International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 22s, 2025
https://theaspd.com/index.php

Antibiofilm and Antibacterial Potential of Phyllanthus Emblica Extract Against Multidrug-Resistant Shrimp Pathogens

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Abstract
Vibriosis remains a significant challenge in shrimp aquaculture. In response, many farmers resort to indiscriminate use of hormones, antibiotics, disinfectants, and other chemicals in feed and culture water to safeguard their stock. In the present study, E. coli and Aeromonas spp. emerged as the predominant bacterial isolates. Herbal agents offer a sustainable alternative in aquaculture for controlling or reducing pathogenic infections. Phytochemical screening of Phyllanthus emblica fruit extract revealed the presence of multiple bioactive compounds. The extract demonstrated antibacterial activity against biofilm-producing shrimp pathogens, with E. coli and Pseudomonas aeruginosa showing the greatest susceptibility. Growth inhibition occurred in a dose-dependent manner. Molecular docking identified β-sitosterol as a potent antibiofilm compound, exhibiting strong binding affinity (-8.4 kcal/mol) with a key biofilm-associated protein. Further in silico analysis was performed using bioactive compounds identified through GC–MS profiling.

Keywords: *phyllanthus emblica, phytochemical analysis, Vibriosis, shrimp,*

1. INTRODUCTION

Shrimp farming is a key sector of aquaculture in many tropical countries, with production expanding rapidly. However, this industrial growth has brought about significant environmental challenges, adversely affecting both shrimp health and their aquatic ecosystems. The intensification of aquaculture has led to a rise in disease prevalence (Shariff et al., 2001), often driven by pathogenic microbes such as Aeromonas spp., Pseudomonas spp., Vibrio spp., Escherichia coli, Salmonella spp., Klebsiella spp., Staphylococcus aureus, and Shigella spp. (Rahman et al., 2012; Abraham et al., 2013; Pooja and Singh, 2022).

Historically, chemicals and antimicrobials have been used extensively to control pathogens in shrimp hatcheries. For instance, chlorine, a common disinfectant in ponds and hatcheries, has been shown to facilitate the spread of antibiotic resistance genes among bacteria (Balcazar et al., 2006). The emergence of antibiotic-resistant pathogens poses a major public health threat, fueling concerns over the prudent use of antibiotics across veterinary, nutritional, and agricultural sectors. In shrimp farming, the improper and excessive use of antibiotics has accelerated resistance development, with resistant bacteria capable of transmission to humans through shrimp consumption (Zanetti et al., 2001). This not only compromises food safety but also results in severe pathological impacts on infected shrimp populations, leading to high mortality rates, reduced growth, lower product quality, economic losses, and increased governmental costs.

Given the growing resistance of foodborne pathogens to multiple drugs, there is an urgent need to identify novel, natural antimicrobial agents. Medicinal plants, used in traditional medicine for thousands of years (Bulfon et al., 2015), have demonstrated diverse bioactivities in aquaculture, including growth promotion, immune stimulation, and antibacterial, antiviral, antifungal, antiparasitic, and appetite-enhancing effects (Syahidah et al., 2015). These effects are largely attributed to secondary metabolites, whether isolated or in combination (Radulović et al., 2013), which can also serve as leads for new drug discovery (Savoia, 2012).

One such medicinal plant is amla (Phyllanthus emblica L.), widely distributed in tropical and subtropical regions (Gantait et al., 2021) and renowned in Ayurvedic texts such as Charaka Samhita and Sushruta Samhita for its therapeutic properties (Kumar et al., 2018; Doan et al., 2022). P. emblica is rich in bioactive compounds, including vitamin C, iron (III), and phenolic compounds, which contribute to its diverse biological activities (Jantan et al., 2019; Irsyam et al., 2020). While numerous studies have reported

its antibacterial potential, its role in improving growth performance and combating pathogens in aquaculture remains underexplored.

This study investigates the antibacterial activity of crude aqueous extracts of P. emblica against multidrug-resistant, biofilm-forming shrimp pathogens. Additionally, molecular docking analyses were conducted to elucidate the potential mechanisms underlying its antimicrobial action.

2. MATERIALS AND METHODS

2.1 Collection of shrimp samples and isolation of bacterial pathogens

Black tiger shrimp (Penaeus monodon) were collected from a shrimp farm in Madipakkam, Chennai, Tamil Nadu, India. To prevent contamination, samples were transported in sterile bags placed inside insulated boxes containing ice packs, maintaining a temperature of 4–6 °C until arrival at the laboratory. Approximately 100 g of shrimp tissue was aseptically homogenized. For preparation of a homogeneous suspension, 10 g of homogenate was mixed with 90 mL of sterile normal saline (0.9% NaCl) in a conical flask. The mixture was vortexed thoroughly and serially diluted prior to microbiological analysis.

Diluted samples were inoculated onto various selective media, including MacConkey agar, eosin methylene blue (EMB) agar, mannitol salt agar (MSA), Salmonella–Shigella agar, cetrimide agar, thiosulfate citrate bile salts sucrose (TCBS) agar, and chromogenic agar. Plates were incubated at 28 °C for 24 h, after which colony morphology and physiological characteristics were recorded. Confirmed isolates were transferred to nutrient agar slants and incubated, followed by storage at 4 °C for further studies.

2.2 Antibiotic Resistance Profiling of Bacterial Pathogens

Antibiotic susceptibility testing was carried out using the Kirby–Bauer disc diffusion method (Bauer et al., 1966). Zones of inhibition were measured and interpreted according to the manufacturer's standard antibiotic chart.

2.3 Screening for Biofilm-Producing Bacterial Pathogens

Biofilm production was detected using Congo red agar (CRA) as described by Freeman et al. (1989). The CRA medium was prepared by adding brain–heart infusion (BHI) powder (37 g), sucrose (50 g), agar (10 g), and Congo red (0.8 g/L) to 1 L of distilled water, followed by autoclaving at 121 °C for 15 min. The sterilized medium was poured into Petri plates and allowed to solidify. Test isolates were streaked onto the plates and incubated at 37 °C for 24 h. Colonies appearing black were considered biofilm producers, whereas pink to red-orange colonies were classified as non-biofilm producers.

2.4 Plant Material Collection and Extraction

Fruits of Phyllanthus emblica were collected from Namakkal district, Tamil Nadu, shade-dried, and powdered. The powdered material was subjected to Soxhlet extraction using ethanol and chloroform solvents. Extracts were dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C for phytochemical and antimicrobial analysis.

2.5 Phytochemical Screening

Qualitative phytochemical analysis of the P. emblica fruit extract was carried out following the methods described by Solomon et al. (2013) to detect the presence of major secondary metabolites.

2.6 Determination of Antibacterial Activity

Antibacterial activity was assessed according to Jahir and Saurabh (2011). Mueller–Hinton agar (MHA) plates were inoculated with bacterial suspensions (10^8 CFU/mL) using a sterile cotton swab, rotating the plate 60° between streaks for even distribution. Wells (6 mm diameter) were made using a sterile cork borer, and varying concentrations of the ethanolic extract were added. Ampicillin (10 µg/mL) served as the positive control, while 100 µL of DMSO served as the negative control. Plates were incubated at 37 °C for 24 h, and the diameter of the inhibition zones was measured in millimetres.

2.7 GC–MS Analysis

Secondary metabolites were identified by gas chromatography–mass spectrometry (GC–MS) following the method of Deepakumari and Chitra (2022). The relative abundance of each compound was calculated based on its average peak area relative to the total chromatographic area, and spectra were analysed using the TurboMass software.

2.8 Molecular Docking Analysis

2.8.1 Protein Model Preparation

The three-dimensional structure of bacterial cellulose synthase BcsB in complex with the polyalanine BcsA model from Escherichia coli was retrieved from the RCSB Protein Data Bank (PDB). The protein structure was energy-minimized using SPDB Viewer and refined using the GalaxyRefine server (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE).

2.8.2 Ligand Preparation

Compounds identified from GC–MS analysis—acetanilide, isoquinoline, and β-sitosterol—were selected for in silico antibiofilm screening. Ligands were energy-minimized using the Galaxy server and saved in PDB format via the Open Babel web server (http://www.cheminfo.org/Chemistry/Cheminformatics/FormatConverter/index.html).

2.8.3 Docking Procedure

Ligand structures were retrieved from the PubChem database. Docking was performed using AutoDock Vina. The protein structure was prepared by removing water molecules, adding polar hydrogens, and assigning charges before saving as a PDBQT file. Ligands were also converted to PDBQT format. The docking grid box was defined using the MGLTools suite, and docking was executed through command-line prompts. Output files were processed with VinaSplit, and protein–ligand interactions were visualised using BIOVIA Discovery Studio. Amino acid interactions and binding energies were recorded.

2.8.4 Molecular Visualization

The best docking poses were analysed for hydrogen bonds, hydrophobic interactions, and π–π stacking using BIOVIA Discovery Studio. Interacting amino acid residues for each ligand were documented (Table 3).

3. RESULT AND DISCUSSION

A total of 26 bacterial isolates belonging to 10 genera were recovered from five shrimp samples. Vibrio spp. and Aeromonas spp. were the most prevalent, whereas Staphylococcus aureus and Pseudomonas spp. had the lowest occurrence. Species-level identification and percentage of maximum identity are presented in Figure 1. A similar diversity of bacterial species in Penaeus monodon was reported by Kusumaningrum and Zainuri (2015). Among these, Vibrio spp. are regarded as primary causative agents of shrimp diseases, leading to severe production losses. Vibriosis remains a critical problem in shrimp farms, with Vibrio parahaemolyticus concentrations as low as 1.0×10^6 CFU/mL capable of causing 90% mortality in abalone post-larvae (Cai et al., 2006) and LD_{50} values of less than 1.0×10^3 CFU/mL (Haldar et al., 2007; Uma et al., 2008).

The detection of coliforms indicates inadequate sanitation measures and poor handling practices, as highlighted by Nilla et al. (2012), who linked contamination to water sources and unhygienic processing conditions. Poor-quality water and improper storage can further increase microbial loads (Hossain et al., 2012). In an attempt to prevent crop losses, shrimp farmers frequently apply hormones, antibiotics, disinfectants, and other chemicals to feed and culture water. However, prolonged and indiscriminate use of these agents has contributed to the emergence of resistant bacterial strains (Watts et al., 2017).

In the present study, the highest antibiotic resistance was recorded in E. coli (67%), followed by Vibrio spp. (57.5%), while S. aureus showed the lowest resistance (40%) (Fig. 2). Similar trends were reported by Hossain et al. (2012) and Talukder et al. (2021) in P. monodon. Resistance to ampicillin was most prevalent (88.4%), followed by tetracycline (61.5%). Of the 26 isolates tested, 10 were resistant to more than five antibiotics, predominantly β-lactam antibiotics (Fig. 3). These findings align with Tricia et al. (2006) and Hossain et al. (2012), both of whom reported high ampicillin resistance in E. coli from shrimp, a trend attributed to its frequent use in aquaculture (Lim Mui Hua and Kasing Apun, 2013).

Multidrug-resistant (MDR) isolates are particularly problematic when they are also biofilm producers. Biofilm formation involves bacterial encasement within an extracellular matrix composed of proteins, polysaccharides, nucleic acids, and lipids, which provides protection against host immune responses and antimicrobial agents. This structural defense contributes to persistent infections and complicates treatment (Verderosa et al., 2019; Bassetti et al., 2018). In this study, 80.7% of isolates were biofilm producers, with E. coli and Aeromonas spp. as the most common.

Given these challenges, attention has turned to medicinal plants as eco-friendly alternatives for pathogen control in aquaculture. Herbs can enhance disease resistance, suppress pathogenic growth, and improve overall fish and shrimp health (Lim Mui Hua and Kasing Apun, 2013).

International Journal of Environmental Sciences
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Table 1 Antibacterial activity of Phyllanthus emblica against shrimp isolates

S. No	Isolates	Different con. of plant extract (mg) Zone of inhibition in mm					Ampicillin
		2.5	5	7.5	10	DMSO	
1.	E.coli	-	10	13	15	-	-
2.	Proteus spp	-	-	10	13	-	-
3.	K.pneumoniae	-	-	-	10	-	-
4.	E.feacalis	10	11	13	16	-	-
5.	Salmonella sp	-	-	-	-	-	-
6.	S.aureus	-	-	-	10	-	-
7.	Vibrio sp	11	13	16	19	-	-
8.	Shigella sp				12	-	-
9.	Aeromonas sp	11	13	16	19	-	-
10.	Pseudomonas spp	13	14	16	20	-	-

Table 2. Chemical structure of Ligands

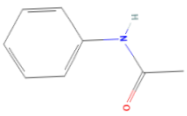
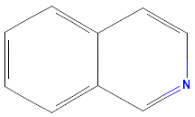
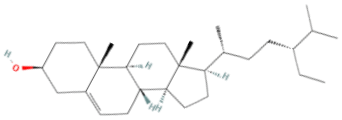
S.No.	Compound Names	Chemical Structure
1.	Acetanilide	
2.	Isoquinoline	
3.	Sitosterol	

Table 3. Minimum binding energies

S.No.	Ligands	Minimum binding energy	Amino acids interaction
1.	Acetanilide	- 5.7	GLN A: 462, MET A: 465, PRO A:475, LYS A:461, HIS A:443
2.	Isoquinoline	- 6.0	MET A:465, ILE A:388, ASP A:442, ALA A:464, HIS A:443
3.	Sitosterol	- 8.4	ARG B:533, ILE B:527, LEU B:404, PHE B:526, PRO B:528, LEU B:730, ARG B:735

Fig.1 isolation of bacterial isolates on shrimp samples

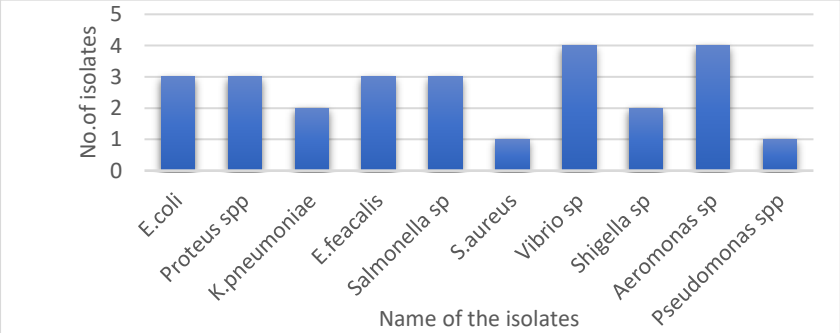


Fig.2 Isolation of antibiotic resistance bacterial isolates on shrimp samples

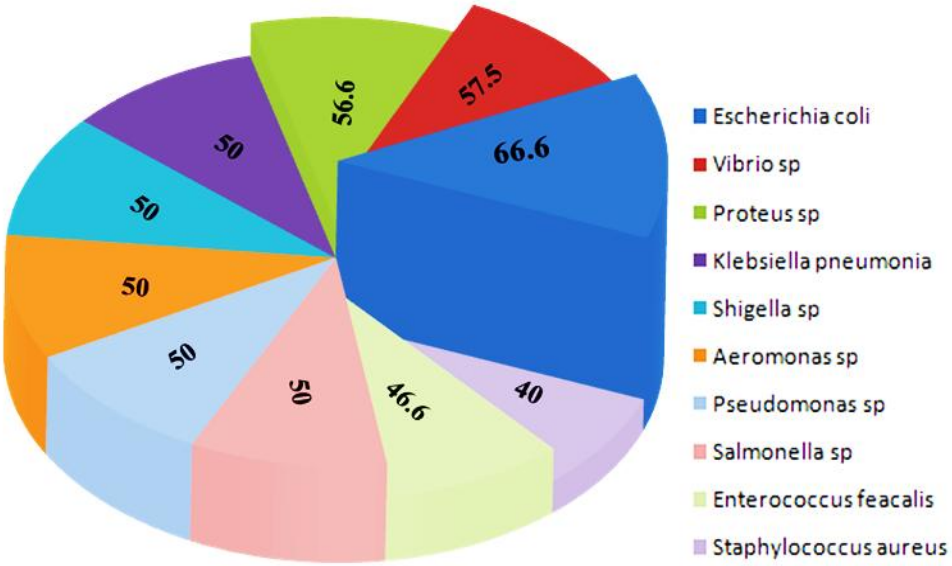


Fig.3 Percentage of antibiotic resistance on shrimp isolates

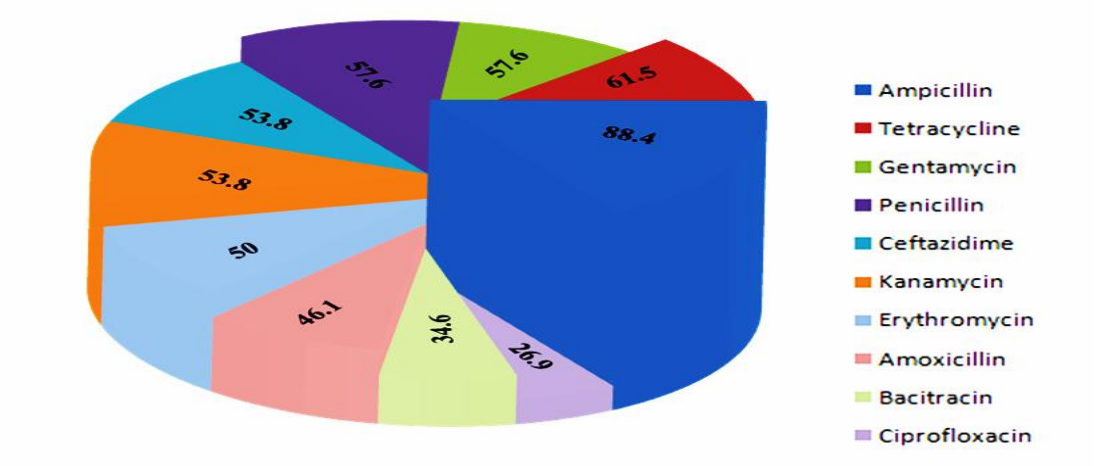
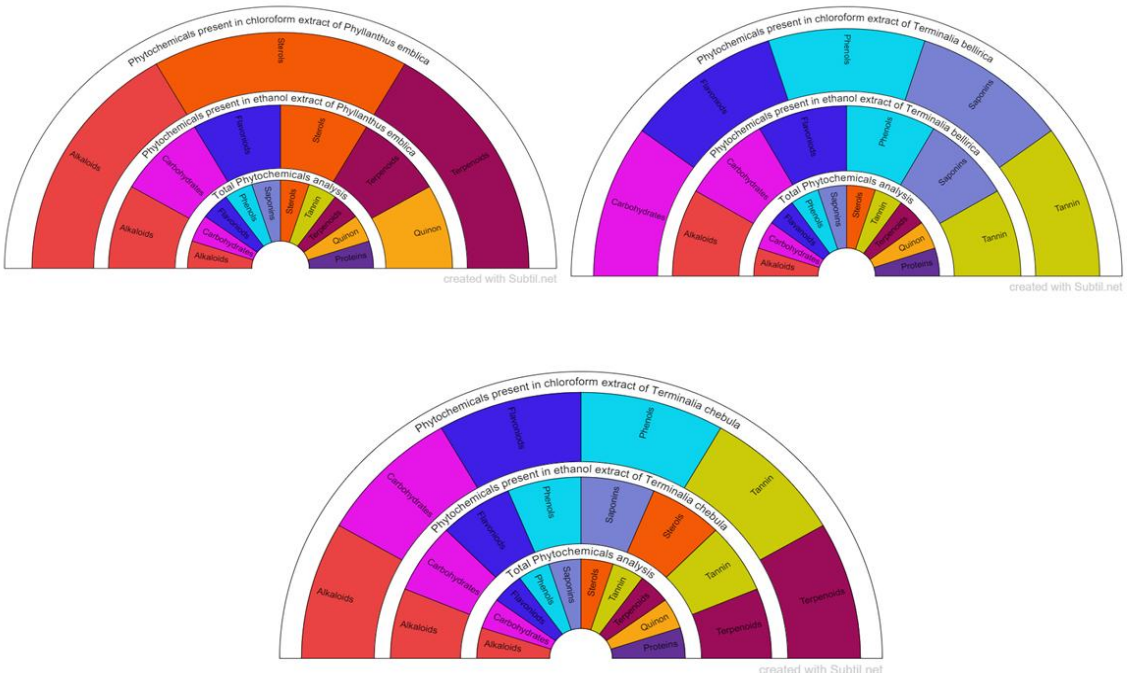


Fig.4 Assessment of preliminary phytochemicals on various plant extracts



International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 22s, 2025
https://theaspd.com/index.php

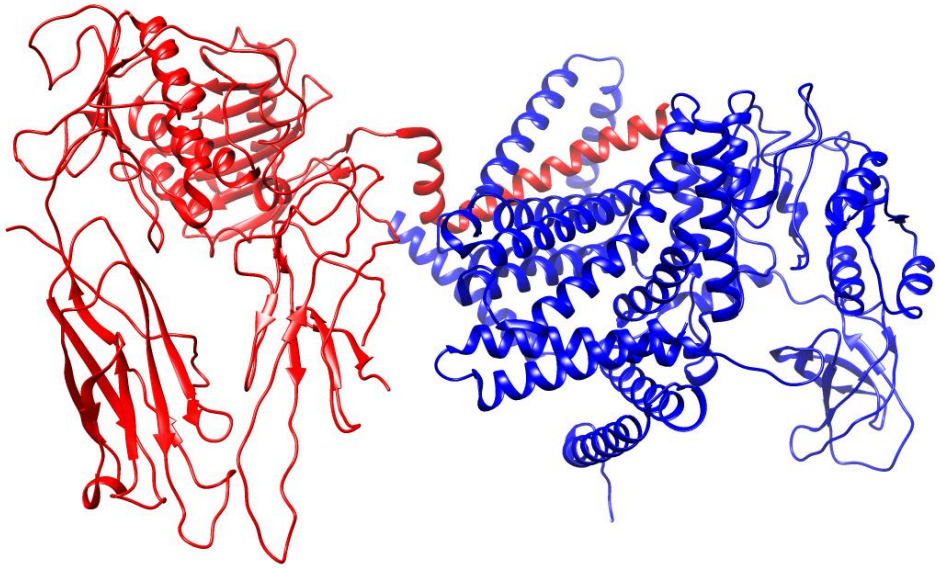


Figure No.1: Three Dimensional Structure of Bacterial cellulose synthase BcsB with polyalanine BcsA model from Escherichia coli. The model showed two chains. Chain A (Blue) and Chain B (Red). The model was visualized using UCSF Chimera software.

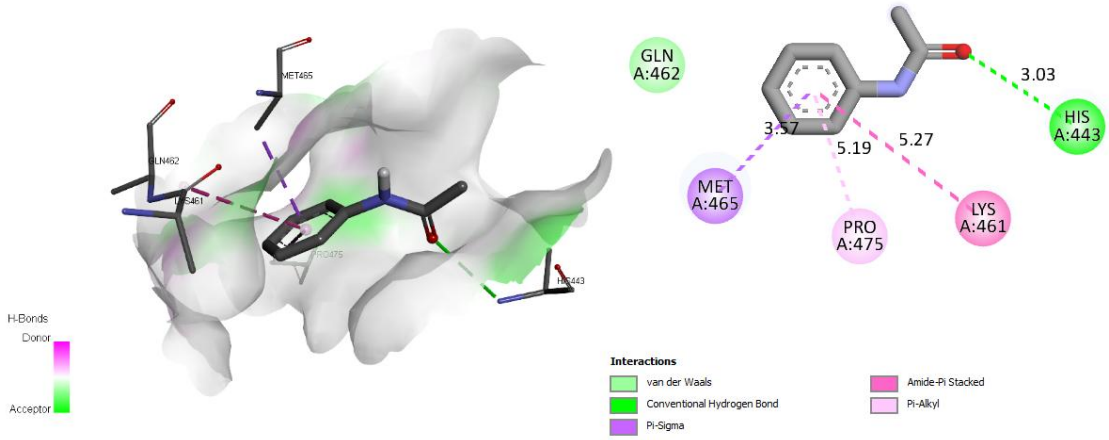


Figure No.2: Bacterial cellulose synthase BcsB with polyalanine BcsA model from Escherichia coli in Complex with Acetanilide.

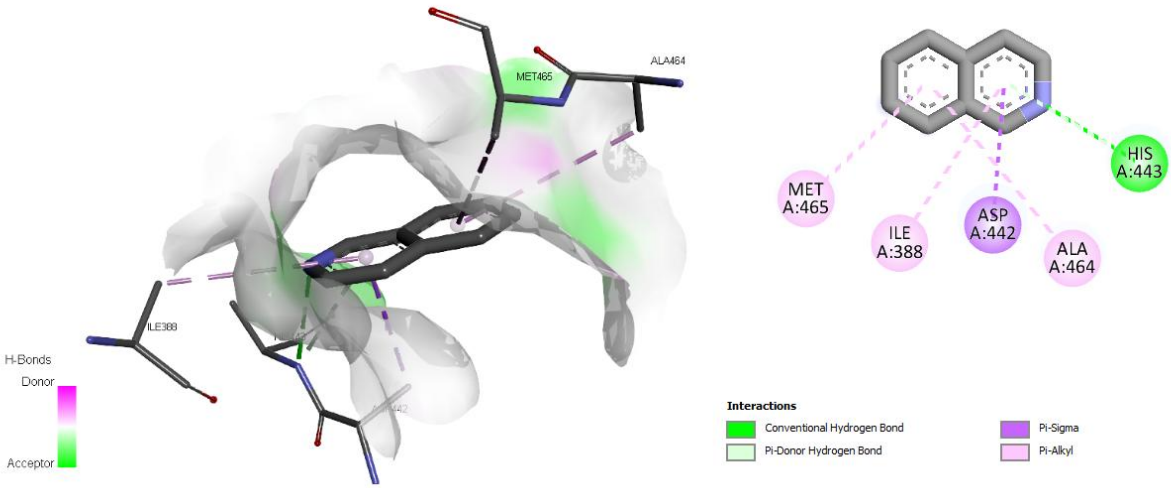


Figure No.3: Bacterial cellulose synthase BcsB with polyalanine BcsA model from Escherichia coli in Complex with Isoquinoline.

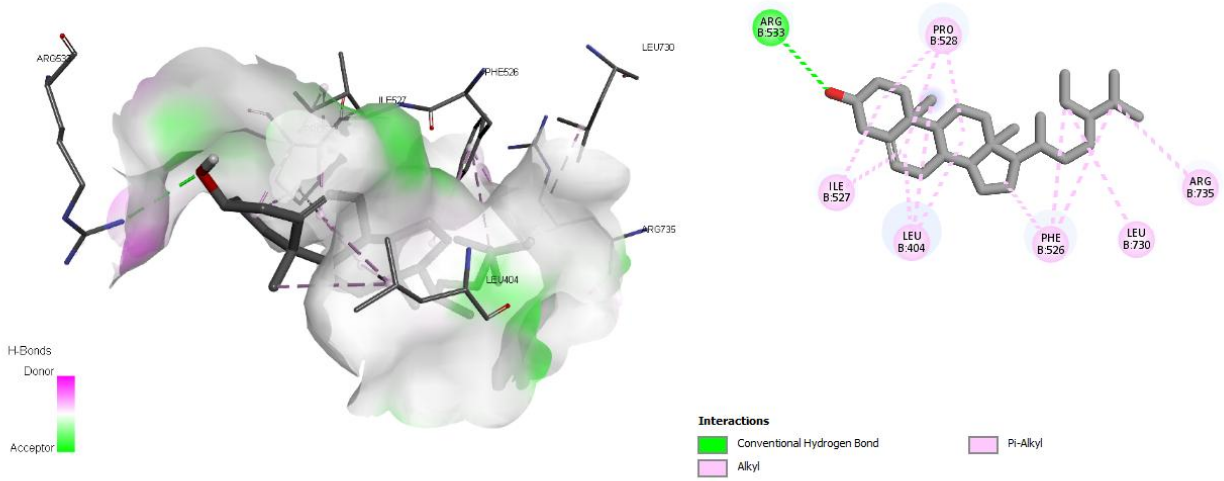


Figure No.4: Bacterial cellulose synthase BcsB with polyalanine BcsA model from Escherichia coli in Complex with Sitosterol.

Phytochemical screening of Phyllanthus emblica fruit extracts revealed the presence of alkaloids, terpenoids, and sterols in both ethanol and chloroform extracts. Additionally, the ethanol extract contained carbohydrates, flavonoids, sterols, and quinones (Table 1). Similar findings have been reported by Mohamed et al. (2022), who confirmed the presence of flavonoids and terpenoids in P. emblica fruit extracts. These bioactive constituents are known for their therapeutic potential against various diseases.

The antibacterial activity assay demonstrated that P. emblica ethanol extract inhibited biofilm-producing shrimp pathogens. Among the ten bacterial genera tested, E. coli and P. aeruginosa exhibited the greatest susceptibility, with inhibition zones of 16–26 mm, while Salmonella spp. showed the lowest sensitivity. The antibacterial effect was dose-dependent, with all genera inhibited at 7.5 mg extract concentration and five genera inhibited at 2.5 mg. The inhibitory activity was comparable for both Gram-positive and Gram-negative bacteria (Table 1).

Previous studies have also demonstrated the efficacy of P. emblica against shrimp pathogens. Hannan et al. (2019) reported inhibition of Vibrio alginolyticus, while Gandhi et al. (2020) observed strong antibacterial and antifungal activities. Notably, this is the first study to report the inhibitory effect of P. emblica against biofilm-forming bacteria in shrimp.

Gas chromatography–mass spectrometry (GC–MS) analysis of the ethanol extract identified several antimicrobial compounds, including acetanilide, β-sitosterol, and isoquinoline, along with fatty acids such as octadecanoic acid and hexadecenoic acid. β-Sitosterol has been previously reported in Phyllanthus spp. (Sparzak et al., 2009) and possesses antimicrobial and antioxidant properties (Alawode et al., 2021). Isoquinoline alkaloids are known for their antimicrobial activity against bacteria, fungi, protozoa, and viruses (Kim et al., 2002).

Molecular docking studies were conducted to explore the interaction between these bioactive compounds and E. coli cellulose synthase BcsA, a protein critical for biofilm formation and encoded by the bcsABZC operon. Biofilms, composed of extracellular polysaccharides such as cellulose and alginate, confer enhanced antimicrobial resistance (Omadjela et al., 2013). In this study, β-sitosterol demonstrated strong antibiofilm potential, with a binding energy of –8.4 kcal/mol, indicating a stable interaction with the target protein.

In conclusion, Vibriosis remains a major threat to shrimp aquaculture, exacerbated by the overuse of antibiotics and chemicals, which fosters multidrug resistance. The present study highlights the potential of P. emblica extract as a natural, sustainable alternative for controlling shrimp pathogens. The extract's broad-spectrum antibacterial activity, combined with its potent antibiofilm effects—particularly from β-sitosterol—positions it as a promising candidate for developing plant-based therapeutics in aquaculture disease management.

Declaration
No funds, grants, or other support was received

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