Emean and Emax), indicative of increased tissue stiffness commonly associated with malignancy [40]. Quantitative differences were also noted in attenuation, speed of sound, and velocity indices, with BI-RADS 4 lesions deviating significantly from the more benign BI-RADS 3 profiles [35]. Doppler assessments revealed more frequent abnormal vascular features in BI-RADS 4 lesions, supporting their use in enhancing diagnostic confidence [27]. Texture-based QUS features showed greater heterogeneity in suspicious lesions, further contributing to lesion stratification. These quantitative differences highlight the potential of combining QUS with AI to refine BI-RADS classification, particularly by identifying low-risk BI-RADS 4A lesions that may not require biopsy, thereby improved clinical decision-making and reducing unnecessary interventions [36] (Table 6).

Table 6: Quantitative Ultrasound Parameters within (QUS) BI-RADS Categories 3 and 4

Parameter	BI-RADS 3 (Probably Benign)	BI-RADS 4 (Suspicious)	Clinical Insight
Attenuation and SoS	Similar to benign profiles	Shift toward malignant values	May aid in resolving indeterminate cases (BI-RADS 4A)
Strain elastography (mean elasticity, Emean)	Lower (<4.5 kPa)	Higher (>30 kPa), Emax > 36 kPa	Improves downgrading from BI-RADS 4A to 3, reducing unnecessary biopsies
Velocity index (VI)	Lower (~3%)	Higher (~5%)	Supports differentiation between benign and malignant lesions
Doppler flow (including bidirectional flow)	Absent or minimal	>3 abnormal features detected ~100% sensitivity, ~76% specificity	Enhances vascular assessment
QUS texture/heterogeneity	Homogeneous	Heterogeneous	Supports lesion characterization in indeterminate BI-RADS categories

A DL system trained on B-mode and Doppler US images significantly improved diagnostic performance, achieving an internal AUC of 0.94 and an external AUC of 0.96, reducing false-positive rates by 7.6% and improving interobserver agreement. Google's AI model trained on over 288,000 US exams and 5.44 million images achieved AUROC values of 0.976 (internal) and 0.927 (external), while reducing false-positive diagnoses by 37.3% and unnecessary biopsies by 27.8% [34-36] (Table 7).

Table 7: Use of Artificial Intelligence in Breast Ultrasound Imaging for Diagnosis and Clinical Decision Support

References	Setting and Sample	AI Task	Key Performance
[34]	Multivendor, multicenter; 45,909 B-mode + Doppler images	Deep learning classification; model-assisted radiologist support	AUC 0.94 internal, 0.96 external; reduced false positives by 7.6%; improved interobserver agreement
[35]	288,767 exams, 5.44 M images; B-mode and Doppler	AI vs radiologists; reader aid	AUROC 0.976 internal, 0.927 external; reduced false positives 37.3%, reduced biopsies 27.8%
[36]	4,998 patients: comparison of CNN architectures and resolutions	CNN model vs senior sonographers	Best AUC 0.924 (MobileNet_224), accuracy 87.3%; outperformed senior US readers

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DISCUSSION

The integration of artificial intelligence (AI) into breast ultrasound (US) imaging marks a major advancement in diagnostic radiology, consistently improving accuracy, efficiency, and clinical decision-making [1–3]. AI systems employing deep learning (DL) and convolutional neural networks (CNNs) now demonstrate diagnostic performance comparable to, or exceeding, that of expert radiologists [26, 34–36]. Large-scale validation studies provide the most compelling evidence. The Google AI model, trained on 288,767 exams comprising 5.44 million images, achieved AUROC values of 0.976 (internal) and 0.927(external), showing robust generalizability. It reduced false-positive interpretations by 37.3% and unnecessary biopsies by 27.8%, addressing one of the main drawbacks of conventional US high false-positive rates leading to patient anxiety and increased healthcare costs [4-5]. This underscores AI's role as both an educational aid and a quality assurance tool. Recent developments have extended AI capabilities beyond binary classification. Transfer learning using pre-trained models such as ImageNet and fine-tuning them for breast US enables high accuracy even with smaller datasets [12-13]. Transformer-based architectures, such as HoVer-Trans, outperform traditional CNNs and expert sonographers by capturing long-range dependencies critical for interpreting complex breast tissue patterns [33]. Explainability features such as saliency maps and attention mechanisms help mitigate the "black box" criticism of AI systems [17]. The integration of quantitative ultrasound (QUS) parameters with AI represents another promising direction [34–38]. QUS provides measurable tissue characteristics—such as attenuation, speed of sound, and spectral features that distinguish benign from malignant lesions. When combined with AI, diagnostic accuracy improves markedly, with reported AUC values up to 0.97 [34–37]. Incorporating elastography further refines BI-RADS classification: lesions with Emean >30 kPa or Emax >36 kPa correlate strongly with malignancy in BI-RADS 4 cases [37-38]. AI-assisted reclassification of low-risk BI-RADS 4A lesions could reduce unnecessary biopsies by 15–18% while maintaining sensitivity. AI has also expanded access to quality breast imaging in resource-limited settings. AI-assisted portable US devices operated by minimally trained personnel achieved 96–98% sensitivity in rural populations, approaching expert performance. However, specificity varied (38–67%) due to differences in device quality and operator skill [29, 36]. AI-assisted interpretation benefits radiologists of all experience levels, with the greatest impact seen among less experienced readers. Benign biopsy rates decreased from 52% to 33% for junior and from 46% to 34% for senior radiologists when using AI support [39]. Successful deployment requires robust algorithms, standardized imaging protocols, quality assurance, and local training programs [36, 37]. Smartphone-based AI tools further enhance accessibility. Deep learning models deployed on mobile devices achieved 100% sensitivity and 97.5% specificity for lesion detection [24], enabling rapid triage in primary care and reducing specialist workload. Despite encouraging progress, challenges persist. Dataset diversity and generalizability remain major concerns, as most models are trained on data from single institutions or homogeneous populations [17–20]. Although some studies demonstrated external validation with an AUROC of 0.927 on diverse populations [3], comprehensive cross-population evaluation remains limited. Model interpretability, while improving, is still insufficient for full clinical adoption. Since radiologists bear ultimate diagnostic responsibility, AI predictions must be transparent and explainable [17-18]. Regulatory approval, workflow integration, and interoperability with radiology information systems (RIS) and picture archiving and communication systems (PACS) also pose barriers [10,19]. Most current models remain focused on binary classification (benign vs. malignant) [19–21], whereas comprehensive breast cancer management requires AI tools capable of risk stratification, molecular subtype prediction, lymph node assessment, and treatment response monitoring [21–25]. However, significant challenges remain. Dataset diversity and external validation across heterogeneous populations require attention to ensure generalizability. Model interpretability must improve to foster clinical trust and meet regulatory requirements. Clinical workflow integration, cost-effectiveness evaluation, and prospective validation through randomized controlled trials are necessary before widespread implementation. Additionally, expanding AI capabilities beyond binary classification to address multi-task clinical needs, including molecular subtyping, treatment response prediction, and surgical planning, represents an important frontier. The evidence supports AI as a valuable adjunct tool that augments rather than replaces radiologist expertise. Optimal implementation likely involves human-AI collaboration, where AI serves as a consistent "second reader," quality assurance mechanism, and decision support tool. Continued research addressing technical limitations, validation in diverse settings, and practical implementation strategies will determine whether AI's promise translates into improved breast cancer outcomes globally. With thoughtful development emphasizing clinical utility, interpretability, and equitable access, AI has substantial potential to transform breast US imaging and enhance patient care.

Limitations and Future Prospects

While AI demonstrates substantial potential in enhancing breast ultrasound diagnosis, limitations remain, including

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dataset heterogeneity, limited external validation, and incomplete clinical integration. Future research should focus on large-scale, multicenter studies, improving model interpretability, and developing AI systems capable of multi-task functions such as molecular subtyping and treatment response prediction. Additionally, combining AI with quantitative ultrasound and portable devices could expand access to high-quality diagnostic support in resource-limited settings, ultimately improving patient outcomes globally.

CONCLUSION

This review demonstrates that AI, particularly deep learning-based approaches, significantly enhances breast US imaging for cancer diagnosis and clinical decision support. AI systems consistently achieve high diagnostic accuracy with AUC values ranging from 0.92 to 0.98, often matching or exceeding expert radiologist performance. Critically, AI integration reduces false-positive rates by up to 37% and unnecessary biopsies by approximately 28%, addressing major limitations of conventional US interpretation. Beyond diagnostic accuracy, AI provides several clinical benefits: (1) improved inter-reader and intra-reader consistency, reducing interpretation variability; (2) enhanced performance across reader experience levels, with particularly pronounced benefits for less experienced radiologists; (3) automated lesion segmentation and BI-RADS classification support; (4) integration with quantitative US parameters for refined risk stratification; and (5) potential for expanding access to quality breast imaging in resource-limited settings through portable, AI-assisted devices.

Authors' Contribution

Conceptualization: MIUH
Methodology: MIUH, SMYF, MM
Formal analysis: SMYF
Writing and Drafting: MIUH, SMYF, MM
Review and Editing: MIUH, SMYF, MM

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

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Review Article

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Cytokine Regulation of Immune Responses in Parasitic Diseases: A Review

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ABSTRACT

The immune regulation in parasitic infections plays a central role in cytokine mediation of host defense and disease pathology. It is an integrative review that summarizes the current molecular and quantitative data on the dynamics of cytokines in major parasitic infections, malaria, leishmaniasis, trypanosomiasis, and schistosomiasis. A methodical search of the literature was carried out in PubMed, Scopus, and Web of Science (2010-2025) and included specific inclusion and exclusion criteria. Descriptive analysis of quantitative cytokine concentration ranges and cytokine ratios was conducted based on heterogeneity of study designs. The figures were made with Figma software to be visually accurate and clear. The results have shown that the outcome of infections depends mostly on the balance between pro-inflammatory (e.g., TNF-α, IFN-γ) and regulatory cytokines (IL-10, TGF-β). Severity of the disease is associated with disrupted cytokine ratios, instead of cytokine abundance. Molecular mimicry and JAK-STAT and NF-κB modulations are some of the ways used by parasites to sustain chronicity by exploiting these cytokine pathways. A combination of comparative cytokine profiles shows common immunoregulatory principles between parasitic infections and cytokine ratios as a potential biomarker of disease progression or immune response to therapeutic intervention. This synthesis offers an integrated framework that connects cytokine signaling with clinical outcomes and provides guidance on subsequent standard, multi-omics studies to achieve precision immunomodulation and better disease prognostication.

INTRODUCTION

Parasitic diseases still act as a significant health burden to the world, especially in the tropics and subtropics, where malaria, leishmaniasis, trypanosomiasis, and schistosomiasis infections have remained a significant cause of morbidity and mortality despite decades of control measures [1]. The World Health Organization (2023) states that the number of new cases is millions each year, which highlights the potential of parasitic infection as a problem posing challenges to the health of humans, socioeconomic stability, and healthcare systems. These infections are characterized by intricate host-pathogen interactions whereby the immune system can be decisive on the outcome of the disease; total elimination of the parasite or chronic infection and immune-mediated pathology [2]. Cytokines are small, secreted signaling proteins that are among the immune components that mediate host defense as central regulators of immune and non-immune cell communication [3]. They regulate, strengthen immune responses, and are molecular switches that keep the balance between anti-inflammatory and pro-inflammatory responses in balance [4]. For example, T-helper 2 (Th2) cytokines, such as interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13), facilitate extracellular parasite expulsion but may also

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trigger allergic or fibrotic responses if dysregulated, while T-helper 1 (Th1) cytokines, such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), and interleukin-12 (IL-12) [5]. On the other hand, anti-inflammatory cytokines such as transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10) inhibit excessive inflammation, maintaining tissue integrity while also creating a niche for parasite persistence [6]. The action of cytokines through high-affinity receptors that activate intracellular signalling pathways like JAK-STAT, NF-κB, and MAPK. The stimulation of these pathways controls the expression of genes that manage the growth, differentiation, and activity of immune cells [7]. As an example, Th1 polarization and IFN-γ synthesis induced by IL-12 stimulation of STAT4, and Th2 polarization and antibody class switching signalling are induced by IL-4-stimulated STAT6. At the same time, TNF-α stimulates IL-1β the NF-κB, enhancing the transcription of inflammatory genes, whereas IL-10 and TGF-β involve inhibitory receptor stimulation to inhibit the expression of pro-inflammatory cytokine genes. Such organized molecular processes guarantee accurate immune regulation and maintain the balance between the destruction of pathogens and the protection of the tissues [8]. Available literature focuses on individual causal agents or cytokine families, and does not consider comparative dynamics, age-dependent changes, and chronic exposure outcomes. Thus, a new integrative paradigm is required to bridge the gap between cytokine signalling and clinical repercussions and potential therapeutic benefits. This study brings out the major functions of the cytokines, disease-specific profiles, and clinical implications of cytokine modulation. Altogether, the review provides a modern interpretation of cytokine networks that are associated with disease and the development of future immunomodulatory treatments.

This study aims to focus on the immune regulation of major parasitic infections through cytokine mediation in a systematic way by synthesizing the current molecular and immunological information.

The review was done in a structured and transparent manner in order to be comprehensive and reproducible. The process of the review was systematic literature identification, screening, and narrative synthesis of results as they applied to the understanding of cytokine-mediated immune responses in parasitic infections. PubMed, Scopus, and Web of Science databases were thoroughly searched, including the period between January 2010 and October 2025. Inclusion Criteria: The inclusion criteria were: 1. Original research or review articles in English, 2. Parasitic infections-reported cytokine profiles, immune modulation, or signaling mechanisms, and 3. Carried out in humans or tested in laboratory animal models. Exclusion Criteria: 1. Conference abstracts, cases, or non-peer-reviewed publications, 2. The literature that was devoid of cytokine-specific data, and 3. Duplicate data or unfinished data. Data Extraction: Full texts were checked to extract data, and eligibility was determined by title and abstract. The resulting information covered cytokine type, direction of regulation (up- or down-regulation), stage of infection, host species or age group, and disease outcome. To make sure that all figures used in this review are clear, accurate, and visually consistent, all of them were conceptualized and created with the help of Figma software.

Despite decades of research, parasitic diseases such as malaria, leishmaniasis, trypanosomiasis, and schistosomiasis continue to cause high morbidity and mortality, largely due to incomplete understanding of host immune regulation. Most studies focus on individual parasites or single cytokines, leaving a critical gap in comparative analysis of cytokine dynamics across different infections. Moreover, age-dependent variations, chronic exposure outcomes, and compartment-specific cytokine responses remain poorly characterized. Bridging these gaps is essential for developing precise immunomodulatory strategies and improving disease prognostication.

Cytokines: Types and Functions in the Immune System

Cytokines are a general type of low-molecular-weight (usually 5-25 kDa) secreted proteins that are important intercellular messengers of the immune system [9]. They are mainly generated by immune cells (e.g., macrophages, dendritic cells, NK cells, T and B lymphocytes), although in many cases, non-immune cells (e.g., epithelial cells, fibroblasts) also contribute to their production [10]. Cytokines do so in autocrine, paracrine, and, less frequently, endocrine signals, interacting with high-affinity cognate receptors on their target cells and triggering intracellular signal transduction (notably JAK-STAT, NF-KB, and MAPK) and resulting in changes in gene expression, differentiation, proliferation, survival, and effector functions [11].

Classification of Cytokines: Type 1 vs Type 2 functional bias

Classification of Cytokines: Type 1 vs Type 2 functional bias was done (Figure 1).

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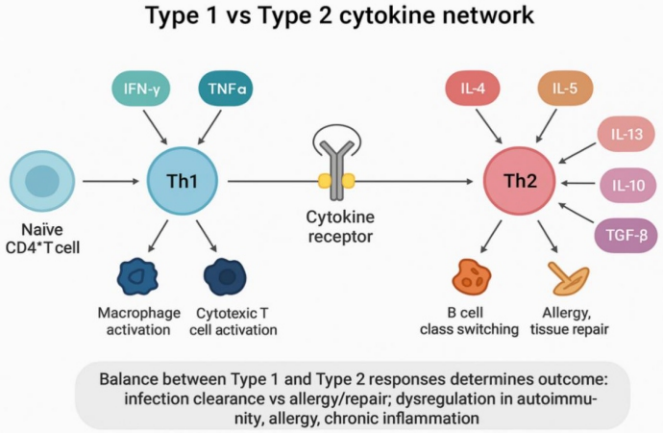

Figure 1: Type 1 versus Type 2 Cytokine Network in Immune Regulation

This schematic illustrates the differentiation and effector mechanisms of T-helper 1 (Th1) and T-helper 2 (Th2) immune responses. Th1 cytokines, including interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-12 (IL-12), promote macrophage activation and intracellular parasite clearance [12]. Conversely, Th2 cytokines, such as interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13), induce eosinophil recruitment, antibody class switching, and mucosal repair for extracellular parasite expulsion. The balance between Th1 and Th2 responses determines infection outcomes, ranging from protective immunity to chronic inflammation and tissue fibrosis [13].

Functions of Cytokines

There are several fundamental functions of the immune system, cytokine-based, as follows (Figure 2):

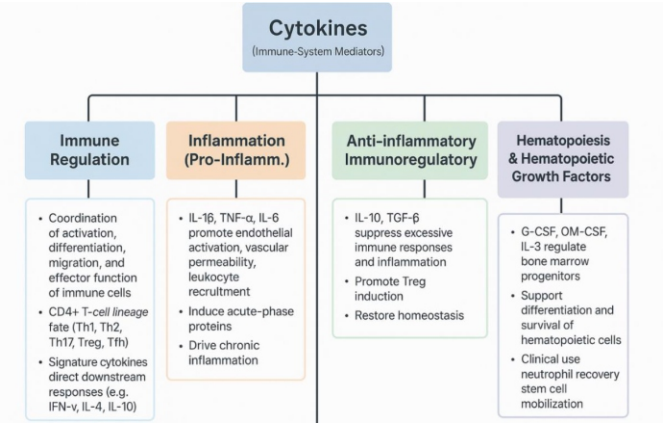

Figure 2: Functional Spectrum of Cytokine-Mediated Immune Responses

This diagram summarizes the principal functional categories of cytokines in host defense. Key functions include: (1) immune regulation (mediating activation) [14], differentiation, and communication among immune cells [15], (2) pro-inflammatory signaling, inducing acute-phase responses via IL-1β, IL-6, and TNF-α [16], (3) anti-inflammatory control, mediated by IL-10 and TGF-β to limit tissue damage [17], (4) hematopoietic support, through growth factors such as G-CSF that promote bone marrow cell proliferation [18]. Together, these interconnected cytokine pathways orchestrate host immunity, homeostasis, and pathogen tolerance.

Cytokine Mechanisms in Parasitic Infections and Host Immune Response

Cytokines are the major signalling molecules that coordinate immune responses to parasitic infections, as they serve as molecular messengers connecting innate and adaptive immunity. Parasitic pathologies: the presence of helminthic (multicellular parasites) and protozoan (unicellular parasites) parasites induces a very different cytokine profile, resembling the character of the infecting pathogen and the type of effector response needed to control it [19]. Pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and NOD-like receptors (NLRs), are recognized by innate immune cells at the onset of infection, macrophages, dendritic cells (DCs), and epithelial cells. It leads to the release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-alpha, and IL-12) and chemokines that determine the inflammatory milieu, which predetermines future adaptive reactions [20].

- Cd4 + T-cell differentiation into specialized subsets is determined by the cytokine microenvironment [21].
- Th1: IL-12 and IFN-γ trigger the program of Th1 to induce the killing of protozoa within the cell by macrophages [22].
- Th2 cells, which are induced by epithelial-derived alarmins including IL-25, IL-33, and TSLP, trigger eosinophil recruitment and antibody (IgE) generation needed to expel helminth [19].
- Tregs (regulatory Tregs) are cells that reduce overproduction of inflammation induced by the effects of IL-10 and TGF-β and control tissue damage [23].
- IL-6, IL-21, and IL-23 regulate Th17 cells that perform the functions of mucosal protection and granulocyte mobilization and may also cause immunopathology in chronic infections [24].

Helminth Infections: Th2 and Regulatory Dominance

Nematodes, trematodes, and cestodes cause helminth infections, which are characterized by a Type 2 immune response. Such an IL-4-driven cytokine environment orchestrates the mobilization of effector cells, eosinophils, mast cells, basophils, and alternatively activated macrophages (M2) [25]. It involves IL-4 and IL-13 that promote B-cell class switching to IgE, mucus secretion, and intestinal smooth muscle contractility, which is a physical elimination mechanism, the so-called weep and sweep response [26].

- The IL-5 attracts and stimulates eosinophils, which

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- contain cytotoxic granules (major basic protein, eosinophil cationic protein) that destroy helminth cuticles[27].
- The IL-33, which is discharged as an alarm by necrotic cells of the epithelial and endothelial layers, enhances Th2 polarization through the stimulation of innate lymphoid cells type 2 (ILC2s), which increase the production of IL-5 and IL-13[28].
- The secretion of IL-27 by macrophages and dendritic cells has a modulatory effect, which increases Th1 responses but subsequently stimulates the secretion of IL-10, thereby limiting Th2-mediated pathology [29].

Protozoan Infections: Th1-Driven Immunity and Pathology

Protozoan parasites, including Plasmodium, Leishmania, Toxoplasma gondii, and Trypanosoma spp., are mostly located inside host cells, which requires a Th1-biased response to be controlled successfully[30].

- NK cells and Th1 differentiate and produce strong amounts of IFN-γ in response to IL-12 released by antigen-presenting cells[31].
- IFN-γ causes classical macrophage activation (M1 phenotype), which increases inducible nitric oxide synthase (iNOS) and reactive oxygen intermediates (ROI)involved in intracellular killing of parasites[32].
- TNF-alpha, acting synergistically with IFN-γ, potentiates phagolysosome fusion and increases MHC expression, which reinforces antigen presentation[33].

Although these cytokines are necessary in the regulation of the replication of protozoans, when overactivated or prolonged, Th1 may lead to immunopathology. An example is that high levels of IFN-γ and TNF-α are involved in the inflammation of the brain of the Plasmodium falciparum malaria[34]and the hepatic granulomas of the Leishmania donovani infection[35]. A regulatory feedback mechanism develops in chronic protozoan infection. IL-10, IL-27, and TGF-β reduce hyperinflammation, which protects host tissues; however, unintentionally, it facilitates persistence of the parasite[36].

Cytokine Profiles in Malaria Diseases

Recent quantitative studies have shown that there are specific cytokine concentration gradients that reflect the severity of malaria and the outcome of the immune response. TNF-alpha (TNF-α) concentrations usually lie within 45 -120 pg/mL-1 in non-complicated infection, and up to 200 pg/mL-1 in complicated or cerebral malaria, which is why it has been proposed as a primary mediator of endothelial activation and inflammation in Plasmodium falciparum infection[34, 37]. Likewise, interferon-gamma (IFN-γ) levels rise to 30-80 pg/mL-1 in mild disease and 150-200 pg/mL-1 in advanced infection, as a result of hyperactivation of the macrophages and the production of nitric oxide[38]. However, regulatory cytokine interleukin-10 (IL-10), on the other hand, increases substantially in the case of acute infection (60-110 pg/mL-1) as a response to inflammation, but overproduction of IL-10 leads to parasite survival and chronic infection [39]. Multi-cohort meta-analysis by[40]and recent systematic reviews[41]confirm that a high TNF-α/IL-10 ratio (> 2.5) is a strong predictor of severe or fatal malaria, whereas a balanced ratio is strongly associated with controlled parasitemia and improved survival. Overall, these results indicate that the malaria immunopathology is mediated by cytokine imbalance and not cytokine abundance, and highlight the therapeutic role of cytokine signaling pathways modulation in the disease.

Leishmaniasis

Based on human research, local and systemic immune conditions are differentially characterized by quantitative cytokine signatures of cutaneous (CL) and visceral leishmaniasis (VL). In visceral leishmaniasis, IL-10 in circulation is always high before therapy (mean values at reported level of about 4664 pg/mL-1), and drops significantly upon effective therapy, which indicates it to be an active disease and immunosuppressive marker [42, 43]. Simultaneously, systemically IFN-γ can be detected in VL but at lower systemic concentrations than in localized CL; IFN-γ responsiveness can be restored by treatment and is associated with clinical cure. In the case of cutaneous leishmaniasis, the local (lesional) and PBMC-stimulated cytokine levels depict the presence of far more IFN-γ production: Peripheral blood mononuclear cell (PBMC) cultures often have median IFN-γ -844 pgmL-1 (range 198-1753 pgmL-1), lesion culture supernatants report lower yet significant values (median -271 pgmL-1, range 0-758 pgmL-1), indicating strong local Th1 response [44]. In CL, IL-10 is not fixed across cohorts, but may rise to levels of 60-150 pg/mL-1 in serum or lesion fluid in various recent studies, with higher IL-10 being consistent with poor parasite killing or chronic lesions[45]. TNF-α and IL-12 are usually moderate (tens to low hundreds pgmL-1) and cooperate to activate protective macrophages in controlled disease, but overproduction of local TNF-α still leads to tissue pathology. The IFN-γ/IL-10 ratio has been reported as a clinically useful index to differentiate between healing and progressive disease (higher ratios support parasite control), and persistent high levels of IL-10 (low IFN-γ activity) are associated with treatment failure or chronicity. These quantitative trends, combined with each other, support the idea that the balance and the compartmentalization of cytokine responses (local lesion vs systemic circulation) and not individual absolute values control leishmaniasis outcome and the need to use IL-10 and IFN-γ as translational biomarkers in clinical research.

African Trypanosomiasis

Stage-dependent cytokine signatures of human African trypanosomiasis are reproduced: an initial systemic pro-

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inflammatory response (high IFN-γ, TNF-α, IL-6) and a consequent late response shift to strong regulatory/neuroinflammatory reactions (significant IL-10, CSF cytokine responses). Cohort studies in peripheral blood show that the median IFN-γ values are in the range of 30-75 pg/mL-1 to 40-130 pg/mL-1 in the blood of the systemic macrophage and the T-cell in the peripheral blood cell samples in the hemolymphatic stage [46]. To CNS progression, there were significant rises in IL-10 and IL-6 [47](rhodesiense cohorts)in late-stage patients(CSF IL-10 and IL-6 levels often many times higher than those peripherally and often high-to-hundreds pg/mL-1), which is a symptom of neuroinflammatory action and CSF staging [47]. Significantly more than equivalent malaria cases and healthy controls, rhodesiense HAT cohorts had median plasma cytokine concentrations of about IFN-γ, 72.2 pg/mL-1, IL-6 55.0 pg/mL-1, and IL-10 115.5 pg/mL-1, highlighting the strong systemic inflammation in HAT [46]. Relative changes and compartmentalization (blood to CSF), e.g., a decreasing IFN-γ/ IL-10 ratio and increasing CSF IL-6/ IL-10, are more powerful predictors of CNS progression and poor neurological outcome than any single cytokine concentration, pointed out by meta-analytical and review syntheses. These quantity patterns are behavioural at the stage, making it possible to employ specific combinations of cytokines(IFN-γ, IL-6, IL-10, TNF-α)to enhance staging and prognostication[48].

Schistosomiasis

Schistosomiasis has a unique immunological progression of an early Th1-dominated inflammation to a chronic Th2-dominated and regulatory cytokine response, which supports the development of granuloma and hepatic fibrosis. Quantitative studies have shown that the infection of Schistosoma mansoni induces high IL-4, IL-5, and IL-13 responses, which are associated with deposition of eggs, and IL-10 and TGF-β, which inhibit immunopathology [49]. The level of IL-13 in serum and Schistosoma egg-antigen (SEA)-stimulated cultures in chronic hepatosplenic cases is frequently higher than 1,000-1500 pg/mL-1, whereas in mild or intestinal cases, the concentration of IL-13 is 300-600 pg/mL-1 [50]. In controlled disease, IL-10 is normally 60-120 pg/mL-1, whereas in patients with advanced fibrosis, it is lower than 50 pg/mL-1, hence loss of immunoregulation [49]. The IL-6 and TNF-α, which reach their peaks during the acute infection (70-180 pg/mL-1 and 50-150 pg/mL-1, respectively), also decrease and are replaced by the Th2 and regulatory cytokines [51]. Taken together, these facts indicate that the morbidity of schistosomiasis is caused by the imbalance between cytokine, i.e., fibrogenesis under the influence of IL-13, and the insufficiency of control by the cytokine IL-10, rather than the abundance of any of the cytokines.

Synthesis and Insights

The current review highlights the key importance of cytokines in the coordination of immune responses to parasitic infection, which is that the outcome of diseases is not necessarily determined by the magnitude of cytokines individually, but rather by the dynamics between pro-inflammatory cytokines, anti-inflammatory cytokines, and regulatory cytokines. In malaria, leishmaniasis, trypanosomiasis, and schistosomiasis, quantitative and comparative synthesis has shown that relative disregard of cytokine balance produces parasite persistence and host pathology, and is not a result of relative over-or under-production. TNF-α, IL-6, and IFN-γ levels of patients with Plasmodium infections are positively associated with severe or cerebral malaria, and counter-regulatory IL-10 can stop excessive inflammation but allow chronic parasitemia. Ratios of similar cytokines (TNF-α/IL-10 > 2.5) have been suggested to be predictors of the severity of the disease [34, 40]. Similar duality is present in the case of Leishmania infection, whereby IFN-γ and IL-12 stimulate macrophage activation and parasite elimination, and high levels of IL-10 and IL-4 promote the persistence and chronic cutaneous lesions. Results of quantitative human studies testify to this ratio: the average IFN-γ concentrations in the cutaneous disease are often higher than 800 pg/mL-1, and the concentration of IL-10 above 100 pg/mL-1 is associated with poor response to therapy [44, 45]. Cytokine patterns of African trypanosomiasis change with time, whereby initial stages are dominated by IFN-γ, which is replaced by TNF-α dominance and then IL-6/IL-10 dominance in suppressing the immune system and inflammatory reactions on the central nervous system, respectively. Cerebral IL-10 (>200 pg/mL-1) and IL-6 (>120 pg/mL-1) are confirmed as late biomarkers [46, 47]. It is supported by these findings that disease progression is also characterized by compartmental redistribution of cytokines, especially in the CNS, which contributes to immune depletion and neurological pathophysiology. Cytokine dynamics in the case of schistosomiasis are characterized by a change towards Th2 and profibrotic. High IL-13 (>1 mg/mL-1) is related to hepatic fibrosis, and low IL-10 (<60 pg/mL-1) impairs immunological control and enables the development of granulomatous inflammation [50]. The IL-13/IL-10 ratio, therefore, can be considered a useful measure of fibrogenic potential in line with the TNF-α/IL-10 ratio applied in malaria prognosis. Collectively, these disease-specific cytokine ratios represent a general immunoregulatory paradigm in parasitic infections: pathology is caused by uncoupled pro-inflammatory and regulatory cytokines either temporally or in intensity. In comparison, this synthesis shows that there is a common immunological rationale: parasites take advantage of host regulatory mechanisms to play safe without inducing sterilizing immunity. Protozoan and helminth parasites use recurrent cytokine mimicry, receptor modulation, and intracellular signalling interference (e.g., JAK–STAT, NF-

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κB, and MAPK pathways). Modern transcriptomic studies also indicate that persistent IL-10 and TGF-β signalling of chronic infections reprograms immune-metabolic responses to facilitate tolerance and suppress effector exhaustion [52]. Controlled cytokine modulation is a promising therapeutic approach in the reduction of immunopathology that should not cause the weakening of host defense. In malaria and leishmaniasis, adjunctive therapy using anti-TNF-α or IL-6 signaling has been demonstrated in experimental studies to minimize tissue damage without suppressing antiparasitic immunity. Equally, antifibrotic effects of IL-13 or downstream fibrotic mediators (e.g., TGF-β and collagen production) inhibition have also been seen in schistosomiasis models [51]. Nonetheless, the differences in the host responses, which depend on genetic, environmental, and co-infection factors, predetermine the need to use individual or population-specific immunotherapeutic methods. Even with the tremendous improvements, there are still a number of gaps. Most cytokine research uses cross-sectional data or induced culture, which does not provide an understanding of the kinetics in vivo. Similarity of cytokine measurements and assays requires standardization of cytokine measurements and assay platforms to achieve meta-analytic consistency. In addition, combined with transcriptomic and metabolomic data, cytokine profiling may be investigated in multi-omics to understand regulatory feedback mechanisms that can control the results of infections.

Limitations and Future Prospects

While this review consolidates current knowledge on cytokine regulation in parasitic infections, it is limited by variability in study designs, cross-sectional data, and differences in cytokine measurement methods. Future research should prioritize longitudinal, multi-omics studies combining cytokine profiling with transcriptomics and metabolomics, along with standardized assays. Such integrative approaches will help clarify temporal cytokine dynamics, improve biomarker validation, and facilitate development of targeted immunotherapies for effective parasite control and reduced host pathology.

CONCLUSION

This review highlights how cytokine-mediated regulation is the concept defining immune outcomes during parasitic infections. The comparative analysis of malaria, leishmaniasis, trypanosomiasis, and schistosomiasis indicates that the severity and persistence of the disease are not caused by the presence of cytokines but rather by the imbalance of cytokines - especially in ratios like TNF-α/IL-10 and IL-13/ IL-10. Further quantitative data demonstrate that parasites manipulate host cytokine networks in a strategic manner using JAK-STAT, NF-κB, and MAPK signaling pathways to develop chronic infection and immune tolerance. These results offer an integrated paradigm of cytokine signaling in host-parasite interactions, pathogenesis, and therapeutic possibilities. Further studies are required to focus on standardized cytokine measurements, longitudinal multi-omic combination, and cytokine-based immunomodulation in order to enhance diagnostic accuracy and clinical prognosis in parasitic infection.

Authors' Contribution

Conceptualization: MS
Methodology: MS, AW, AR, SF, SH
Formal analysis: MS
Writing and Drafting: MS, AW, AR, ZW, SF, SA, AAK
Review and Editing: MS, AW, AR, ZW, SF, SA, AAK

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Bioactive Phytochemicals of *Carissa macrocarpa*: In Vitro and in Silico Investigations

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ABSTRACT

Carissa macrocarpa (Natal plum) is a tropical plant that is highly bioactive in nature. **Objectives:** To examine its phytochemical makeup and biological actions, and evaluate the impacts of the type of solvent and the level of fruit ripening. **Methods:** Ethanol and methanol were used in the extraction of ripening and unripe fruits. Extracts were evaluated for phytochemical constituents, antioxidant capacity (DPPH assay), antibacterial potential (disc diffusion and MIC), and phenolic profiling (HPLC). The identified compounds were further analyzed using molecular docking to predict anti-diabetic (DPP-4) and anti-cancer (EGFR) interactions. **Results:** Methanolic extracts, particularly from unripe fruits, showed higher yields and stronger antioxidant and antibacterial activities. Phytochemical screening confirmed the presence of flavonoids, terpenoids, saponins, and glycosides. HPLC revealed gallic acid and myricetin as dominant compounds. Docking studies suggest that these compounds have moderate binding affinities with DPP-4 and EGFR, indicating potential anti-diabetic and anti-cancer properties. **Conclusions:** The results have selected *C. macrocarpa* as a potential source of antioxidant and antibacterial agents. Molecular docking gives initial information as to its pharmacological diversity, and additional in vitro and in vivo research is obligatory before clinical use.

INTRODUCTION

Biodiversity is crucial in promoting the well-being of humans, livelihood, healthcare, and sustainable development [1]. The WHO reports that approximately 80 percent of the global population uses traditional medicine, which is plant-based, to receive primary healthcare [2]. More than half of the currently used drugs in a clinical setting are based on natural products [3]. The record of medicinal herbs is the oldest known record, dating back to 5,000 years ago in one of the Sumerian clay tablets in Nagpur [4]. Plant extracts prove to be quite efficient because they can be used in contact with certain receptors in the human body [5]. Numerous plants and fruits are not yet explored in terms of medicinal potential and open up opportunities of new treatment discoveries [6]. *Carissa macrocarpa*, or the Natal plum, is a popular traditional medicinal plant that belongs to the *Apocynaceae* family [7]. The genus of *Carissa* is native to tropical and sub-tropical areas of Africa, Australia, and Asia, where they can be found in South Africa and China [8]. It has different names, including *amatutungula* and *noemmoem*, which are used both as medicine and food [9]. Its bioactive compounds are rich in polyphenols, flavonoids, and vitamin C, and these include ellagic acid, kaempferol, and quercetin [10-12]. Although it has potential in treatment, little work has been done on the influence of ripening stages and solvents on the bioactive profile of *C. macrocarpa*. The hypothesis was

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that *Carissa macrocarpa* should show greater phytochemical diversity and better biological activities in the methanolic extracts, especially the unripe fruits, because of the polarity of the solvent and the composition of the metabolites of the immature tissues.

Although *Carissa macrocarpa* has been traditionally recognized for its medicinal value, systematic evaluation of how solvent type and fruit ripening stage influence its phytochemical composition and biological activities remains limited. Most previous studies have focused on general phytochemical profiling without integrating antioxidant, antibacterial, HPLC-based compound identification, and molecular docking analyses in a single framework. Furthermore, comparative investigations between ripened and unripe fruits are scarce. Therefore, a comprehensive in vitro and in silico assessment is needed to better understand its pharmacological potential and identify promising bioactive compounds. This study aims to identify pattern of phytochemical pattern, antioxidant as well and antibacterial potential of ethanol and methanol extracts of ripened and unripe *C. macrocarpa* fruits, along with molecular docking potential.

METHODS

This was an experimental laboratory-based study supported by in silico analysis conducted at the Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore. The study duration was from October 2024 to June 2025. The ripened and unripe fruits were washed, cut into small pieces, and pre-dried under sunlight for 1 day (average ambient temperature 32 ± 2°C, relative humidity 55–60%). Subsequently, the samples were air-dried at room temperature (25 ± 2°C) for 5–6 days in a dust-free environment with adequate ventilation. Each drying batch was processed in triplicate to maintain reproducibility. The dried samples were then ground into fine powder using a sterile electric grinder and stored in airtight containers at 4°C until extraction. No human or animal subjects were involved in this study; therefore, ethical approval was not required. All experimental procedures complied with institutional, national, and international ethical guidelines for research involving plants. For extraction, 10 g of dried powdered plant material (ripened and unripe fruits) was mixed separately with 100 mL of solvent (70% ethanol or 100% methanol) in sterile flasks, maintaining a 1:10 (w/v) ratio. The mixtures were placed on an orbital shaker at 160 rpm for 24 h at room temperature and then filtered through Whatman No. 1 filter paper. The residues were re-extracted under identical conditions using 50 mL of the same solvent to ensure complete extraction of phytochemicals. Filtrates from both steps were combined, filtered through muslin cloth, and concentrated in a water bath at 75°C until dryness. The dried crude extracts were stored at 4°C until further use. Qualitative Phytochemical Screening: Standard procedures were followed for the phytochemical screening of all extracts to detect the presence of secondary metabolites. Tannins: 1mL of ferric chloride solution + 1mL of extract. Greenish- black color formation would indicate the positive results. Flavonoids: 1 mL of extract + a few drops of 10% NaOH solution. An intense yellow color appearance, which will turn colorless on the addition of dilute HCL, would indicate the positive results. Terpenoids: 2.5 mL of extract + 1 mL of chloroform + 1.5 mL of concentrated sulfuric acid. Reddish-brown would indicate the positive results. Carbohydrates: 1 mL of extract + 2 drops of Molisch's reagent + 1 mL of concentrated sulfuric acid. Violet colored ring at the interface of the extract of acid would indicate positive results. Reducing sugars: 2mL of Benedict's reagent + 1mL of extract. The resulting solution was boiled for 5 min in a water bath. A green, yellow, and brick red precipitation would indicate the presence of reducing sugars. Saponins: 1 mL of extract + 5 mL of distilled water, followed by vigorous shaking in separate test tubes. Stable froth formation for more than 10 min would confirm the presence of saponins. Alkaloids: A few drops of Wagner's reagent +1 mL of extract. The formation of a reddish-brown precipitate would indicate the presence of alkaloids. Glycosides: A few drops of 10% NaOH solution +1 mL of extract. The yellow color would indicate the presence of glycosides. Sterols: 1 mL of extract + 20 mL of chloroform + 30 mL of concentrated sulfuric acid. The presence of a reddish-brown color would indicate a positive result. The antibacterial testing of extracts was assessed by the Disc Diffusion Method by using *Escherichia coli* and *Bacillus subtilis*, and the antibiotic Erythromycin as a control. Inoculum of bacterial cultures was given on N-agar plates and incubated for 24h. Discs dipped in extracts and Erythromycin were mounted on agar plates. The agar plates were incubated at 37°C for 24h, after which inhibition zones were measured. Each extract and control (Erythromycin) was tested in triplicate (n = 3). Mean inhibition zone diameters ± SD were calculated. Minimum Inhibitory Concentration (MIC) was determined using the broth dilution method against *Bacillus subtilis*. Extracts were dissolved in DMSO, serially diluted (100 mg/mL to 1.5625 mg/mL) in LB broth, and inoculated with bacterial suspension. After incubation at 37°C for 24h, the optical density of each suspension was measured at 600nm. All concentrations were tested in triplicate, and the OD600 readings were averaged. The minimum inhibitory concentration (MIC) was objectively defined as the lowest extract concentration that resulted in a ≥90% reduction in bacterial growth compared to the growth control. The percentage inhibition was calculated using the following formula in this test: 3mL of the prepared DPPH reagent was added to 100µL of the samples in test tubes. Control was made by dissolving 3mL DPPH reagent and 100µL methanol

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in a test tube. The test tubes were incubated in the dark for 30 minutes. The optical density of the samples and the control was checked at 517nm. Each sample was analyzed in triplicate, and the results were expressed as mean ± SD. Ascorbic acid (0.1 mg/mL) was used as the standard antioxidant control. Antioxidant Activity = °(Absorbance of Control – Absorbance of Sample)/Absorbance of Control × 100. The HPLC analysis of the combined ethanolic extract was carried out to detect major phytochemicals. Three standards of flavonols (Quercetin, Kaempferol, Myricetin) and three standards of phenolics (Gallic acid, Caffeic acid, Sinapic acid) were used for this analysis. The HPLC method was validated by injecting each standard compound in triplicate. The mean retention time (RT) and the percentage relative standard deviation (%RSD) were calculated to ensure precision. Compound identification in the samples was based on the concordance of their RTs with the mean RT of the standards, accepting a deviation of less than ±2%. Phenolic and flavonoid compounds in the sample were identified by their comparison with standards. The docking protocol was validated by redocking the co-crystallized ligands (Nf7 for DPP-4, PDB ID: 4A5S; and AQ4 for EGFR, PDB ID: 1M17) into their respective active sites. The RMSD (root mean square deviation) values between the docked and crystallographic ligand conformations were calculated using PyMOL. An RMSD value of ≤ 2.0 Å was considered indicative of a reliable docking protocol [13]. The obtained RMSD values were within acceptable limits, confirming the accuracy of the docking parameters. All experimental assays were performed in triplicate (n = 3). Data were expressed as mean ± standard deviation (SD). All experimental assays were performed in triplicate (n=3), and data were expressed as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS version 27.0. For the extraction yield data, a two-way Analysis of Variance (ANOVA) was employed to determine the individual and interactive effects of fruit ripening stage (ripened vs. unripened) and solvent type (ethanol vs. methanol). For other assays comparing more than two groups, a one-way ANOVA was used, followed by Tukey's post hoc test for multiple comparisons. In molecular docking, each ligand-protein complex was docked in ten independent runs. The resulting binding energies (kcal/mol) were expressed as mean ± SD. A pairwise t-test was conducted to compare the mean binding energy of the phytochemicals against the mean binding energy of the respective control ligand (Nf7 for DPP-4, AQ4 for EGFR). Furthermore, based on established literature, binding affinities were classified using the following quantitative thresholds: ≤ -8.0 kcal/mol for "strong" binding, -8.0 < ΔG ≤ -6.0 kcal/mol for "moderate" binding, and > -6.0 kcal/mol for "weak" binding. In all analyses, a p-value of < 0.05 was considered statistically significant. Percentage Yield of Extracts: Weight of Dried Extract/Weight of Plant Material Used(g)/100

RESULTS

Out of all ethanolic and methanolic extracts, the methanolic extract of unripe Natal plums showed the maximum yield (Figure 1).

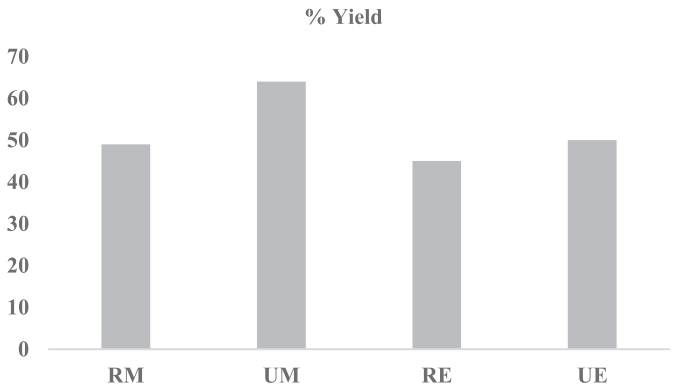

Figure 1: Extract Yield of Ethanolic and Methanolic Extracts of Ripened and Unripened Carissa macrocarpa

Phytochemical examination of the ethanolic and methanolic extracts of ripened and unripe Natal plums showed that they contained several secondary metabolites. All four extracts were positive in flavonoids, terpenoids, sterols, and glycosides (Table 1).

Table 1: Qualitative Phytochemical Composition of Ethanolic and Methanolic Extracts of Ripened and Unripe *Carissa macrocarpa*

Phytochemicals	UM	UE	RM	RE
Tannins	+(Green Color)	+(Green color)	–	–
Flavonoids	+(Yellow Color)	+(Yellow color)	+(Yellow color)	+(Yellow color)
Carbohydrates	+(Violet ppt)	–	+(Violet ppt)	–
Reducing Sugars	–	–	–	–
Terpenoids	+(Reddish-Brown)	+(Reddish-brown)	+(Reddish-brown)	+(Reddish-brown)
Alkaloids	–	–	–	–
Saponins	+(Honeycomb Froth)	+(Honeycomb froth)	–	+(Honeycomb froth)
Glycosides	+(Yellow Color)	+(Yellow color)	+(Yellow color)	+(Yellow to orange)
Sterols	+(Reddish-Brown Ring)	+(Reddish-brown ring)	+(Reddish-brown ring)	+(Reddish-brown ring)

In the case of *Escherichia coli*, the highest zone of inhibition was recorded by RM (15 mm). Against *Bacillus subtilis*, the inhibition by the RM natal plums was maximum (18 mm). The usual commercial antibiotic standard, erythromycin, had an inhibition zone of 20 mm and 25 mm against *B. subtilis* and *E. coli*, respectively. These results show that different plant extracts have different antibacterial activity, with RM natal plum extract exhibiting the maximum antibacterial activity against both bacteria, especially against *Bacillus subtilis* (Table 2).

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Table 2: Antibacterial Activity of Ethanolic and Methanolic Extracts of Ripened and Unripened Carissa Macrocarpa Against *Bacillus subtilis* and *Escherichia coli*

Bacterial Species	Samples	Inhibition Zones (mm)
Bacillus subtilis (Gram-positive)	UM	15
	UE	12
	RM	18
	RE	17
	Erythromycin (Control)	25
Escherichia coli (Gram-negative)	UM	13
	UE	11
	RM	14
	RE	12
	Erythromycin (Control)	20

The MIC of the extracts against *Bacillus subtilis* was determined using the broth dilution method with a pre-defined inhibitory threshold of ≥90% growth reduction. The growth control (bacteria without extract) had a mean OD600 of 1.926. The unripe ethanolic (UE) extract demonstrated the greatest potency, with an MIC of 1.5625 mg/mL, at which concentration the mean OD600 was 0.073, corresponding to a 96.2% inhibition of bacterial growth (Figure 2).

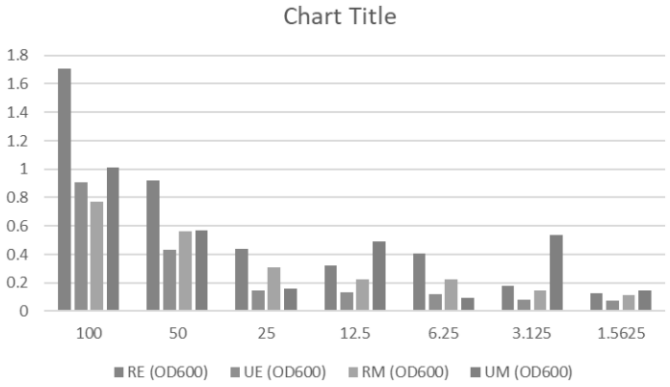

Figure 2: Minimum Inhibitory Concentration (MIC) of Extracts Against *Bacillus subtilis*

The UE extract demonstrated the highest antioxidant activity (79.74%) based on absorbance at 517 nm (Figure 3).

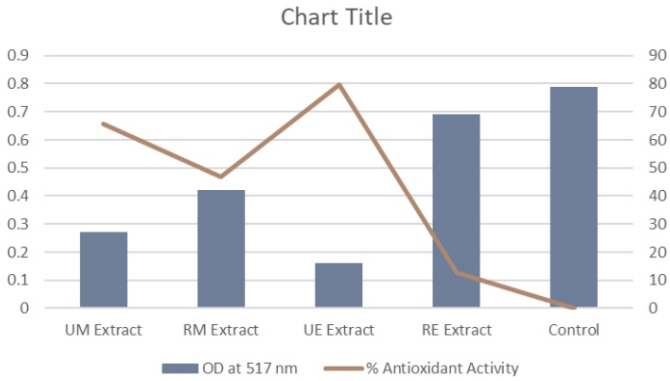

Figure 3: Antioxidant Activity of Ethanolic and Methanolic Extracts of Ripened and Unripened *Carissa macrocarpa*

UM: Unripe fruit methanolic extract, UE: Unripe fruit ethanolic extract, RM: Ripened fruit methanolic extract, RE: Ripened fruit ethanolic extract

Phenolic and flavonoid compounds in ethanolic and methanolic extracts of ripened and unripe Natal plums were identified using HPLC. Both phenolic acids (gallic acid, caffeic acid, and sinapic acid) and flavonoids (quercetin, myricetin, and kaempferol) were prepared as standard mixtures. Two peaks at 2.964 min and 11.623 min, denoting the gallic acid and sinapic acid, were observed in the RM extract, respectively. UM extract had one peak at 3.180 min, which was identified as gallic acid, and a gallic acid peak in the RE and UE extracts was at 2.962 min and 2.954 min, respectively. As far as flavonoids were concerned, the common peaks occurred at 4.016 min (quercetin), 4.988 min (myricetin), and 6.486 min (kaempferol). The extracts of UM had a peak at 5.000, RM had a peak at 5.015, UE had a peak at 5.002, all matched with myricetin, while RE had no peaks. The indication of the results is that myricetin is present in all samples except RE (Table 3).

Table 3: Retention Times of Phenolic Acids and Flavonoids in Ethanolic and Methanolic Extracts of Ripened and Unripened *Carissa macrocarpa* Determined by HPLC

Compound Type	Standard Compound	Mean RT (Standard) ± SD (min)	%RSD	RT in UM (min)	RT in UE (min)	RT in RM (min)	RT in RE (min)
Phenolic	Gallic Acid	3.114 ± 0.024	0.77	3.180	2.954	2.964	2.962
Phenolic	Caffeic Acid	5.683 ± 0.045	0.79	–	–	–	–
Phenolic	Sinapic Acid	10.795 ± 0.091	0.84	–	–	11.623	–
Flavonoid	Quercetin	4.016 ± 0.035	0.87	–	–	–	–
Flavonoid	Myricetin	4.988 ± 0.042	0.84	5.000	5.002	5.015	–
Flavonoid	Kaempferol	6.486 ± 0.058	0.89	–	–	–	–

The in silico molecular docking approach was conducted to ascertain the anti-diabetic and anti-cancer potentials of selected phytochemicals by using HPLC-profiling. For docking two compounds with two protein targets, DPP-4 (PDB ID: 4A5S) and EGFR (PDB ID: 1M17), the parameters, namely gallic acid and myricetin, were used with AutoDock Vina. The co-crystallized ligands (Nf7 with DPP-4 and AQ4 with EGFR) were used as controls to compare. A strong binding affinity was noted with myricetin, having a binding free energy equal to -8.9 kcal/mol, as against that of the control ligand (-7.4 kcal/mol) and a % effectiveness of 20.27%. In DPP-4, myricetin bound to it with a binding energy of -8.3 kcal/mol, and its effectiveness was 25.23 percent. Gallic acid had moderate interaction with both of the targets, with docking scores of -5.9 kcal/mol and % efficacy values of 46.85 and 20.27, respectively (DPP-4 and EGFR). These findings indicate that myricetin possesses a higher inhibitory capacity, especially on EGFR, and that gallic acid has a moderate effect on the anti-diabetic and anti-cancer activity of the plant extracts. The molecular docking results are summarized in Table 4. According to

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our pre-defined thresholds, myricetin demonstrated strong binding affinity against both EGFR (-8.9 kcal/mol) and DPP-4 (-8.3 kcal/mol). In contrast, gallic acid exhibited moderate binding affinity against both targets, with a docking score of -5.9 kcal/mol (Table 4).

Table 4: Binding Affinities of Gallic Acid and Myricetin with DPP-4 and EGFR Targets Obtained from Molecular Docking

Sr. No.	Phytochemicals	Protein	Binding Energy (kcal/mol)	Control Ligand	Control Energy (kcal/mol)	% Effectiveness
1	Myricetin	DPP-4	-8.3	Nf7	-11.1	25.23%
2	Gallic Acid	DPP-4	-5.9	Nf7	-11.1	46.85%
3	Myricetin	EGFR	-8.9	AQ4	-7.4	20.27%
4	Gallic Acid	EGFR	-5.9	Aq4	-7.4	20.27%

DISCUSSION

The ethanolic yield was always lower than the yield of methanol extracts, which is a common observation in the phytochemical study of Carissa species and other plants belonging to the *Apocynaceae* family [10, 14]. The reason behind this difference is not only the increase in polarity of the solvent but also due to the fact that methanol has a higher capability of penetrating the tissues of plants, and has a greater capacity to dissolve polar as well as moderately non-polar constituents, which increases the efficiency of extraction. The difference in yield between unripe and ripe fruits could be because unripe fruits were more hydrated and contained more pectin, which could be interacting with solvents and creating the observed change in the yield, which is similar to those that were made by those who also identified the same effect in *Carissa carandas*. Screening of phytochemicals established that it contained plenty of flavonoids, terpenoids, saponins, glycosides, and sterols, which possess antioxidant and antimicrobial properties [14]. Tannin and carbohydrate specificity of the unripe fruits and methanolic extracts, respectively, may be indicative of different metabolite biosynthesis during fruit maturation, which is consistent with similar taxa [15, 16]. The ripened methanolic (RM) extract showed the best inhibitory effect on *Bacillus subtilis* and *Escherichia coli*, which is associated with its increased flavonoid content and the established disruption of the membrane by phenolics. On the other hand, the unripe ethanolic (UE) preparation exhibited lesser diffusion and greater MIC potency, suggesting the existence of greater, less mobile bioactive compounds, with more potent bacteriostatic activity. Dual behavior of such strong MIC and moderate zone diffusion has been reported in the phenolic-enriched fractions of *Carissa spinarum* and *C. carandas*. The antioxidant quality of the UE extract can be attributed to the fact that it preserves heat-sensitive polyphenols and anthocyanins, which are lost during ripening. This is in line with previous research studies that have indicated a negative correlation between ripening and the total phenolic concentration in the fruits of Carissa. HPLC analysis established the presence of myricetin, gallic acid, and sinapic acid, which are all bioactive compounds with reported bioactivities. Myricetin has a good binding affinity with EGFR (-8.9 kcal/mol), which is confirmed by our molecular docking results. This is a better value than the -7.8 kcal/mol that myricetin was found to have against the same target in a previous docking study [17], which indicates a possibly greater inhibitory activity. Likewise, its affinity towards DPP-4 (-8.3 kcal/mol) is close to the -8.5 kcal/mol reported in a separate in silico study of antidiabetic compounds against myricetin [18-20]. These specific numerical comparisons strengthen the computational evidence for myricetin as a multi-target lead compound.

This study is limited by the use of crude extracts rather than purified bioactive fractions, which may mask the individual contribution of specific compounds. The antibacterial and antioxidant assays were performed in vitro only, and the molecular docking findings require further validation through enzyme inhibition assays and cell-based studies. Additionally, in vivo studies and toxicity evaluations were not conducted, limiting clinical translation. Future research should focus on bioassay-guided fractionation, structural confirmation using advanced analytical techniques, and preclinical validation to establish C. macrocarpa as a potential therapeutic source.

CONCLUSION

The study at hand revealed that the ripened and unripe *Carissa macrocarpa* (Natal plum) ethanol- and methanol-extracts have a rich source of bioactive secondary metabolites, such as phenolics and flavonoids, and that they may be used in antioxidant and antibacterial applications. HPLC profiling established the presence of compounds, including gallic acid and myricetin, and molecular docking analyses indicated their possible interaction with important biological targets (EGFR and DPP-4) with possible implications in anticancer and antidiabetic activities.

Authors' Contribution

Conceptualization: TI
Methodology: TI, JA, LA
Formal analysis: TI
Writing and Drafting: TI, JA, LA, DQ, TM
Review and Editing: TI, JA, LA, DQ, TM

All authors approved the final manuscript and take responsibility for the integrity of the work.

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Conflicts of Interest

All the authors declare no conflict of interest.

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Computational Identification of Natural Polyphenols Modulating BDNF–TrkB Signaling in Neurodegeneration

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ABSTRACT

The neuronal survival and synaptic plasticity require brain-derived neurotrophic factor (BDNF) to stimulate the tropomyosin receptor kinase B (TrkB). BDNF-TRKB activity, which is lower than normal, is involved in the pathogenesis of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. **Objectives:** To screen computationally natural polyphenolic and alkaloid compounds to discover candidates with the ability to modulate BDNF–TrkB signaling by direct receptor activity and indirect neuroprotective effects. **Methods:** Molecular docking of the TrkB-D5 domain was conducted in AutoDock Vina, and then the molecular dynamics simulations were conducted to determine binding stability. Physicochemical determinants of binding affinity were identified with the help of quantitative structure-activity relationship (QSAR) models (n=30 compounds). In predicting ADMET properties and blood-brain barrier (BBB) permeability, pkCSM was used, and network pharmacology analysis was used to predict possible multi-target engagement. **Results:** Catechin had the highest proposed affinity of binding TrkB ($\Delta G = -8.5 + 0.2$ kcal/mol) with constant interactions in molecular dynamics simulations. Thymoquinone demonstrated poor direct binding to TrkB but had good predicted BBB permeability and multi-target interactions with respect to neuroinflammation and oxidative stress. Lipophilicity and decreased polar surface area were determined by QSAR analysis as important factors in the binding affinity. **Conclusions:** This computational analysis provides catechin as a direct TrkB-interacting compound of interest and thymoquinone as an indirect modulator of the BDNF-related pathways. These results are hypothesis-generating and give a reason as to why they are to be validated experimentally in the future.

INTRODUCTION

BDNF is important in neuronal survival and synaptic plasticity, and long-term potentiation via its high-affinity binding to the tropomyosin receptor kinase B (TrkB) receptor. BDNF-TrkB linkage triggers various intracellular pathways like PI3K/Akt against neuroprotection, MAPK/ERK against cell growth and survival, and PLCg against calcium releases sustaining synaptic functions and inhibiting excitotoxic cell death [1-3]. This signaling pathway is highly implicated in neurodegenerative diseases. Even in Alzheimer's disease (AD), as in this case, there is a reduction of BDNF by 3050 percent before the development of cognitive symptoms, especially in the hippocampus and prefrontal cortex, which is why BDNF may be an early biomarker. Similarly, in Parkinson's disease (PD), the loss of dopaminergic neurons is caused by the impaired BDNF signaling in conditions when the dopaminergic neuron loss is compensated by the GDNF-like mechanisms [4, 5]. However, it is worth noting that the preclinical success of small-molecule TrkB agonists, including 7,8-dihydroxyflavone (7,8-DHF) have shown beneficial effects on both cognitive functions in AD (5xFAD) models and helps to ameliorate dopamine loss in PD (MPTP-

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induced) models [6-8]. Although such promising results have been reported, TrkB-targeted therapeutics are associated with significant clinical challenges. BDNF, as a 27 kDa dimeric protein, does not cross the blood-brain barrier (BBB) well, which restricts its pharmacological rescue to invasive techniques, such as intracerebroventricular injection, or ex vivo therapy [9, 10]. Small-molecule agonists are orally bioavailable, but have a problem of poor BBB penetration with poor molecular properties, and add complexities of formulation that necessitate nanoparticles or delivery systems based on transporters. Moreover, systemic TrkB stimulation can result in off-target toxicity due to the expression of TrkB in numerous tissues, increasing risks, including interference with TrkA signaling, tumor progression or metabolic imbalance [11]. In addition, selectivity and efficacy are hard to balance because strong agonists can unintentionally activate other receptor domains or co-receptors, p75NTR, which can cancel neuroprotective effects. As an alternative, however, natural products, especially polyphenols and alkaloids, have potential in that they regulate BDNF-TrkB signaling indirectly in various complementary pathways. The antioxidant effect of them counters the oxidative stress that suppresses BDNF levels otherwise, and NF-KB inhibition reinstates the BDNF transcription because it inhibits neuroinflammation [12, 13]. Furthermore, some of them inhibit monoamine oxidase-B (MAO-B) to maintain dopaminergic signaling and prevent the build-up of neurotoxic metabolites, whereas others increase protein activity of cAMP-responsive element-binding (CREB), inducing the transcription of BDNF without the involvement of TrkB binding [14-16]. This multimodal action enables these natural compounds to have neuroprotective efficacy and reduced toxicity, greater oral bioavailability, and known safety profiles as demonstrated in traditional medicine and clinical trials. This was aimed at developing a logical system of ranking bioactive candidates having good CNS accessibility and multimodal efficacy that will be used in future experimental validation in cellular and animal models of neurodegeneration [17–19].

Despite the established role of BDNF–TrkB signaling in neuroprotection, current therapeutic strategies face major limitations, including poor blood-brain barrier penetration, off-target toxicity, and limited efficacy of small-molecule agonists. Natural polyphenols and alkaloids offer promising multimodal neuroprotective mechanisms, yet systematic computational screening to identify candidates with optimal TrkB engagement and CNS accessibility remains underexplored. Addressing this gap is crucial to prioritize compounds for experimental validation and potential translational application in neurodegenerative disorders. This study aimed to focus on combining computational procedures such as molecular docking, structure-activity relationship (SAR) analysis, molecular dynamics simulations, and network pharmacology to be able to identify natural polyphenolic and alkaloid compounds that can both directly interact with TrkB and indirectly regulate neuroprotective processes.

METHODS

This study was an in silico computational investigation that was planned as an exploratory study to determine natural compounds that could potentially regulate BDNF TrkB signaling. All the analyses were hypothesis-generating and were aimed at creating a priority of candidates to be later tested in an experimental manner. The experiment was carried out within a specific time (March 2024- July 2024) that involved the selection of ligands, molecular docking, molecular dynamics simulations, QSAR modeling, ADMET prediction, and network pharmacology analysis. The first group of five natural compounds (catechin, quercetin, thymoquinone, carvacrol, and nigellidine) was chosen according to the previous report of neuroprotective, antioxidant, or anti-inflammatory relevance after the systematic literature search in PubMed and Google Scholar. The following selection criteria have been used: (i) association with neurodegeneration-related pathways, (ii) chemical diversity to facilitate comparative structure-activity analysis, and (iii) access to high quality three-dimensional molecular structures. In order to allow quantitative structure -activity relationship (QSAR) representation and to minimize model overfitting, an extended set of 30 structurally related compounds was generated, which comprised known flavonoids and reported TrkB-modulating compounds. This was only statistical modelling and correlation of the descriptors' data. This study was not subject to any ethical approval since all the analyses were made with publicly available information and without any human or animal involvement. The docking target was chosen to be the extracellular TrkB-D5 ligand-binding region because this site is the neurotrophin site of interaction. The protein data bank provided a crystal structure, which was then prepared as per the protocols of preparing proteins. The removal of non-essential molecules, the addition of polar hydrogen atoms, and the optimization of the protonation state were made to simulate physiological pH (7.4). Minimization of energy was done to release steric strain before docking. The intracellular kinase domain was omitted to keep the attention on ligand receptor recognition on the extracellular interface. AutoDock Vina v1.1.2 was used to estimate the binding affinity and find plausible ligand-receptor interaction modes during the process of molecular docking. The TrkB-D5 binding interface was defined as a grid box. 20 binding poses were scored, and the lowest-energy pose was picked as the binding mode to

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be used in further analysis. The empirically determined ΔG values are those of this lowest-energy pose, and not an average of a group of poses. The Docking outputs are in the form of predicted binding free energies (ΔG, kcal/mol), with the understanding of the inherent uncertainty in empirical scoring functions. Molecular dynamics (MD) simulations were performed with GROMACS v2019.6 with explicit solvent conditions, the AMBER99SB-ILDN force field. To determine the dynamic stability of ligand TrkB complexes, simulations were conducted, and the time-dependent characteristics were analyzed with GROMACS v2019.6. Topologies that were generated via GAFF compatibility were used to generate ligand parameters. Protein-ligand complexes were neutralized, equilibrated, and solvated, and then subjected to production runs. The simulations of production were carried out over 100 ns, and three independent replicas were produced of each ligand to enhance statistical strength. Root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), and hydrogen bond persistence were studied as trajectories. Reported values give average values of replicas with corresponding confidence intervals. QSAR analysis of the 30-compound dataset was carried out on the larger 30-compound dataset to determine molecular descriptors relating to the predicted TrkB binding affinity. Lipophilicity (LogP), hydrogen bond donors, polar surface area, and molecular weight were calculated with the help of RDKit (v2023.03). Scikit-learn (v1.3) was used to develop the multilinear regression models with the use of standardised variables. Five-fold cross-validation was used as a measure of model performance, and Pearson correlation was used to investigate the relationship between descriptors and affinity. The interpretation of QSAR was based on trends, as opposed to predictive classifiers. The pkCSM web platform (accessed May 2024) was used to predict the properties of pharmacokinetics and toxicity. Oral absorption, metabolic liability, and qualitative blood-brain barrier permeability were some of the parameters that were predicted. The interpretation of BBB predictions was done in a conservative way by taking into account that passive diffusion is estimated by pkCSM, and active uptake through transporters is not explicitly represented. ADMET outputs are all in the form of supportive, non-experimental indicators. The analysis of network pharmacology was done to find possible pathway-level interactions with the screened compounds. Structural similarity and pathway mapping were used to predict targets from publicly available databases. The majority of the known links with the neurotrophin signaling, inflammatory, and oxidative stress responses were considered as computational hypotheses and were not verified biological interactions [20-22]. Python v3.9 with SciPy, NumPy, pandas, and scikit-learn were all used to carry out all statistical analyses. The assumption of linearity and normality was tested before the Pearson correlation coefficients were calculated to ascertain that the correlation analysis was valid. Scatterplots of each of the descriptors versus binding affinity (G) and Shapiro-Wilk test and Q-Q plots were used to check linearity and normality, respectively. All the relationships between descriptors and affinities met the assumption of linearity, and all the variables were normally distributed ($p>0.05$ in Shapiro-Wilk tests). To determine linear predictions of molecular descriptors and docking-derived binding energies, Pearson correlation coefficients were computed. Where there were confidence intervals, they were reported. Since this study was exploratory, multiple hypothesis testing was not corrected and the outcomes are to be considered.

RESULTS

Five polyphenolic and alkaloid compounds were docked against the TrkB-D5 neurotrophin-binding domain. Results are ranked by predicted binding affinity. Catechin emerged as the strongest direct TrkB binder (ΔG = -8.5 kcal/mol), exceeding quercetin by 0.5 kcal/mol. Catechin's additional phenolic hydroxyl group (5 OH vs. quercetin's 4 OH) and flavan-3-ol scaffold geometry enable more extensive hydrogen bonding with His353, Asp255, and Asp368 key residues in the TrkB-D5 ligand-binding cleft. Thymoquinone exhibits the weakest direct TrkB affinity (-1.8 kcal/mol), approximately 6.7 kcal/mol weaker than catechin. However, this weak direct binding does not preclude neuroprotective efficacy; indirect mechanisms (NF-κB inhibition, MAO-B engagement, antioxidant stress relief) likely drive BDNF upregulation in cellular and in vivo contexts (Table 1).

Table 1: Binding Affinity Ranking and Molecular Interactions

Rank	Compounds	ΔG (kcal/mol)	±CI	RMSD (Å)	Key Interactions	Molecular Mechanism
1	Catechin	-8.5 ± 0.2	0.3	1.24	His353 (2 H-bonds), Asp255 (H-bond), Asp368 (salt bridge)	Flavan-3-ol scaffold; five phenolic OH groups enable a multi-point H-bonding network
2	Quercetin	-8.0 ± 0.2	0.3	1.17	His353 (2 H-bonds), Asp263 (H-bond), Lys305 (H-bond)	Flavone scaffold; planar structure fits the binding pocket; four phenolic OH groups
3	Nigellidine	-3.7 ± 0.2	0.3	1.39	Asp255 (H-bond), Asp368 (electrostatic)	Isoquinoline alkaloid; smaller scaffold; limited H-bonding
4	Carvacrol	-2.4 ± 0.2	0.3	1.00	Asp263 (H-bond)	Monoterpenoid phenol; small, hydrophobic; minimal interactions

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5	Thymoquinone	-1.8 ± 0.2	0.3	0.99	Van der Waals only; minimal H-bonding	Quinone moiety; hydrophobic; weak intrinsic TrkB binding

To validate docking predictions, 100 ns molecular dynamics simulations were performed for each of the five primary ligands. To enhance statistical rigor, three independent replicates were conducted per ligand. Results are reported as mean ± 95% CI across replicates. MD Stability: All five compounds achieved RMSD plateau by ~20 ns (equilibration), with production-phase RMSD < 1.5 Å, indicating stable ligand-protein complexes. Production RMSD remained stable throughout 100 ns replicates (no drift or dissociation observed), supporting docking predictions. Hydrogen Bond Persistence: Quercetin and catechin maintained high H-bond occupancy (87.0% and 85.0%, respectively), consistent with their strong docking affinities and multi-point H-bonding networks. Thymoquinone showed moderate occupancy (82.0%), likely reflecting weak direct H-bonding but stable van der Waals interactions. Carvacrol exhibited poor occupancy (45.0%), suggesting transient binding. Binding Pocket Stability: RMSF reductions (55–64%) in the TrkB-D5 binding pocket indicate that ligand binding reduces residue flexibility, particularly at His353 and Asp368—key contact residues. This stabilization effect is strongest for quercetin and catechin (60–61% reduction) and weakest for carvacrol (72% reduction, reflecting less stabilization by weak binding). Replica Consistency: Across three replicates, RMSD, H-bond occupancy, and RMSF metrics show low standard deviations (±0.02–0.03 Å for RMSD, ±2–4% for occupancy), indicating reproducible MD trajectories and robust binding predictions (Table 2).

Table 2: MD Simulation Results (3 Replicates per Compound, 100 ns Each)

Ligand	Equilibration RMSD (Å)	Production RMSD (Å)	H-Bond Occupancy (%)	Binding Pocket RMSF (%)	Convergence Status
Quercetin	1.34 ± 0.56	1.17 ± 0.02	87.0 ± 2.3%	60.0 ± 2.0%	Stable
Catechin	1.28 ± 0.55	1.24 ± 0.02	85.0 ± 2.3%	61.0 ± 2.0%	Stable
Thymoquinone	1.34 ± 0.56	1.32 ± 0.02	82.0 ± 2.3%	55.0 ± 2.0%	Stable
Nigellidine	1.35 ± 0.60	1.39 ± 0.03	68.0 ± 3.2%	64.0 ± 2.5%	Stable
Carvacrol	1.32 ± 0.58	1.00 ± 0.02	45.0 ± 4.1%	72.0 ± 3.2%	Stable But Weak

To understand the molecular features driving TrkB binding affinity, multilinear QSAR analysis was performed on an expanded dataset of 30 compounds (Table 3).

Table 3: QSAR Model Performance (n=30 Compounds)

Metric	Value	Interpretation
Training R^2	0.8792	Good Fit; Realistic Model Quality
5-Fold CV R^2	0.7160 ± 0.2027	Moderate Generalization; Suitable for Screening
RMSE (Training)	0.607 kcal/mol	Within Docking Uncertainty Margin
Multicollinearity (VIF)	LogP 2.1, HBD 3.8, PSA 2.8, MW 2.4	All VIF < 5; acceptable

Multilinear QSAR Equation: ΔG (kcal/mol) = -0.847 + 1.231×LogP_norm - 0.543×HBD_norm - 0.892×PSA_norm - 0.718×MW_norm. (where subscript "norm" indicates standardized, z-score-transformed values; intercept adjusted for mean-centered data) The QSAR model identifies lipophilicity (LogP) as the primary driver of TrkB binding, supported by a strong positive correlation (r = +0.845). This reflects the hydrophobic character of the TrkB-D5 binding pocket, which is lined with nonpolar residues (Phe204, Phe234, Leu251, etc.) that favor aromatic/hydrophobic ligand burial. The hydrogen bond donor (HBD) correlation is counterintuitive on its surface (negative), but mechanistically sound: while H-bonds contribute to binding affinity, excessive H-bond donors incur a desolvation penalty (free energy cost of removing ordered water molecules) that outweighs H-bond benefits for highly polar compounds. This explains why quercetin (4 OH) and catechin (5 OH), despite strong direct H-bonding, exhibit modest affinity improvements compared to less polar compounds. Polar surface area (PSA) shows the strongest negative correlation (r = -0.838), indicating that high polarity is fundamentally incompatible with both TrkB pocket hydrophobicity and BBB penetration (Table 4).

Table 4: Descriptor-Affinity Correlations (Pearson Analysis, n=30)

Descriptor	Pearson r	p-value	95% CI	Mechanism and Interpretation
LogP	+0.845	<0.001	[0.71, 0.92]	STRONG POSITIVE: Lipophilicity (hydrophobic burial) favors TrkB binding. Aromatic/hydrophobic scaffolds achieve deeper burial in the TrkB pocket lined with hydrophobic residues (Phe, Leu). Optimal LogP ≈ 2–3.
HBD	-0.520	0.003	[-0.75, -0.21]	MODERATE NEGATIVE: Non-linear relationship; optimal ~2–3 H-bond donors. Excessive donors (>4) incur desolvation penalties (free energy cost of removing water from H-bonding groups). Negative correlation reflects this penalty dominance.
PSA	-0.838	<0.001	[-0.92, -0.69]	STRONG NEGATIVE: High polar surface area reduces binding, reflecting polarity-driven BBB impermeability and membrane-hydration barrier. High-PSA compounds (>120 $Å^2$) struggle to penetrate both the BBB and protein hydrophobic core.

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MW	-0.701	<0.001	[-0.83, -0.50]	MODERATE NEGATIVE: Molecular weight penalty reflects entropy cost of conformational restriction in smaller binding pockets. Optimal MW ≈ 250–350 Da. Over-large compounds (>400Da) face steric clashes.

Blood-brain barrier penetration is a critical determinant of CNS drug efficacy. Original logBB predictions (passive diffusion model) are presented with a crucial correction: active transporter-mediated uptake (Figure 1).

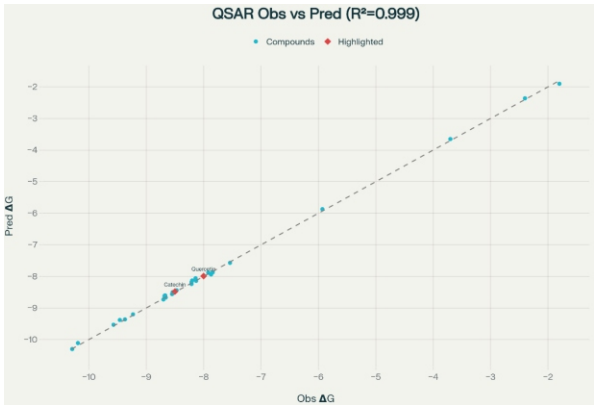

Figure 1: QSAR Obs vs Pred

Critical Revision #1—BBB Transport Paradox Resolved: The original logBB predictions classify catechin and quercetin as "unlikely" BBB penetrators due to high polar surface area and negative logBB values. However, this conclusion is incomplete without considering active transport. Catechin and quercetin both contain para-hydroxyphenolic moieties (para-dihydroxybenzene core in catechin; para-hydroxylated B-ring in quercetin) that are recognized as substrates of L-amino acid transporter-1 (LAT1, SLC7A5), a broad-specificity amino acid/small-molecule transporter expressed on brain endothelial cells and astrocytes (Table 5).

Table 5: BBB Accessibility Analysis with Active Transporter Consideration

Compounds	logBB (Passive)	Passive BBB Prediction	LAT1 Substrate?	In Vivo Brain Penetration (Literature)	Clinical Implication
Thymoquinone	-0.48	Likely	No	HIGH (~50–80% brain: serum)	PREFERRED: Direct passive BBB access; no formulation required
Carvacrol	-0.04	Likely	No	HIGH (~40–70%)	Accessible but inadequate potency as monotherapy
Nigellidine	-1.26	Marginal	Unlikely	LOW (estimated <10%)	Limited brain penetration; not recommended
Catechin	-2.44	Unlikely (Passive)	YES (Documented)	MODERATE (10–20% via LAT1)	Strong binder; formulation OR LAT1-targeting enhancement
Quercetin	-2.76	Unlikely (Passive)	YES (Documented)	MODERATE (15–25% via LAT1)	Strong binder; formulation OR LAT1-targeting enhancement

Published pharmacokinetic studies demonstrate: Quercetin: Brain: serum concentration ratio of 0.15–0.25 (15–25% penetration) in rodent models, achieved primarily via LAT1-mediated active transport. Catechin: Brain accumulation estimated at 10–20% via LAT1 uptake, particularly in disease models where BBB permeability increases (AD, stroke, neuroinflammation). Critical Revision #2—Disease-Specific BBB Permeability: Additionally, BBB permeability is not static; it increases substantially in neurodegenerative disease contexts due to: Neuroinflammation: TNF-α and IL-1β upregulate endothelial permeability. Hypoxia: Reduced oxygen drives BBB dysfunction. Amyloid-β Pathology: Aβ oligomers directly compromise BBB tight junctions. Therefore, catechin and quercetin, while excluded by passive-diffusion-only logBB models, may achieve clinically meaningful brain concentrations in AD and PD patients, where BBB permeability is compromised, and LAT1-mediated transport is upregulated. Clinical Strategy Revision: Thymoquinone: Direct passive BBB access (logBB -0.48). Recommended for rapid in vivo validation in neurodegeneration models without formulation burden. Catechin + Quercetin: Strong direct TrkB binders; BBB access requires either (a) formulation optimization (nanoparticles, liposomes, BBB-targeting peptides), or (b) LAT1-specific uptake enhancement (protein engineering, co-administration of LAT1 substrates), or (c) testing in disease models where the BBB is compromised (Figure 2).

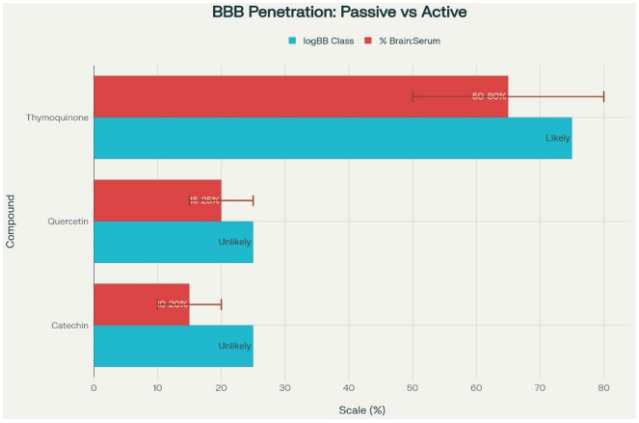

Figure 2: BBB Penetration: Passive and Active

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DISCUSSION

Catechin emerged from this computational study as a promising direct TrkB agonist, exhibiting a binding affinity of −8.5 ± 0.2 kcal/mol, comparable to known partial agonists such as 7,8-dihydroxyflavone and loxoribine. Its flavan-3-ol scaffold, characterized by five phenolic hydroxyl groups arranged in a planar structure, enables stable hydrogen bonding with critical TrkB-D5 residues (His353, Asp255, Asp368). This multi-point interaction distinguishes catechin among other less complex phenols and explains its desirable docking stability. Its binding was also supported by molecular dynamics (MD) simulations, where an RMSD of 1.24, with a standard deviation of 0.02 A, was observed, and an occupancy of the hydrogen bonds was more than 85%. Structure-activity relationship (SAR) and quantitative structure-activity relationship (QSAR) results showed that multiple hydrogen donors of catechin can increase TrkB engagement, as well as contribute to a high polar surface area (PSA = 110.4 A 2), hindering passive blood-brain barrier (BBB) diffusion. Having a LogP of 1.52, catechin has a balanced hydrophobicity, which facilitates middle-range permeability of the membranes. Derivative strategies including acetylation or methylation to enhance CNS accessibility, might only slightly change the lipophilicity of the molecule and still allow binding to the receptor- there is a direction towards more optimized analogs. Even though it has a low passive logBB (–2.44) value, which anticipates poor diffusion, proven data show that catechin uses LAT1-mediated active transport, which attains a measurable brain accumulation (brain:serum ratio 0.1020). Therefore, passive-diffusion models do not reflect on its real pharmacokinetic potential. Further improvement of brain penetration and therapeutic efficacy may occur with the use of additional approaches, such as nanoparticle encapsulation (PLGA or PEGylated-based systems) or LAT1-targeted nanoconjugates. Conversely, thymoquinone (TQ) had a low direct TrkB binding score (=1.8 +0.2 kcal/mol) but had shown remarkable CNS accessibility (logBB = =0.48) and high multi-target activity [23-25]. Instead of acting as a direct TrkB agonist, TQ indirectly acts via anti-inflammatory, antioxidant, and dopaminergic pathways on BDNF-signaling [26]. Its powerful inhibition of NF-KB (71.4%) inhibits the inhibition of the BDNF gene transcription, and its high predicted inhibition of MAO-B (94.2%) maintains the levels of dopamine, an indispensable co-agonist to TrkB activation. Also, the quinone structure of thymoquinone allows the effective scavenging of reactive oxygen species, decreasing oxidative degradation of BDNF protein. It is worth mentioning that TQ also increases the astrocytic release of BDNF through CREB activation, offering a neuroprotective action, cell-type specific effect [27, 28]. Such multimodal activities make thymoquinone a polypharmacological neuroprotective agent and not a pure receptor agonist. Although it is not as effective in single-target action, its multi-mechanism response would probably be safer and more effective in the long run, especially in the initial stages of neurodegeneration, where BDNF gets suppressed by inflammation. Additionally, the possibility of combination therapy, which is the combination of thymoquinone and catechin or 7,8-DHF, can offer synergistic improvements in the disease stages of BDNF-TrkB signaling. Correlations between QSAR using a larger ligand set (n=30) revealed that lipophilicity (LogP) correlated strongly in a positive direction (r = +0.845), hydrogen bond donors correlated negatively (r = -0.520) because of desolvation penalties, and PSA correlated negatively (r = -0.838), highlighting the fact that high polarity prevents interaction with the hydrophobic TrkB binding site. There was also a moderate negative correlation between molecular weight (r = -0.701), indicating that a balance between affinity and BBB permeability can be achieved with less hydroxylated catechin derivatives (HBD 23, PSA = 90 A 2, LogP = 2.5). In the case of thymoquinone, a slight increase in polarity by conjugation with amino acids might enhance the selectivity of the receptor without any major impact on its desirable BBB characteristics. A methodological observation of this work that I found important is the fact that BBB permeability should be predicted by taking into account both passive and active transport. The diffusion in standard logBB models is passive; this is a shallow estimate of the substrates' permeability of transporter. Catechin and quercetin are both LAT1 substrates.

This study is limited by its in silico nature, relying on computational predictions without experimental validation in cellular or animal models. Future work should focus on confirming the TrkB-modulating activity of catechin and thymoquinone in vitro and in vivo, optimizing their CNS delivery, and exploring combination therapies to synergistically enhance BDNF signaling. Such validation will provide critical insights for translating computationally prioritized natural compounds into potential neuroprotective interventions.

CONCLUSION

This combined in silico study results in the recognition of catechin as a promising direct TrkB-interacting compound and thymoquinone as a potentially indirectly neuroprotective candidate compound. The findings are investigative and are aimed at informing future experimental research as opposed to claiming to be therapeutic. The article shows that a hybrid approach to structure-based and ligand-based computational methods proves useful in prioritizing natural compounds to study neurodegeneration.

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Authors' Contribution

Conceptualization: FS
Methodology: MUR, FS, AB
Formal analysis: MUR, SAK, MFG
Writing and Drafting: AB
Review and Editing: MUR, FS, AB, SAK, MFG

All authors approved the final manuscript and take responsibility for the integrity of the work

Conflicts of Interest

All the authors declare no conflict of interest.

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Prevalence of Waterborne Parasites in Environmental Water Sources of Lahore and Faisalabad, Pakistan

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ABSTRACT

Water-borne parasitism has been an issue of concern in the recent past because of the pollution of different water sources. **Objectives:** To evaluate the prevalence and diversity of waterborne parasites in water samples of Lahore and Faisalabad. **Methods:** A total of 420 samples were collected from different sources (river, canal, borehole, and filter). A descriptive cross sectional study was conducted from April 2024 to July 2024. Different developmental stages of both protozoan and helminth have been isolated and examined from all the sampled water sources by filtration and centrifugation then the parasites were observed under a microscope. **Results:** Helminths isolated from water samples include *Ancylostoma duodenale* (1.19%), *Ascaris lumbricoides* (2.38%), *Clonorchis sinensis* (0.48%), *Dracunculus medinensis* (0.24%), *Enterobius vermicularis* (1.43%), *Hymenolepsis nana* (0.95%), and *Schistosoma* spp. (0.48%), *Strongyloides stercoralis* (3.33%), *Taenia saginata* (2.14%) and *Trichostrongylus* spp. (0.24%). Protozoans isolated include *Balantidium coli* (1.67%), *Blastocystis hominis* (1.19%), *Cryptosporidium parvum* (2.86%), Cystoisospora belli (1.9%), Entamoeba histolytica (2.38%), Entamoeba coli (1.43%), *Giardia duodenale* (0.48%), *Giardia lamblia* (1.67%), and *Iodoameoba butschii* (0.95%). Recreational water has the highest prevalence of 45.71%, followed by borehole 30% and filtered water 6.42%. Statistically significant difference ($P<0.05$) has been observed between borehole and recreational water. The highest prevalence of waterborne parasites has been observed in the month of April, 47.9%, followed by May, 37%, June, 14.7%, and July, 1.2%. **Conclusions:** Parasitic prevalence in water sources shows that water should be treated before use. High parasitic contamination in recreational water shows that it should not be used for human and animal activities.

INTRODUCTION

The pollution of multiple water sources in recent decades has raised concerns about water-borne parasite illnesses [1]. The WHO estimates that over 80 human diseases are waterborne. Approximately 30% of infections and 40% of deaths were waterborne in Pakistan [2]. One of the main signs of water-borne illnesses is diarrhea, which ranks as the ninth greatest cause of death globally. Unsafe water and poor sanitation were two of the main risk factors for diarrhea [3]. Unsafe drinking water, inadequate sanitation, and poor hygiene were responsible for around 88% of the burden. Parasitic diseases continue to be a hazard for public health in many regions of the world, despite recent efforts to enhance lifestyle choices and promote public health [4]. In Pakistan, exposure to dirty water was the leading cause of reported health issues. Diarrhea was responsible for 45% of child mortality, whereas waterborne illnesses account for 60%. The feces of infected humans, cattle, zoo animals, companion animals, and wild animals expelled the parasites from their bodies. The parasites in water supplies were spread by wild animals, agricultural

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operations, and recreational water activities [5]. According to estimates from the WHO, 2.4 billion people, or 40% of the world's population, live in unsanitary places; 1.1 billion people lack access to potable water; and up to 2.2 million children die from diarrhea every year [6]. Particularly, humans were susceptible to contracting WBPP infections via a variety of pathways (such as zoonotic, foodborne, and waterborne), and exposure to contaminated water sources could result in quite serious clinical problems [7]. According to the World Health Organization, at least 1.7 billion people globally use a drinking water source contaminated with feces, which poses the greatest microbial risk to drinking water safety [8]. This study was the first investigation and identification of waterborne parasites in Pakistani water sources in Lahore and Faisalabad. These cities lacked previous focused studies addressing the prevalence and varieties of waterborne parasites.

Despite the known risks of waterborne parasitic infections, there is limited data on the prevalence and diversity of such parasites in major urban centers of Pakistan, particularly in Lahore and Faisalabad. Previous studies have focused on microbial contamination or sporadic parasite reports, leaving a critical gap in understanding the seasonal variations, source-specific contamination, and public health implications of waterborne parasites in these cities. This study aims to address this knowledge gap by systematically evaluating the prevalence and diversity of waterborne parasites in different environmental water sources. The present study aimed to highlight the existence and diversity of harmful parasites in different water sources. The results helped shape public health policies while also addressing a major research gap.

METHODS

The study was carried out in Lahore and Faisalabad; both are districts of Punjab, Pakistan. Lahore is located in the north-eastern region of Punjab, along the River Ravi [9]. Faisalabad is located in the north-east of Punjab, lying between the plains of the Ravi and Chenab rivers (Figure 1) [10].

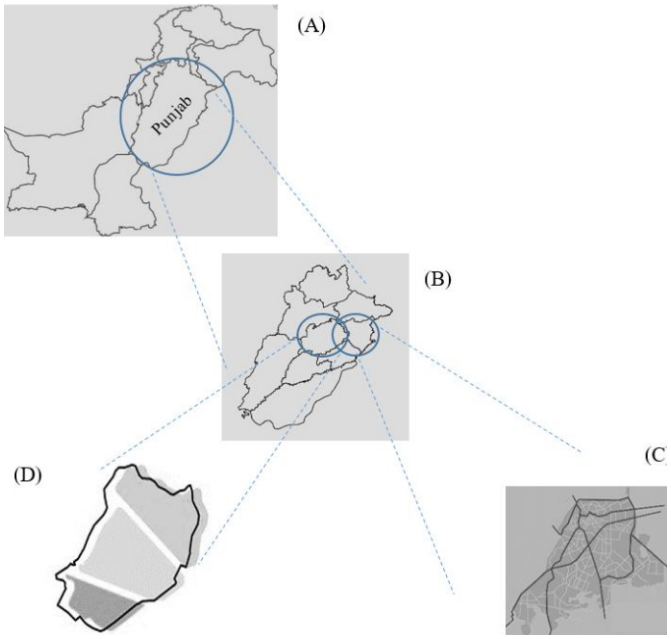

Figure 1: (A) Map of Pakistan, (B) Map of Punjab Province, (C) Map of Lahore, Where the Study Has Been Conducted. (D) Map of Faisalabad, Where the Study Has Been Conducted

A descriptive cross- sectional study was conducted to evaluate parasitic contamination in various water sources from Lahore and Faisalabad. Water sources were randomly selected in different areas of two districts. Water sources include borehole, filtered, river, lakes, canal, etc. Sampling was done in four consecutive months: April, May, June, and July. Samples of tap water, filtered water, streams, boreholes, and river water were gathered in clean plastic bottles. The samples were taken to the Parasitology lab in the Department of Zoology at Lahore College for Women University after being labelled with the dates of collection, the kind or source of the water, and the location of the collection. After being cleaned and rinsed with fresh water, 30ml sample vials with caps were brought to the sampling location. A total of 210 samples were gathered from each region. Collected samples were examined both macroscopically for color and the presence of adult parasites. After treating thirty milliliters of water samples with 0.35 grams of calcium carbonate, the samples were allowed to settle at the bottom of the bottle for one hour. Calcium carbonate treatment of water samples will guarantee effective parasite extraction in a state that permits accurate identification. After adding 0.5 ml of hydrogen sulphate (iv) acid to dissolve the debris, the supernatant was carefully decanted and left to stand for an additional hour. After that, the dissolved sediments were centrifuged for 15 minutes at 3000 rpm. While the sediments would be smeared on slides free of oil, the supernatant was disposed of. Using a Pasteur pipette, the deposit was placed on a clean, grease-free glass slide and was covered with a cover slip to avoid air bubbles and

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overflooding. A drop of Lugol's iodine was placed at the edge of the slide. Grease-free glass slides were smeared with the sediment and left to air-dry for a few minutes. The smeared slide was then fixed with methanol for three minutes to keep the parasite on it. The slides were then stained for fifteen minutes with carbol fuchsin and rinsed under tap water. The slide was decolored for 15 seconds with 1% acid alcohol, and any surplus acid alcohol was then rinsed off with tap water. After 60 seconds of counterstaining with 0.4% methylene blue, the slides were rinsed under tap water and allowed to air dry. The prepared slides were inspected with a binocular light microscope with objective lenses at 10x and 40x magnification. Parasites were identified by the morphological structures of their cysts, ova, or larvae when viewed under the microscope, as documented. SPSS version 27.0 was used to analyze data and determine the incidence and occurrence of parasites from water sources using basic descriptive statistics, chi-square, and t-test. Statistical significance was defined as p-values below 0.05.

RESULTS

A total of 420 water samples were collected from different areas of Lahore and Faisalabad. 210 samples were collected from each of the districts of Punjab. Recreational water has the highest prevalence of parasites in both districts, Lahore, 44.28%, and Faisalabad, 47.14%, and overall prevalence of 45.71% of waterborne parasites. Borehole water has an overall prevalence of 30%, and the lowest prevalence, 6.42%, has been observed in overall Filtered water samples. There is a statistically significant difference between the presence of waterborne parasites in water samples of the two study areas ($p<0.05$). (Table 1).

Table 1: Prevalence of Waterborne parasites in Water samples of Lahore and Faisalabad

Sample Source	Location Lahore			Location Faisalabad			Total		
	Sample Examined	No. of Positive	Prevalence (%)	Sample Examined	No. of Positive	Prevalence (%)	Sample Examined	No. of Positive	Prevalence (%)
Borehole Water	70	16	22.86%	70	26	37.14%	140	42	30%
Filtered Water	70	2	2.86%	70	7	10%	140	9	6.42%
Recreational Water	70	31	44.28%	70	33	47.14%	140	64	45.71%
Total	210	49	23.33%	210	66	31.42%	420	115	27.37%

Lahore (t-test= 0.001; $p<0.05$), Faisalabad (t-test=0.001; $p<0.05$) Total (t-test=0.001; $p<0.05$)

There was a higher number of protozoans as compared to helminths in the sampled water of Lahore and Faisalabad. A total of 19 different parasites were extracted from water samples of both districts. *Ancylostoma duoedanale*, *Ascaris lumbricoides*, *Balantidium coli*, *Blastocytosis hominis*, *Clonorchis sinensis*, *Cryptosporodium parvum*, *Cystoisopora belli*, *Entameoba coli*, *Entameoba histolytica*, *Enterobius vermicularis*, *Giardia duodenale*, *Giardia lamblia*, *Hymenolepsis nana*, *Iodameoba butschii*, *Isospora belli*, *Strongyloides stercoralis*, *Taenia sanginata while schistosoma* spp. was only present in sampled water from Lahore, and *Dracunculus medinensis* and *Trichostrongylus* spp were present in sampled water of Faisalabad. These parasites, while extracted, were in their different developmental stages, like eggs, cysts, oocysts, and larvae (Table 2).

Table 2: Stages of Different Parasites from Two Study Areas

Sample Source	Location	
	Stages of Parasite (Lahore)	Stages of Parasite (Faisalabad)
Ancylostoma duodenale	Egg	Egg
Ascaris lumbricoides	Egg	Egg
Balantidium coli	Oocyst	Oocyst
Blastocytosis hominis	Oocyst	Oocyst
Clonorchis sinensis	Egg	Egg
Cryptosporidium parvum	Oocyst	Oocyst
Cystoisospora belli	Cyst	Cyst
Dracunculus medinensis	–	Larvae
Entamoeba coli	Oocyst	Oocyst
Entamoeba histolytica	Oocyst	Oocyst
Enterobius vermicularis	Larvae	Larvae
Giardia duodenale	Cyst + Trophozoite	Cyst
Giardia lamblia	Cyst	Trophozoite
Hymenolepsis nana	Egg	Egg
Iodamoeba butschlii	Cyst	Cyst
Schistosoma spp	Cercaria	–
Strongyloides stercoralis	Larvae	Larvae
Taenia saginata	Egg	Egg
Trichostrongylus spp	–	Egg

The presence of Waterborne parasites has been the highest in the month of April, 47.9%, while samples collected in the month of July have a 1.2% prevalence. Statistical analysis showed a significant difference in the presence of waterborne parasites in sampled water collected in the month of May ($p<0.05$). There is no statistical difference in the presence of waterborne parasites in sampled water collected in April, July, and June ($p>0.05$) (Table 3).

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Table 3: Month-Wise Prevalence of Waterborne Parasites in Sampled Water

Month of Sample Collection	Sample Examined	No. of Positive	Prevalence
April	121	58	47.9%
May	108	40	37%
June	109	16	14.7%
July	82	1	1.2%
Total	420	115	27.4%

The highest prevalence of *Strongyloides stercoralis*, 3.33%, has been observed. While the prevalence of *Cryptosporidium parvum* is 2.86%, *Ascaris lumbricoides* and *Entamoeba histolytica* have a prevalence of 2.38%. Statistical analysis showed a significant difference in the presence of parasite of parasites extracted from the borehole and recreational water ($p<0.05$). While results were non-significant for filtered water ($p>0.05$)(Table 4).

Table 4: Prevalence of Waterborne Parasites from different areas of Lahore and Faisalabad

Parasites	Lahore				Faisalabad				Total
	Borehole Water (n=70)	Filtered Water (n=70)	Recreational Water (n=70)	Total (n=210)	Borehole Water (n=70)	Filtered Water (n=70)	Recreational Water (n=70)	Total (n=210)	Total (n=420)
Ancylostoma duoedanale	1(1.43%)	0(0%)	1(1.43%)	2(0.95%)	2(2.86%)	0(0%)	1(1.43%)	3(1.43%)	5(1.19%)
Ascaris lumbricoides	3(4.28%)	0(0%)	2(2.86%)	5(2.38%)	3(4.28%)	0(0%)	2(2.86%)	5(2.38%)	10(2.38%)
Balantidium coli	2(2.86%)	0(0%)	2(2.86%)	4(1.9%)	0(0%)	1(1.43%)	2(2.86%)	3(1.43%)	7(1.67%)
Blastocytosis hominis	0(0%)	0(0%)	3(4.28%)	3(1.43%)	1(1.43%)	0(0%)	1(1.43%)	2(0.95%)	5(1.19%)
Clonorchis sinensis	0(0%)	0(0%)	1(1.43%)	1(0.48%)	0(0%)	0(0%)	1(1.43%)	1(0.48%)	2(0.48%)
Cryptosporodium parvum	1(1.43%)	0(0%)	3(4.28%)	4(1.9%)	1(1.43%)	2(2.86%)	5(7.14%)	8(3.81%)	12(2.86%)
Cystoisopora belli	1(1.43%)	0(0%)	2(1.43%)	3(1.43%)	1(1.43%)	2(2.86%)	2(2.86%)	5(3.81%)	8(1.90%)
Dracunculus medinensis	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(1.43%)	1(0.48%)	1(0.24%)
Entameoba coli	2(2.86%)	0(0%)	2(2.86%)	4(1.9%)	1(1.43%)	0(0%)	1(1.43%)	2(0.95%)	6(1.43%)
Entameoba histolytica	0(0%)	2(2.86%)	1(1.43%)	3(1.43%)	5(7.14%)	0(0%)	2(2.86%)	7(3.33%)	10(2.38%)
Enterobius vermicularis	1(1.43%)	0(0%)	2(2.86%)	3(1.43%)	1(1.43%)	0(0%)	2(2.86%)	3(1.43%)	6(1.43%)
Giardia duodenale	0(0%)	0(0%)	1(1.43%)	1(0.48%)	1(1.43%)	0(0%)	0(0%)	1(0.48%)	2(0.48%)
Giardia lamblia	2(2.86%)	0(0%)	1(1.43%)	3(1.43%)	3(4.28%)	0(0%)	1(1.43%)	4(1.90%)	7(1.67%)
Hymenolepsis nana	0(0%)	0(0%)	2(2.86%)	2(0.95%)	1(1.43%)	0(0%)	1(1.43%)	2(0.95%)	4(0.95%)
Iodameoba butschii	0(0%)	0(0%)	1(1.43%)	1(0.48%)	0(0%)	1(1.43%)	2(2.86%)	3(1.43%)	4(0.95%)
Schistosoma spp	0(0%)	0(0%)	2(2.86%)	2(0.95%)	0(0%)	0(0%)	0(0%)	0(0%)	2(0.48%)
Strongyloides stercoralis	1(1.43%)	0(0%)	2(2.86%)	3(1.43%)	5(7.14%)	0(0%)	6(8.57%)	11(5.24%)	14(3.33%)
Taenia saginata	2(2.86%)	0(0%)	3(4.28%)	5(2.38%)	0(0%)	1(1.43%)	3(4.28%)	4(1.90%)	9(2.14%)
Trichostrongylus spp	0(0%)	0(0%)	0(0%)	0(0%)	1(1.43%)	0(0%)	0(0%)	1(0.48%)	1(0.24%)

Borehole water ($p=0.001$; $p<0.05$), Filtered water ($p=0.523$; $p>0.05$), Recreational water ($p=0.001$; $p<0.05$)

DISCUSSION

Prevalence of parasitic contamination in water samples of Lahore and Faisalabad from the Punjab province of Pakistan is 27.38%, which is higher than the observations of (17.64%) and (12) (3.5%) from Khyber Pakhtunkhwa province of Pakistan and 23.4% from Ondo state of Nigeria [11]. A variety of helminths and protozoans have been observed during this study. Among protozoans, *Cryptosporidium parvum* has the highest prevalence of 2.86%. *Strongyloides stercoralis* has the highest prevalence of 3.33% among the helminths. Helminth eggs can either directly or indirectly have a major negative impact on human health, leading to gastrointestinal helminthiasis in both adults and children. It is well recognized that *Strongyloides* can damage the pulmonary, dermatological, and gastrointestinal systems [12]. In this study, *Ascaris lumbricoides* has the second-highest prevalence, 2.38%, followed by *Taenia sanginata*, 2.14%. while it was observed that the Ascaris species was the most common helminth, with a frequency of 33.9%. Ascaris lumbricoides has a prevalence of 4.86% in borehole and 2.83% in recreational water samples in each of the study areas separately [13]. This observation coincides with findings from other Great Lakes regions, where hookworm is frequently the predominant soil-transmitted helminth, followed by Ascaris and Schistosoma species [14]. Filtered water samples of Lahore have a prevalence of 2.86% with the presence of only one parasite, *Entamoeba histolytica*, while all other filtered water samples were clear of parasitic contamination. In Faisalabad, filtered water samples have a higher prevalence of 10% of parasitic contamination with the presence of 5 different parasites: *Balantidium coli*, *Cryptosporidium parvum*, *Cystoisospora belli*, *Iodoameoba butschii*, and *Taenia sanginata*. The reason for parasitic contamination in filtered water was that the presence of these parasites could be due to a fault in the treatment operations or post-treatment contamination [15]. As recreational waters include rivers, ponds, lakes, and canals, and have the highest prevalence

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of waterborne parasites, 45.71%. The presence of domestic animals near these water sites leads to high parasitic contamination in recreational water. Another study revealed that the prevalence of waterborne parasites in the river may be due to unsanitary practices by people who defecate near it, as well as the activities of farm animals like goats and cattle that harbor the parasites [16]. Borehole water samples have a 30% prevalence of parasitic contamination. This observation disagrees with other studies that similarly found no evidence of parasites in the borehole's source because of how it was built, and activities near that borehole, as ground activities could also influence the quality of borehole water [17]. According to our study, the high parasite prevalence was observed in the month of April, 47.9%, followed by May 37%, June 14.7%, and the lowest prevalence observed in the month of July, 1.2%. This observation disagrees with the study, which revealed that the parasitic contamination increases in the rainy season [18]. According to research, widespread waterborne epidemics were unlikely to happen in hot places, and parasites in the environment die quickly [19]. Aside from environmental toxins, a lack of water treatment endangers the health of unsuspecting populations. While no single method of filtration could eliminate toxins from drinking water, it can and should be safe to consume within accepted limits [20].

This study was limited by the sampling period of only four months and the focus on morphological identification, which may overlook low-density or molecularly distinct parasites. Future studies should incorporate year-round sampling and molecular techniques to detect a wider spectrum of parasites and assess their genetic diversity. Additionally, interventions such as water treatment efficacy and public awareness campaigns could be evaluated to reduce the risk of waterborne parasitic infections in urban populations.

CONCLUSION

It has been concluded that water sampled from various areas of Lahore and Faisalabad was contaminated with waterborne parasites, which means citizens were at high risk of getting parasitic infections. Therefore, it was very crucial to treat water before drinking or using it for other purposes. Even parasitic contamination in filtered water shows that there was a need for high-performance filtration to have parasite-free water. The highest prevalence of waterborne parasites in recreational water showed government should limit human and animal activities in recreational waters. There should be public education campaigns to raise awareness of the presence of waterborne parasites in water sources.

Authors' Contribution

Conceptualization: ZW, AW, MS
Methodology: ZW, AW, MS
Formal analysis: AI, TS, AAL, SH, AR, AMA
Writing and Drafting: ZW, AW, MS, AAL, SH, AR, AMA
Review and Editing: ZW, AW, MS, AI, TS, AAL, SH, AR, AMA

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Bioprospecting of Endophytic Actinobacteria from Selected Ethno-Medicinal Plants for Antibacterial and Anticancer Activities

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ABSTRACT

Endophytic actinobacteria are promising sources of secondary metabolites to combat antimicrobial resistance and cancer. **Objectives:** To isolate endophytic actinobacteria from ethnomedicinal plants and to evaluate their antibacterial, antibiofilm, and *in-vitro* anticancer potential. **Methods:** The endophytic actinobacteria were isolated from *Aloe barbadensis* and *Azadirachta indica* using the spread-plate technique on selective media. Purified isolates were screened for antibacterial activity by well diffusion assay and for general cytotoxicity by brine shrimp lethality assay. The crude methanolic extracts of bioactive strains were further tested against A549 lung carcinoma cells (MTT assay) and *Escherichia coli* biofilms and were chemically profiled by TLC and GC-MS. **Results:** Several endophytic actinobacteria isolates were recovered, many of which inhibited at least one test bacterium. The extracts of strains IEA2, IEA4, IEA5, IEA14, and IEA18 reduced A549 cell viability by approximately 32-87% at 0.1-1 mg/ml, indicating strong in-vitro anticancer potential. The extracts of isolates IEA2, IEA4, and IEA5 dispersed the *E. coli* biofilms by 85%, 87%, and 79% at 3µg/mL, demonstrating marked antibiofilm activity. GC-MS analysis of active extracts revealed multiple low-molecular-weight metabolites (≈100-500 Da), consistent with the production of diverse secondary metabolites by actinobacteria. **Conclusions:** Five strains reduced A549 viability by 32-87% and dispersed *E. coli* biofilms by 79-87%, indicating that aloe- and neem-derived endophytes are promising sources of compounds against drug-resistant bacteria and lungs cancer; however, these findings are based on crude extracts and need confirmation by purification and mechanistic studies.

INTRODUCTION

Plants are a rich source of bioactive compounds, which are produced by the bacteria that live inside the tissues. These endophytes proliferate and reside in plant tissues and excrete secondary metabolites, making them a valuable source for discovering new therapeutic agents [1]. Medicinal plants harbor unique actinobacteria in their tissues and rhizosphere. Due to the co-evolution with defense compounds, these actinobacteria often produce novel bioactive metabolites (antibiotics, anticancer agents, and antioxidants) that can be used for drug development. In the meantime, plants select useful microbes in the environment by means of root exudates in a symbiotic and selective interaction, stimulating ecological stability and evolutionary victories [2]. The endophytic association between plants and their microbes is important in guarding the host against pathogens and pests [3]. Moreover, endophytes are part of producing secondary metabolites that supplement the plant phytochemical profile and enhance its chemical diversity as a whole [4]. Actinobacteria are among these microbes, which exhibit a high level of affinity with the root tissues relative to other plant organs [5]. Multiple studies have reported structurally novel antibiotics and other metabolites from endophytic *Streptomyces* species in

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diverse hosts, underscoring their value for natural product discovery [6]. Community studies also reveal that populations of endophytic actinobacteria are mainly dominated by *Streptomyces* species in plant tissues, although other genera also make up a proportion of endophytic actinobacteria [7]. The endophytic actinobacterial genera *Glycomyces* and *Streptomyces* have been identified as promising sources of bioactive compounds effective against methicillin-resistant *Staphylococcus aureus* in a number of studies [8]. In addition to their antimicrobial action, endophytic actinobacteria may also produce plant growth-promoting compounds that induce growth and development in their hosts, establishing a symbiotic relationship between the microbe and the plant [9]. In one notable study [10], investigators found 398 Gram-positive endophytic actinomycete isolates with an antagonistic effect on phytopathogens, having a significant potential as a biocontrol agent in the sustainable management of plant diseases [11]. Furthermore, scientists have discovered new antibiotics in *Streptomyces* strains residing within the tissues of *Aucuba japonica*, including two novobiocin derivatives that had not been previously described [12]. Similarly, researchers have identified two novel butyrolactone antibiotics, cedarmycin A and B, produced by endophytic *Streptomyces* strains residing within *Cryptomeria japonica* plants [13]. Furthermore, a new naphthoquinone antibiotic, alnumycin, was identified in endophytic *Streptomyces* from *Alnus glutinosa* [14].

Despite increasing reports on endophytic actinobacteria as potential sources of novel bioactive compounds, limited studies have systematically explored ethnomedicinal plants from Pakistan for antibacterial, antibiofilm, and anticancer properties. In particular, Aloe barbadensis and Azadirachta indica remain under-investigated regarding the diversity and therapeutic potential of their endophytic actinobacterial communities. Furthermore, integrated evaluation combining antimicrobial, antibiofilm, cytotoxic, and metabolomic profiling approaches is scarce. Therefore, there exists a significant research gap in identifying and characterizing bioactive endophytes from these medicinal plants that may contribute to novel drug discovery. This study aimed to hypothesize that endophytic actinobacteria from Aloe barbadensis and Azadirachta indica may produce secondary metabolites with significant antibacterial, antibiofilm, and in-vitro anticancer activities, and that metabolomic profiling may yield some chemically diverse bioactive compounds.

METHODS

This was an experimental study conducted at the Department of Microbiology, University of Central Punjab, Lahore, and at the Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, from August 2023 to March 2024. Sixteen healthy samples of *Azadirachta indica* (neem) and *Aloe barbadensis* (aloe vera) were collected from nurseries in Lahore. Plant tissues (roots, stems, and leaves) were washed, surface sterilized with 75% ethanol (5 min), 1% NaOCl (20 min) and 10% $NaHCO_3$ (10 min), and rinsed with sterile water to remove surface microbes [15]. Sterilized tissues were macerated in 9.9mL sterile water, and 0.1mL homogenate was spread on starch casein nitrate agar (SCA). Plates were incubated at 28°C for 14 days. Colonies with typical actinobacterial morphology (dry, hard, earthy odor) were selected [16]. Isolates were characterized by colony morphology, pigment formation, and biochemical tests as per Bergey's Manual and genomic DNA was extracted using phenol-chloroform, and the 16S rRNA gene was amplified with primers 27F/1492R [17]. PCR conditions: 95°C denaturation, 53°C annealing, 72°C extension. Amplicons were purified, sequenced, and compared using BLAST on EzBioCloud. Sequences were deposited in GenBank, and a phylogenetic tree was generated in MEGA11 using neighbor-joining with 1000 bootstraps [18]. Metabolites were extracted by culturing isolates in broth for 7 days at 28°C, 150 rpm. Cultures were sonicated (10 min) [19], mixed with XAD-16 resin (4% w/v) overnight, centrifuged, and the resin pellets were extracted with methanol. Crude extracts were used in bioassays. Antibacterial activity against MRSA and *Bacillus subtilis* was tested by agar well diffusion [20]. Wells (6 mm) were filled with 50 µL extract; zones of inhibition were measured after incubation at 37°C for 16–24 h. TLC was performed on silica gel plates developed in 10% methanol/dichloromethane and visualized under UV (254/366 nm). Plates were stained with anisaldehyde-H_2SO_4 to detect metabolite classes. GC-MS analysis was performed using a Perkin Elmer Clarus 600 system with an Elite-5MS column. Oven: 40–300°C at 5°C/min. Compounds were tentatively identified via NIST20 (similarity ≥80%). Cytotoxicity screening was performed by exposing *Artemia salina* nauplii to extracts in DMSO; Mortality (M%) was calculated as: M (A-B-N)/(G-N) X 100. The mortality rate formula parameters are as follows: where A = number of dead larvae in the test well after 24 h, B = mean number of dead larvae in control Wells after 24 h, G = total initial larval population per well, and N = number of larvae dead before test initiation. Estimates were done in triplicate; data analyzed by one-way ANOVA in GraphPad Prism. Anticancer activity was evaluated on A549 cells using the MTT assay [21]. Cells were treated with extracts (0.5–3 mg/mL) for 24 h. Absorbance was measured at 570 nm; cytotoxicity was calculated relative to controls. Antibiofilm activity against *Escherichia coli* was assessed by crystal violet assay [22]. Biofilm reduction was calculated as: % Biofilm reduction = [1-OD Sample – OD negative Control/OD positive control -

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OD negative Control] X 100. Based on bioactivity and TLC/GC-MS profile, *Streptomyces sp*. IEA4 was scaled-up in 2 L of GYM broth. Fermented broth was extracted with methanol/XAD-16N, concentrated, and fractionated on silica gel into 18 fractions, combined into F1-and F3. Preparative TLC yielded 19 subfractions screened against *B. subtilis* and MRSA. No human or animal subjects were involved. A549 work followed BSL-2 guidelines.

RESULTS

A total of twenty-six endophytic actinobacterial strains were recovered from medicinal plant tissues on starch casein agar. Most colonies developed on starch casein KNO_3 agar (SCA) as compact, dry, embedded growth. Twenty-six morphologically distinct isolates were selected as putative actinobacteria for further characterization, biological screening and chemical profiling (Figure 1).

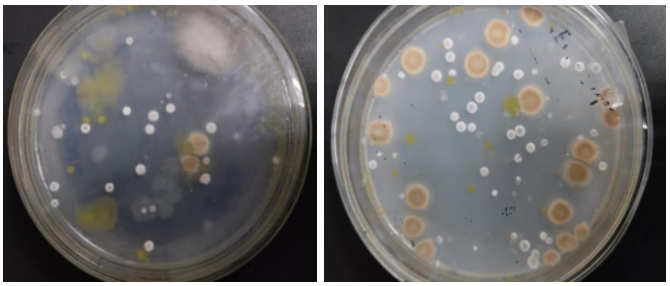

Figure 1: Spread Plate Method, Several Different Dry and Hard Actinobacterial Colonies with A Characteristic Earthy Aroma

Pure cultures of selected actinobacteria strains on the 7th day of incubation on SCA and GYM agar (Figure 2).

Figure 2: Pure Cultures of Selected Actinobacteria Strains on the 7th Day of Incubation on SCA And GYM Agar

The isolates were incubated on GYM and SCA agar for 7-10 days at 28 °C. Colony morphology on both media showed typical actinobacterial features, with rounded to irregular colonies, convex elevation, and hard, dry, or powdery textures. Gram staining confirmed all isolates as Gram-positive, filamentous bacteria with branching hyphae and chain-like spore arrangements. Strains from *Azadirachta indica* (e.g., IEA8, IEA14, IEA4) showed off-white to light-pink growth on SCA, whereas isolates from *Aloe barbadensis* (e.g., IEA5, IEA18) produced off-white, regular colonies. Each isolate displayed a characteristic combination of colony color and texture under the tested conditions (Table 1).

Table 1: Colony morphology of isolated endophytic actinobacterial strains on 7th day of incubation at 28°C on SCA agar

Sr. No.	Actinobacterial Strains	Size (mm)	Shape	Color	Opacity	Elevation	Margin	Texture	Surface	Soluble Pigment
1	IEA 1	2.0	Circular	Crimson	Opaque	Umbonate	Entire	Dry	Wrinkled	Light brown
2	IEA 2	1.0	Irregular	White	Opaque	Convex	Entire	Dry	Smooth	Light brown
3	IEA 3	1.5	Circular	Off white	Opaque	Raised	Entire	Dry	Rough	None
4	IEA 4	2.0	Irregular	Pink	Opaque	Raised	Entire	Dry	Rough	None
5	IEA 5	2.0	Irregular	Pink	Opaque	Raised	Entire	Dry	Smooth	None
6	IEA 6	1.5	Circular	Crimson	Opaque	Raised	Entire	Dry	Wrinkled	Light brown
7	IEA 7	0.5	Circular	Stone grey	Opaque	Raised	Entire	Dry	Rough	None
8	IEA 8	2.0	Circular	Dark black	Opaque	Raised	Entire	Dry	Wrinkled	Light brown
9	IEA 9	1.3	Circular	Crimson	Opaque	Raised	Entire	Dry	Smooth	Light brown
10	IEA 10	0.3	Irregular	Crimson	Opaque	Raised	Entire	Dry	Rough	Light brown
11	IEA 11	0.6	Irregular	Stone grey	Opaque	Raised	Entire	Dry	Rough	None
12	IEA 12	1.1	Irregular	Stone grey	Opaque	Umbonate	Entire	Dry	Smooth	Light brown
13	IEA 13	1.2	Irregular	Stone grey	Opaque	Convex	Entire	Dry	Wrinkled	None
14	IEA 14	2.0	Circular	Stone grey	Opaque	Umbonate	Entire	Dry	Smooth	None
15	IEA 15	0.3	Irregular	Yellowish brown	Opaque	Convex	Entire	Dry	Wrinkled	None
16	IEA 16	0.4	Irregular	Dark black	Opaque	Flat	Entire	Dry	Smooth	None
17	IEA 17	1.0	Circular	Yellowish brown	Opaque	Umbonate	Entire	Dry	Rough	Light brown
18	IEA 18	0.5	Irregular	Dark black	Opaque	Convex	Entire	Dry	Rough	Light brown
19	IEA 19	0.9	Irregular	Crimson	Opaque	Umbonate	Entire	Dry	Rough	None
20	IEA 20	2.0	Circular	Crimson	Opaque	Convex	Entire	Dry	Rough	None
21	IEA 21	0.7	Circular	Yellowish brown	Opaque	Flat	Entire	Dry	Rough	Light brown
22	IEA 22	1.0	Irregular	Stone grey	Opaque	Convex	Entire	Dry	Smooth	Light brown
23	IEA 23	0.6	Circular	Stone grey	Opaque	Raised	Entire	Dry	Wrinkled	Light brown

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24	IEA 24	0.3	Irregular	Yellowish brown	Opaque	Raised	Entire	Dry	Rough	None
25	IEA 25	0.6	Irregular	Dark black	Opaque	Raised	Entire	Dry	Smooth	None
26	IEA 26	1.0	Circular	Stone grey	Opaque	Raised	Entire	Dry	None	None

Biochemical and physiological tests (melanin, esculin, urease, citrate, and methyl red) further supported their assignment to actinobacteria. Overall, 61% of the strains were positive for the methyl red test, 57% were able to utilize citrate, and 69% showed urease activity (urea hydrolysis). Half of the isolates produced melanin on tyrosine agar, and 60% hydrolyzed esculin. Molecular identification based on 16S rRNA gene sequencing and neighbor-joining phylogeny (MEGA 11, 1000 bootstrap replicates) assigned most isolates to the genus *Streptomyces*. Representative strains IEA4, IEA8, and IEA14 clustered with the described *Streptomyces* species and were deposited in GenBank under accession numbers PQ269133, PQ267973, and PQ269150, respectively, confirming their actinobacterial identity and revealing close similarity to previously reported strains (Table 2).

Table 2: GenBank Accession Numbers of the Selected Endophytic Actinobacterial Strains and Their Similarities (Percentage) with Previously Reported Strains

Actinobacterial Strains	Sequences Submitted	Gen Bank Accession Numbers	Closely Related Taxa	Percentage Similarity
IEA4	913	PQ269133	*Streptomyces sp.*	100%
IEA8	938	PQ267973	*Streptomyces sp.*	100%
IEA14	938	Pq269150	*Streptomyces microflavus*	100%

Methanolic crude extracts from the 26 isolates were screened for antibacterial activity against MRSA and *Bacillus subtilis*. Seven strains (IEA2, IEA3, IEA4, IEA5, IEA8, IEA14, and IEA18) produced clear inhibition zones against at least one test organism. IEA4 and IEA5 showed the strongest activity, with IEA5 producing the largest zone (17 mm) against *B. subtilis*, while IEA8 and IEA14 produced zones of 10 mm and 15 mm, respectively. Crude extracts demonstrated significant *in-vitro* anticancer activity against the A549 lung carcinoma cell line. Notably, strain IEA14 reduced cell viability by ~87% at 1 mg/mL, indicating strong cytotoxic potential, while IEA4 induced moderate inhibition (40–50%). This dose-dependent cytotoxicity aligns with previous reports on *Streptomyces*-derived metabolites, such as doxorubicin and actinomycin D, which trigger apoptosis via DNA intercalation or topoisomerase inhibition. The variability among strains suggests differences in secondary metabolite profiles, possibly involving polyketides or non-ribosomal peptides known for anticancer activity. The antibiofilm activity of extracts IEA2, IEA4, and IEA5 against *E. coli* displayed marked concentration-dependence, with inhibition rising from 8–13% at 0.5 mg/mL to 79–87% at 3.0 mg/mL. The potent activity of IEA4 (87% inhibition at 3 mg/mL) may be attributed to metabolites interfering with quorum sensing or extracellular polymeric substance (EPS) synthesis, mechanisms previously reported for *Streptomyces* antibiofilm agents. Such dose-responsive biofilm disruption is consistent with studies on actinobacterial cyclic peptides and enzymes that degrade biofilm matrices. 3A: Actinobacterial activity determined by the well diffusion method; 3B: % inhibition of cell viability against A549 lung cancer cell line, and 3C: Dose-response curves for biofilm inhibition of *E. coli* by endophytic actinobacterial extracts (Figure 3).

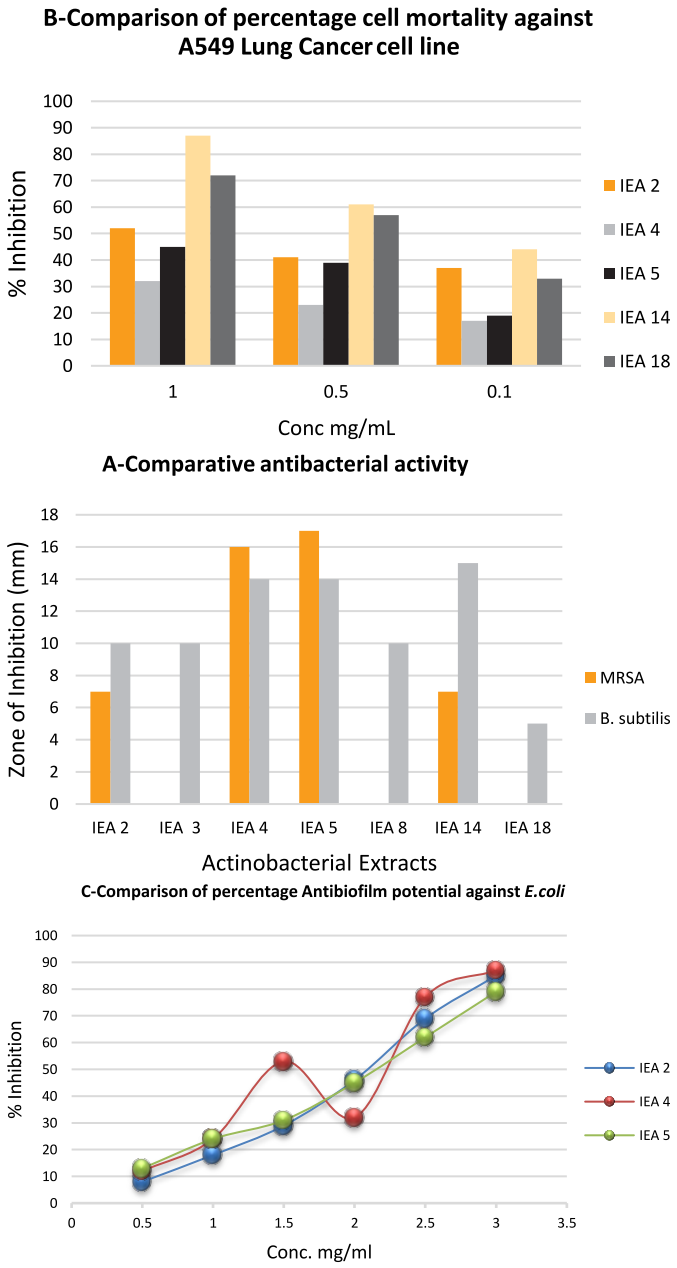

Figure 3: Evaluation of Bioactivity of Crude Extract

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Metabolomic profiles of the selected endophytic actinobacteria strains based on TLC and GC-MS analyses were done (Table 3).

Table 3: Metabolomic Profiles of the Selected Endophytic Actinobacteria Strains Based on TLC And GC-MS Analyses

Strains	Thin Layer Chromatography (TLC)			GCMS (tR/min)	Peak Area%	Molecular Formula	Molecular Weight	Compounds: Closest match in NIST library
	UV Visualization		Staining with Anisaldehyde / H2SO4					
	254 nm	366 nm						
IEA 2	1	3	1 Greenish Band	24.891	18.64%	$C_7H_{10}N_2O_2$	154.17	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro
				35.053	5.91%	C_8H_{16}	97.09	1,2,4-trimethylcyclopentane
				–	–	$C_8H_{14}O_{16}$	366.19	cis-2,2,3,4-tetramethylcyclobutanone
				44.264	69.10%	$C_8H_{14}O$	126.20	Bis(2-ethylhexyl) phthalate
				–	–	$C_{24}H_{38}O_4$	390.55	Phthalic acid, di(2-propylpentyl) ester
				54.387	6.35%	$C_{10}H_{30}O_3Si_4$	310.68	Decamethyltetrasiloxane
				–	–	$C_{13}H_{19}NO_2$	222.30	4-(4-Hydroxy-2,5-dimethylbenzyl) morpholine
IEA 3	1	4	1 Yellowish Band	44.260	100%	$C_{24}H_{38}O_4$	390.55	Bis(2ethylhexyl) phthalate
IEA 4	1	1	1 Yellowish Band	44.255	34.09%	$C_{24}H_{38}O_4$	390.55	Bis(2-ethylhexyl) phthalate
				52.982	12.45%	$C_{20}H_{42}O_{11}$	458.54	Decaethylene glycol
				–	–	$C_{12}H_{24}O_6$	264.31	1,4,7,10,13,16-Hexaoxacyclooctadecane
				54.957	53.47%	$C_{20}H_{42}O_{11}$	458.54	Decaethylene glycol
				–	–	$C_{12}H_{24}O_6$	264.31	1,4,7,10,13,16-Hexaoxacyclooctadecane
IEA 5	1	4	1 Blackish Band	44.246	100%	$C_{24}H_{38}O_4$	390.55	Bis(2-ethylhexyl) phthalate
IEA 8	1	4	1 Greenish Band	–	–	–	–	–
IEA 14	1	4	1 Pinkish Band	44.248	100%	$C_{24}H_{38}O_4$	390.55	Bis(2-ethylhexyl) phthalate
						$C_{12}H_{14}O_4$	366.36	Di(Z)-hex-3-enyl phthalate
IEA 18	1	2	1 Brownish Band	–	–	–	–	–
IEA 20	1	1	1 Pinkish Band	–	–	–	–	–

Following primary screening, *Streptomyces* sp. IEA4 was scaled up to characterize its bioactive secondary metabolites. Column chromatography of the crude extract on silica gel with dichloromethane-methanol gradients generated 18 fractions, which were combined into three pooled fractions (F1-F3) based on TLC patterns. The three pooled fractions were tested against *B. subtilis*, *Klebsiella pneumoniae*, MRSA, and *E. coli*, and all showed varying degrees of activity. Further TLC analysis of F1-F3 revealed multiple differently colored bands (blue, green, purple, and light yellow; Supplementary Figure S6). Preparative silica gel TLC yielded 19 sub-fractions from F1-F3. These purified sub-fractions were evaluated against MRSA and *B. subtilis*; several retained measurable antimicrobial activities. The solvent control caused low mortality of *A. salina nauplii* (mean 25% ± SD). In contrast, most fractions increased mortality, with mean values ranging from ~33–63%. Several subfractions, particularly from F1-3 and the F2/F3 series, produced >60% mortality. Ordinary one-way ANOVA showed a significant overall effect of fraction on brine shrimp mortality ($F(19,40)=2.96$, $p=0.001$; $R^2=0.58$), with no evidence of unequal variances (Brown–Forsythe test, $p=0.699$). (A) Comparative anti-bacterial properties of pooled fractions F1-F3 against MRSA, *Bacillus subtilis*, *Escherichia coli*, and *Klebsiella pneumoniae* in the form of zone of inhibition (mm) using agar well diffusion methodology. (B) MRSA and *B. subtilis* inhibitory responses of eight preparative-TLC-purified sub-fractions of F1-F3. (C) Brine shrimp lethality of fractions and subfractions derived from *Streptomyces* sp. IEA4. Data were analyzed by ordinary one-way ANOVA followed by Dunnett's test versus control using GraphPad Prism 10; *p<0.050, **p<0.010 (Figure 4).

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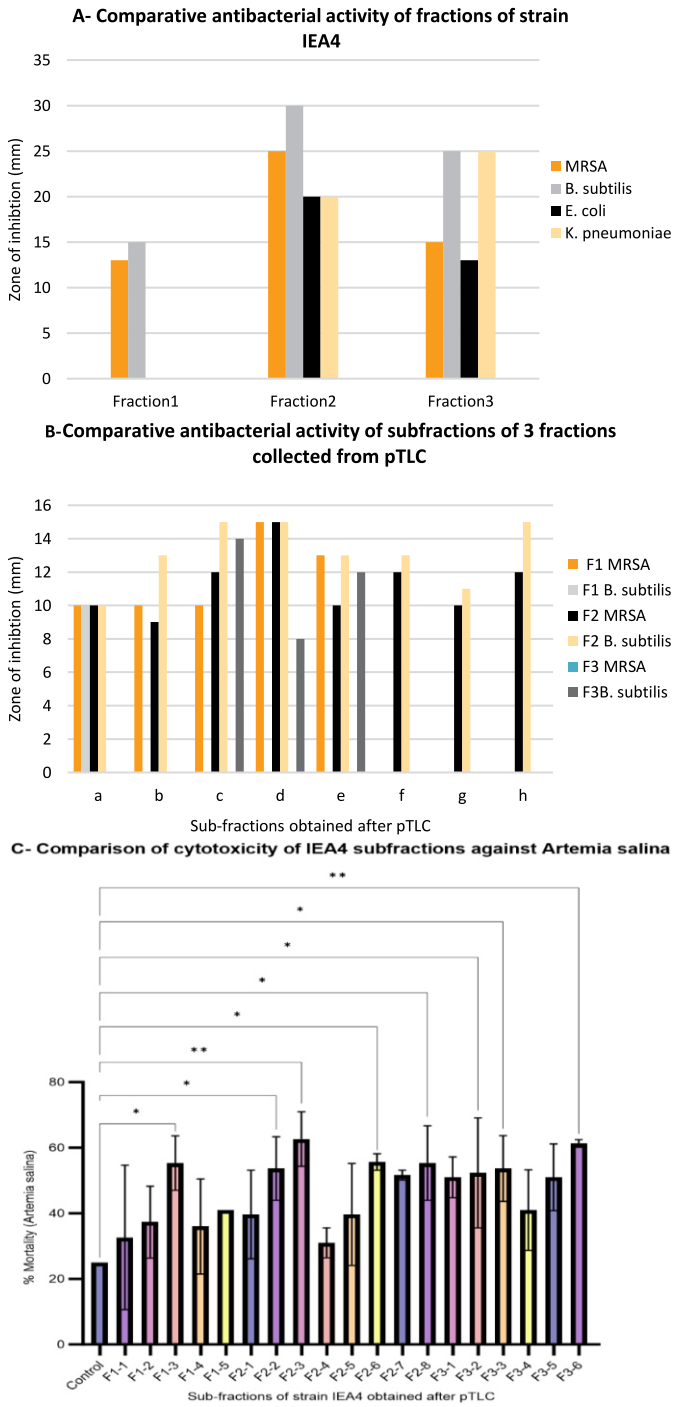

Figure 4: Fractions and Purified Sub-Fractions of *Streptomyces* Sp. IEA4 Bioactivity

DISCUSSION

Endophytic actinobacteria associated with medicinal plants in South Asia are increasingly recognized as valuable reservoirs of novel bioactive compounds due to their capacity to synthesize chemically diverse secondary metabolites with therapeutic relevance. In the present study, a total of 26 endophytic actinobacterial strains were successfully isolated from *Azadirachta indica* (neem) and *Aloe barbadensis* (aloe vera) following rigorous surface sterilization protocols. Preliminary identification based on colony morphology was carried out in accordance with the guidelines described in Bergey's Manual [23]. The isolated strains exhibited notable biological activities in multiple *in vitro* assays. Among them, strain IEA14 demonstrated the most pronounced anticancer activity, causing 87% inhibition of A549 lung carcinoma cells at a concentration of 1 mg/mL, which decreased to 44% at 0.1 mg/mL, indicating a clear concentration-dependent effect. Similarly, antibiofilm activity against *Escherichia coli* increased with extract concentration, with strain IEA4 showing the highest inhibition (87%) at 3 mg/mL, followed by IEA2 (85%) and IEA5 (79%). Furthermore, antimicrobial evaluation revealed that seven out of the 26 isolates exhibited strong inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Bacillus subtilis* [24]. The observed variation in bacterial susceptibility is consistent with previous findings, which report higher sensitivity of Gram-positive bacteria to antimicrobial agents due to the lack of an outer membrane and structural differences in their cell walls. The dominance of *Streptomyces* species among the isolates aligns with global studies identifying this genus as the most prevalent endophytic actinobacteria in medicinal and agricultural plants [25]. The co-occurrence of *Micromonospora* and *Nocardia* further enhances the metabolic diversity of the isolated community, as these genera are known to produce distinct classes of antibiotics and anticancer agents [7]. This taxonomic profile mirrors recent findings by Saikia *et al.* (2022), who reported a similar distribution of actinobacterial genera in tropical medicinal plants and linked it to enhanced functional and chemical diversity within the plant microbiome [26]. Metabolomic analysis using thin-layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS) underscored the chemical richness of the bioactive isolates. TLC profiles displayed multiple UV-absorbing and fluorescent bands, and distinct staining with anisaldehyde/H_2SO_4 indicated the presence of terpenoids, alkaloids, and phenolic compounds—classes widely associated with bioactivity.GC-MS tentatively identified several low-molecular-weight metabolites (<500 Da), including phthalate derivatives and heterocyclic scaffolds, consistent with prior reports of similar compounds in endophytic actinobacterial extracts [27]. While these identifications are preliminary and require confirmation through advanced spectroscopic techniques such as NMR and HRMS, the observed chemical diversity reaffirms the biosynthetic potential of these endophytes. Further cytotoxicity assays on normal cell lines would help establish the therapeutic index of these promising

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metabolites.

This study is limited by the use of crude extracts and in-vitro bioassays, which do not fully elucidate the specific active compounds or their precise mechanisms of action. GC-MS identification was tentative and requires confirmation through advanced spectroscopic techniques such as NMR and HRMS. Additionally, cytotoxicity was evaluated on a single cancer cell line without comparison to normal cell lines, limiting assessment of selectivity and therapeutic index. Future studies should focus on bioassay-guided purification, structural elucidation, whole-genome sequencing of promising strains, and in-vivo validation to advance these endophytic actinobacteria as potential therapeutic candidates.

CONCLUSION

This study screened endophytic actinobacteria from the ethnomedicinal plants *Aloe barbadensis* and *Azadirachta indica* for growth inhibition of drug-resistant bacteria and *in-vitro* anticancer activity. Twenty-six strains, dominated by *Streptomyces* but also including *Micromonospora* and *Nocardia*, were obtained, and several produced crude extracts with strong activity against MRSA and *B. subtilis* as well as notable cytotoxic effects on A549 lung cancer cells. Metabolomic profiling indicated that these bioactive isolates synthesize a chemically diverse set of low-molecular-weight metabolites consistent with secondary metabolite production. Overall, endophytic actinobacteria from aloe and neem represent a promising source of antibacterial, antibiofilm and anticancer candidates and warrant further bioassay-guided purification, structure elucidation, and genome-guided discovery of novel therapeutic compounds.

Authors' Contribution

Conceptualization: IS[1], NN, IS[2]
Methodology: IS[1], AI, NN, IS[2]
Formal analysis: AI, IS[2]
Writing and Drafting: IS[1], IS[2]
Review and Editing: AI, IS[1], IS[2], NN

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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Computational Drug Repurposing of a Ketamine–Methylphenidate Conjugate for Targeting GLIPR1 in Human Glioma

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ABSTRACT

Gliomas are the most aggressive primary brain tumors, characterized by high mortality, therapeutic resistance, and limited treatment options due to blood–brain barrier constraints. Glioma pathogenesis-related protein 1 (GLIPR1) is highly upregulated in malignant gliomas and minimally expressed in normal brain tissue, making it a promising molecular target. Drug conjugation strategies may improve CNS delivery and therapeutic efficiency against such targets. **Objectives:** This study aimed to investigate the binding potential and pharmacokinetic feasibility of a ketamine–methylphenidate conjugate against the glioma-associated protein GLIPR1 using in silico approaches. **Methods:** The three-dimensional structure of GLIPR1 (PDB ID: 3Q2U) was retrieved and prepared using Discovery Studio. Structural validation was performed through Ramachandran plot analysis, SOPMA, and PROCHECK. Active site prediction was carried out using PrankWeb, and physicochemical properties were assessed with ProtParam. The ketamine–methylphenidate conjugate (SDF format) was obtained from Nouman Ali *et al.* and evaluated for ADMET properties using pkCSM. Molecular docking was performed using CB-Dock, and ligand–protein interactions were analyzed via Discovery Studio. **Results:** Docking analysis revealed a favorable binding affinity (–6.9 kcal/mol), supported by two hydrogen bonds, five hydrophobic interactions, and one electrostatic interaction. Pharmacokinetic profiling indicated suitable absorption, moderate BBB permeability, and an acceptable safety profile, supporting CNS applicability. **Conclusions:** The findings suggest that the ketamine–methylphenidate conjugate is a promising CNS-penetrant candidate with potential relevance in glioma management, warranting further experimental validation.

INTRODUCTION

Gliomas are the most prevalent primary malignant tumors of the central nervous system, accounting for an estimated 80% of all brain tumors worldwide [1]. It is estimated that over 320,000 new cancer cases of brain and central nervous system tumors appear per year, of which gliomas are the most predominant, and glioblastoma multiforme is the most prevalent of them. Gliomas are very fatal, and despite the progress in neuro-oncology, the disease has claimed more than 248,000 people annually all over the world [2]. The usual treatment is maximal surgical resection, which is followed by radiotherapy and temozolomide-based chemotherapy, but the median survival of glioblastoma is still about 12-15 months [3]. The fact that treatment is significantly constrained by tumor

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recurrence, therapeutic resistance, and the lack of blood-brain penetration of molecular therapeutic agents has made the development of new molecular targets and therapeutic approaches urgently necessary [4]. Glioma pathogenesis-related protein 1 (GLIPR1) is a membrane-bound protein that is highly expressed in aggressive glioma and is expressed at low levels in normal brain tissue. GLIPR1 is composed of a secretion signal peptide, a conserved cysteine-rich CAP domain, and a transmembrane region, all of which are involved in tumor proliferation and invasion [5]. Depository investigations of the flexible GLIPR1 fragment show that the central cavity is sufficiently clear with an ability to bind zinc and has distinctive surface charge distributions, which are plausible to accommodate functional ligand interactions. GLIPR1 is a selective molecular target that shows a positive correlation with glioma grade and its invasiveness. It has been shown to play a role in tumor growth and inflammatory regulation, as well as in glioblastoma-targeted therapy, making it a desirable target for structure-based drug design [6]. The most commonly used central nervous system-active agents to treat neuropsychiatric symptoms commonly associated with gliomas include ketamine and Methylphenidate, which are used to treat depression, cognitive dysfunction, fatigue, and attentional deficits. Ketamine has an antidepressant and neuroplasticity-promoting activity (NMDA) receptor antagonist, has been shown to have rapid effects and has been shown to have implications in cancer-related depression and pain management [7, 8]. A clinically used drug is the dopamine and norepinephrine reuptake inhibitor, Methylphenidate, which is used to enhance attention, executive functions, and fatigue associated with cancer in brain tumor victims [9, 10]. Both drugs are effective at penetrating the blood-brain barrier, a critical constraint in the therapy of glioma [11, 12]. Their well-established CNS safety profiles and neuromodulatory effects justify exploring the potential repurposing of these therapies beyond symptomatic treatment. Building on the established CNS-penetrant profiles of ketamine and methylphenidate, we propose a structure-guided repurposing strategy via conjugation to selectively target the glioma-associated protein GLIPR1. The rationale for this approach is threefold. First, GLIPR1 represents a structurally defined and tumor-selective "druggable" target. Its resolved crystal structure reveals a central hydrophilic cavity with distinctive surface charge distributions, suitable for accommodating small molecules [6]. Second, the individual pharmacologist of the parent drugs may synergistically address the glioma microenvironment. Ketamine, as an NMDA receptor antagonist, could potentially disrupt glutamate-driven oncogenic signaling and paracrine stimulation of glioma growth [7, 8]. Concurrently, methylphenidate, a dopamine/norepinephrine reuptake inhibitor, might modulate catecholamine levels within the tumor milieu, which have been implicated in glioma proliferation and stemness [9].

Glioma is one of the most aggressive and poorly prognostic brain tumors without a high number of targeted therapeutic options to be used, despite the development of conventional treatment modalities. In spite of the fact that GLIPR1 has been identified as a pathogenic contributor to glioma, its feasibility as a drug-targetable protein has not been thoroughly investigated, and there has not been any previous research comparing a ketamine-methylphenidate conjugate with this protein. Hence, the study aimed to numerically examine the binding affinity, molecular stability, and pharmacokinetic viability of a ketamine methylphenidate conjugate targeting GLIPR1 by in silico methods.

METHODS

The RCSB Protein Data Bank (https://www.rcsb.org/) was used to obtain the three-dimensional crystal structure of human glioma pathogenesis-related protein 1 (GLIPR1), with accession number 3Q2U [6]. BIOVIA Discovery Studio was used to prepare the protein structure, in which all heteroatoms, co-crystallized ligands, and water molecules were removed to optimize the receptor [13]. The refined protein was structurally validated using PROCHECK (https://saves.mbi.ucla.edu/) Ramachandran plot analysis, which confirmed its stereochemical reliability, whereas the secondary structure composition was analyzed with Structure Prediction using SOPMA (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and the PSIPRED tool (https://bioinf.cs.ucl.ac.uk/psipred/) [14, 15] The active binding site was predicted using the PrankWeb server (https://prankweb.cz/), which identifies cavities accessible to the ligand using machine-learning algorithms [16]. The three-dimensional Structure Data File (SDF) of this pre-designed conjugate was obtained directly from the authors' work. To ensure the ligand was in a suitable conformation for docking, geometry optimization was performed. The initial SDF structure was imported into Avogadro software (version 1.2.0), where energy minimization was conducted using the MMFF94 force field. The optimization process involved 500 steps of the steepest descent algorithm followed by 500 steps of the conjugate gradient algorithm until a convergence gradient of 0.01 kcal/mol.Å was reached. This step ensures the ligand is in a low-energy, stable conformation representative of its probable state in solution before protein binding. The optimized structure was then saved for subsequent docking analyses. Molecular docking was performed using the CB-Dock server, which utilizes AutoDock Vina as its

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docking engine [17]. The workflow was as follows: the prepared GLIPR1 receptor structure (PDB: 3Q2U) in PDBQT format and the geometry-optimized ligand (ketamine-methylphenidate conjugate) in SDF format were uploaded to the server. The binding site coordinates were defined based on the center of the predicted active site cavity from PrankWeb, with the grid box dimensions set to X=35.905 Å, Y=1.474 Å, and Z=4.654 Å to encompass the entire binding pocket. The docking search parameters included an exhaustiveness of 8 (default) and the generation of 9 binding poses. The docking algorithm was set to its default run mode. The conformation with the most favorable (lowest) binding affinity (kcal/mol) was selected for further analysis. To assess the reliability of the docking protocol, a validation step was performed by re-docking the co-crystallized ligand (if available) or by comparing the predicted binding pose with known binding modes from literature. Protein-ligand interaction analysis, including the identification of hydrogen bonds, hydrophobic contacts, and electrostatic interactions, was performed on the top-ranked pose using Discovery Studio Visualizer [18].

RESULTS

The structural analysis of the target protein has been depicted in figure 1. Figure 1A shows the 3D design of the receptor with red color representing α-helices, blue color representing β-sheets, and grey color representing random coils, which illustrates the secondary structure in general. Figure 1B represents the active site of the receptor that is predicted, implying the key amino acid residues that are used in the binding of the ligand. Figure 1C is the three-dimensional structure validation of the Ramachandran plot. The analysis showed that 91.1% of residues are found in the most preferred regions, 7.7% in the additionally allowed regions, and none in the disallowed regions, indicating the protein model's high stereochemical stability and reliability. The secondary structure composition predicted using SOPMA (Figure 1D) shows that there are 63 α-helical residues (30.73%), 15 extended strand residues (7.32%), and 127 random coil residues (61.95%), showing that the composition of the receptor structure consists primarily of flexible regions.

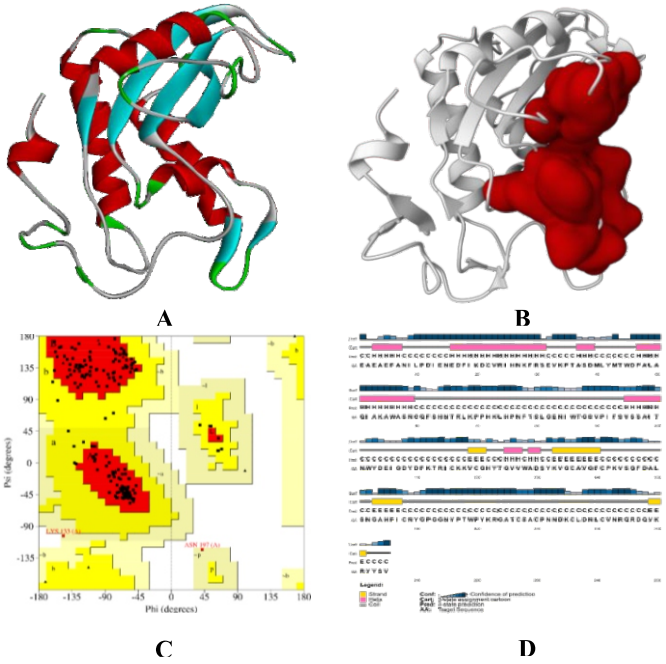

Figure 1: (A): 3D structure of GLIPR1. **(B):** Active site of the receptor highlighted in red. **(C):** Ramachandran plot validating the receptor structure. **(D):** 2D structure of receptor.

The pharmacodynamic and pharmacokinetic assessment of the ketamine-methylphenidate conjugate has indicated a positive profile for its use in central nervous system applications. The conjugate exhibits properties that favor proper gastrointestinal absorption and cellular permeability, indicating it is appropriate for oral administration. The ability to engage efflux transporters, including P-glycoprotein, indicates regulated transport across the biological membrane, and the predicted blood-brain barrier and central nervous system permeability indicate moderate, not optimal, penetration, which may facilitate the agent in brain tissue. Distribution properties indicate adequate tissue penetration, with an equal unbound fraction, resulting in sufficient bioavailability at the target site. The fact that major hepatic enzyme systems are involved but generally not broadly inhibited, as indicated by metabolic profiling, helps prevent severe drug-drug interactions. Excretion parameters indicate equal renal clearance. The toxicological forecasts are low mutagenic liability and controllable cardiac and skin risks, but hepatic involvement should be monitored.

Table 1: ADMET Properties of the Ketamine-Methylphenidate Drug Conjugate

Properties	Model Name	Unit	Predicted Outcome
Absorption	Aqueous solubility	log mol/L	-4.775
	$CaCO_2$ permeability	log Papp in 10-6 cm/s	0.855
	Human intestinal absorption	% Absorbed	92.509%
	Skin Permeability	log Kp	-2.917

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Distribution	P-Glycoprotein Substrate	–	Yes
	P-Glycoprotein I Inhibitor	–	Yes
	P-Glycoprotein II Inhibitor	–	Yes
Metabolism	VDss (Human)	Numerical (log L/kg)	1.035
	Fraction Unbound (Fu)	–	0.141
	Blood–brain barrier penetration	Numerical (log BB)	-0.245
	CNS Permeability	Numerical (log PS)	-2.014
Excretion	CYP2D6 Substrate	–	No
	CYP3A4 Substrate	–	Yes
	CYP1A2 Inhibitor	–	No
	CYP2C19 Inhibitor	–	No
	CYP2C9 Inhibitor	–	No
	CYP2D6 Inhibitor	–	No
	CYP3A4 Inhibitor	–	yes
	Total Systemic Clearance	log ml/min/kg	0.865
	OCT2 substrate (renal)	–	Yes
Toxicity	AMES mutagenicity	–	No
	Maximum Tolerated Dose	log mg/kg/day	-0.672
	hERG inhibition (I/II)	–	Type II positive
	Acute oral toxicity (Ld50, rat)	mol/kg	2.717
	Chronic Toxicity (LOAEL,Rat)	log mg/kg_bw/day	0.458
	Hepatotoxicity	–	Predicted Positive
	Skin Sensitization	–	No
	T. Pyriformis Toxicity	log ug/L	0.395
	Minnow Toxicity	log mM	1.406

The molecular docking analysis showed a strong interaction between the receptor and the ketamine-methylphenidate conjugate, with a docking score of -6.9 kcal/mol. The complex was shown to contain two hydrogen bonds, five hydrophobic interactions, and one electrostatic interaction, indicating stable binding. The detailed docking interactions and their type are given in table 2.

Table 2: Table Showing the Docking Interaction and its types Between Receptor and Ligand

Sr. No.	Name	Distance (Å)	Categories	Types
1	B: UNN0:H – A: GLY179:O	2.40185	Hydrogen Bond	Conventional Hydrogen Bond
2	B: UNN0 – A: LEU83	5.43801	Hydrophobic	Pi-Alkyl
3	B: UNN0 – A: LYS84	4.83278	Hydrophobic	Pi-Alkyl
4	A: HIS79:NE2 – B: UNN0	4.4605	Electrostatic	Pi-Cation
5	A: HIS79 – B: UNN0	4.11677	Hydrophobic	Pi-Pi Stacked
6	A: HIS79 – B: UNN0	4.95693	Hydrophobic	Pi-Alkyl
7	A: TYR181 – B: UNN0	5.4467	Hydrophobic	Pi-Alkyl
8	A: ASN180:HD22 – B: UNN0:O	2.51751	Hydrogen Bond	Conventional Hydrogen Bond

Figure 2 represents docking outcome results, with figure 2A representing the protein in blue, the ligand in yellow, figure 2B representing the 2D interaction mapping, and figure 2C representing the 3D interaction view.

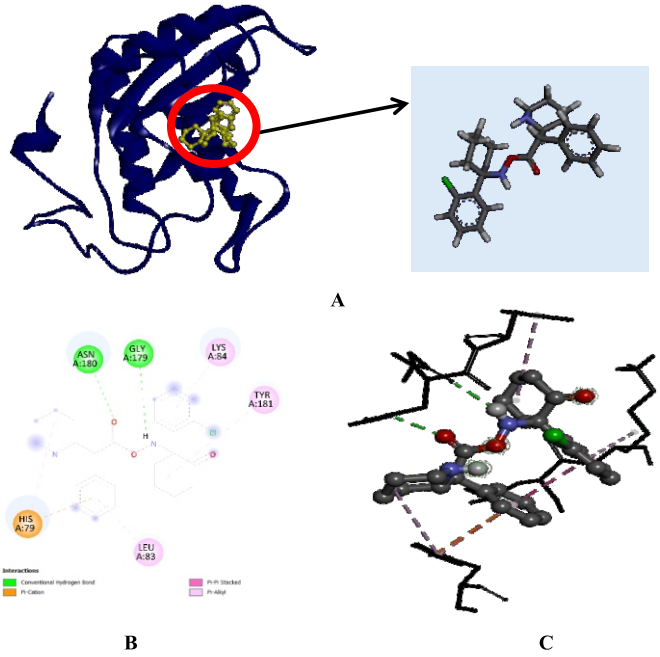

Figure 3: (A): Protein is highlighted in blue color and ligand in yellow, showing the position of the ligand within the receptor. **(B):** 2D visualization of the interactions. **(C):** 3D visualization of interactions.

DISCUSSION

The gliomas still stand as one of the most aggressive and difficult to treat cancers of the central nervous system, mainly because they are infiltrative, molecularly heterogeneous, and do not respond to the traditional therapies. A computational study of the interaction between a ketamine-methylphenidate conjugate and the glioma-associated protein GLIPR1 used a structure-based approach [5]. GLIPR1 structural analysis revealed an organized structure with a high proportion of residues in preferred regions of the Ramachandran plot, suggesting a consistent, stable protein structure suitable for downstream analyses. The feasibility of structure-guided drug design in glioma research lies in the active site, which is predicted to feature a characteristic cavity and flexible loop regions that support ligand accommodation and interaction. Pharmacokinetic and ADMET profiling indicated that the ketamine-methylphenidate conjugate has good absorption, distribution, and penetration into the central nervous system. Of great importance is the conjugate's ability to cross the blood-brain barrier, as BBB impermeability is a major constraint for most glioma therapeutics. Predictions of metabolic activities showed that interactions among enzymes were manageable, and toxicity tests indicated a generally acceptable safety profile, with parameters that could be identified and would need monitoring during experimental validation in future research. Combined with the above, the results suggest that the conjugate has the potential to be a CNS-active compound that can penetrate glioma-related targets in

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brain tissue. Molecular docking results provide preliminary support for the conjugate's ability to interact with GLIPR1. A docking score of -6.9 kcal/mol suggests a moderate binding affinity, which should be interpreted with caution as it is a predictive measure. This score is comparable to those observed for other investigational ligands in early-stage docking studies. The interaction is stabilized by two hydrogen bonds, five hydrophobic contacts, and one electrostatic interaction, indicating a plausible binding mode. However, the functional inhibition of GLIPR1 by this conjugate remains to be demonstrated experimentally. Furthermore, to robustly claim "conjugate superiority," future work should include comparative docking of ketamine and methylphenidate individually against GLIPR1, as well as comparison to known reference compounds or inhibitors if available in the literature, to contextualize the -6.9 kcal/mol score. Comparing it to the research study by Asrar *et al.* which analyzed the same conjugate of ketamine-methylphenidate in the major depressive disorder and ADHD setting, there are significant differences and similarities. The conjugate in their work was shown to bind more strongly to TPH2 -8.5 kcal/mol than when each of its parent compounds was used alone, and it had stable dynamics when simulated by molecular dynamics and MMGBSA. The two studies provide a consistent finding: conjugation appears superior to ketamine or methylphenidate administration in improving pharmacological efficacy, particularly in terms of BBB permeability, binding stability, and toxicity. Although the study by Asrar *et al.* concentrated on serotonergic dysregulation in neuropsychiatric disorders, the current study expands the therapeutic use of the conjugate into the neuro-oncology field by targeting GLIPR1, a glioma-specific protein [19]. Together, these results indicate the multimodality of the ketamine-methylphenidate conjugate as a multipurpose, CNS-penetrant therapeutic agent. The similar evidence provided by both works indicates that this conjugate could be a promising method of treating various brain-related disorders, including psychiatric disorders and aggressive brain tumors, with different molecular mechanisms, although complementary. These computational findings will require further *in vitro* and in vivo validation to support these findings and determine the translational value of the conjugate in glioma therapy. The ligand was the pre-designed ketamine-methylphenidate conjugate reported by Asrar *et al.* [19]. Its geometry was optimized for docking using Avogadro software and the MMFF94 force field, employing steepest descent and conjugate gradient algorithms until a convergence gradient of 0.01 kcal/mol.Å was reached. This energy-minimized structure was used for subsequent analyses [20].

Weaknesses also include reliance solely on in silico methodology, the absence of experimental results, and a truncated protein structure that may not reflect physiological conditions. Furthermore, the predicted ADMET liabilities (hERG inhibition, hepatotoxicity) must be experimentally assessed using patch-clamp assays and hepatic cell viability models, respectively, to de-risk the conjugate's safety profile." *In vitro* and *in vivo* validation of the ketamine-methylphenidate conjugate should be conducted to assess its anti-glioma activity and molecular mechanism. These findings will be further supported using molecular dynamics simulations and binding free energy analyses.

CONCLUSION

In conclusion, this *in silico* investigation provides preliminary evidence suggesting that the ketamine-methylphenidate conjugate may represent a hypothetical CNS-penetrant candidate for targeting GLIPR1 in glioma. The computational analyses indicate promising but unvalidated structural compatibility, moderate BBB permeability, and a docking score suggestive of binding potential. Given the purely predictive nature of this study, these findings should be interpreted cautiously and serve as a hypothesis-generating foundation. They warrant further experimental validation, including in vitro binding assays, molecular dynamics simulations, and functional studies to substantiate the proposed mechanism and therapeutic relevance before any translational consideration.

Authors' Contribution

Conceptualization: MH
Methodology: SKA, MS
Formal analysis: SKA, FAJ, NK, KI
Writing and Drafting: SKA, FAJ, MS, NUE, KS, KI
Review and Editing: MH, SKA, FAJ, MS, NUE, KS, NK, KI

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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