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Exploring The Antimicrobial Potential of *Phyllanthus Emblica L.* (Amla) Using Molecular Docking Studies Against Shrimp Pathogens

Mariam Ali K[1], Prakash Balu[1]*

[1]*Department of Biotechnology, School of Life Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai, Tamil Nadu, India*

***Corresponding Author:** Prakash Balu*
Email: prakazbt@gmail.com

Abstract

Vibriosis is a major problem in shrimp farms. Farmers indiscriminately use hormones, antibiotics, disinfectants and other chemicals in fish feed and culture water to protect their crops. *E. coli* and *Aeromonas sp* were highly predominant isolates. Herbs act as agents in aquaculture to control or reduce pathogen infections. The results of phytochemicals screening of fruit extract of *Phyllanthus emblica* showed the presence of various phytchemicals. *Phyllanthus emblica* extract shows anti-bacterial properties against biofilm producing shrimp pathogens. Among the 10 bacterial genera, E. coli and *P.aeruginosa* were highly suppressed. The extracts exhibited the bacteria growth inhibitory activity in a dose-dependent manner. The compound sitosterol has potent antibiofilm activity was showed by molecular docking, which revealed a significant binding energy and interaction (-8.4 Kcal/mol) between it and key biofilm-forming protein. Diving analysis was done using the chemical compounds found in GCMS analysis.

Keywords: vibriosis, shrimp, phyllanthus emblica, phytochemical analysis, docking

1. Introduction

Shrimp farming is one of the most important aquaculture sectors in many tropical countries, and production is expanding significantly. However, such industrial expansion leads to a variety of environmental impacts that are harmful to shrimp and their aquatic environment. Due to the expansion of aquaculture activities, the prevalence of diseases in the industry has increased (Shariff *et al*., 2001). This situation has also been affected by pathogenic microbes, especially following bacterial isolates that affected shrimp, namely *Aeromonas* spp., *Pseudomonas* spp., *Vibrio spp., Aeromonas spp., E. coli, Salmonella spp.*, *Kliebsiella spp., Staphylococcus aureus*, and *Shigella spp. (Rahman et al., 2012; Abraham et al., 2013; Pooja and Singh, 2022).*
Chemicals and antimicrobials have historically been used to control pathogen problems in shrimp hatcheries. For example, chlorine, which is often used in ponds and hatcheries, has the potential to promote the establishment of several antibiotic resistance genes in bacteria (Balcazar et al., 2006). The development of antibiotic resistance in pathogenic bacteria has become a major public health concern and has sparked a heated debate on the prudent use of antibiotics, especially in the veterinary, nutritional, and agricultural fields.
Antibiotic resistance has resulted from improper and overuse of antibiotics in shrimp, and antibiotic-resistant bacteria are spread to humans by shrimp (Zanetti et al., 2001). Such pathogens are present in shrimp, making them unfit for human consumption and dangerous. Due to the pathogen, it has a significant pathological impact

on the infected population. Also, it leads to big economic losses, more deaths, slower growth rates, lower product quality, and higher costs for running the government. In this situation, it is very important to find a new natural antibiotic as soon as possible because pathogens in food are becoming more resistant to many drugs. Medicinal plants are known worldwide and have been used as traditional medicines for thousands of years (Bulfon et al., 2015). So, medicinal plants have been tested on fish and shellfish and have been shown to be effective as growth promoters, immune stimulants, antibacterial, antiviral, antifungal, antiparasitic, and appetite stimulants (Syahidah et al., 2015). Secondary metabolites from medicinal plants helped with these biological processes, whether they were pure or mixed (Radulovi et al., 2013). They may also be a source for new drugs to treat diseases (Savoia, 2012). In that way, the medicinal use of amla in fish farming is high. Ayurvedic texts like Charaksanhita and Sushrutsanhita (Kumar et al., 2018; Doan et al., 2022) talk about how amla can be used as a medicine. Amla (Phyllanthus emblica), found in tropical and sub-tropical countries (Gantait et al., 2021), is a beneficial plant that has been proven to show many biological activities (Jantan et al., 2019). Irsyam et al., 2020, found that P. emblica is good for your health because it has bioactive compounds like vitamin C, iron (III), and phenolic compounds. There have been a lot of studies showing that Phyllanthus emblica kills bacteria. However, in aquaculture, the positive effects of Phyllanthus emblica on growth performance and antimicrobial activity have not been studied as much. This study looks into how well the crude aqueous extract of Phyllanthus emblica L. (amla) kills bacteria that are resistant to multiple drugs and make biofilms. In addition, the anti-microbial activity of the extract was showed by molecular docking studies.

2. Materials and Methods

2.1 Collection of shrimp samples and isolation of bacterial pathogens

The black tiger shrimp were collected from the shrimp farm in Madipakam, Chennai, Tamil Nadu, India.To avoid further contamination, samples are shipped in special sterile bags in insulated boxes filled with ice to keep the temperature around 4 to 6 °C during transport from the source to the laboratory. Samples of shrimp weighing 100 g were blended aseptically. To make a homogeneous suspension, 10 g of sample (the blending part) and 90 ml (about 3.04 oz) of sterile normal saline (0.9% w/v NaCl) solution were placed in a conical flask. Samples were thoroughly mixed and serially diluted using a vortex mixture before being examined for microbiological contamination.

The serially diluted samples were inoculated into various selective media, including MacConkey agar, EMB, MSA, Salmonella shigella agar, Cetrimide agar medium, TCBS agar, and chromogenic agar media. The plates were incubated at 28 °C overnight. After the incubation period, we observed the morphological and physiological characterizations of each isolate on culture media. Then confirmed isolates were inoculated into nutrient agar slants, and after the incubation period, the culture was stored at 4 oC for further study.

2.2 Isolation of antibiotic resistance in bacterial pathogens

Bacterial resistance to antimicrobial agents was performed by the disc diffusion method using guidelines established by Bauer et al. [9] (1966). The zone of inhibition was correlated with the manufacturer's standard antibiotic chart.

2.3 Isolation of biofilm-producing bacterial pathogens

The BHI powder (37 g), sucrose (50 g), agar (10 g), and Congo red (0.8 g/L) were added to 1 litre of distilled water to prepare the agar medium. The mixture was then autoclaved for 15 minutes at 121 °C. Then the media was poured into the petri dishes and allowed to solidify. Once the media had solidified, the plates were inoculated with the microorganisms and incubated at 37°C for 24 h. The plates were seen the next day, and the organisms were considered positive (biofilm producers) when they produced black colonies on the agar and negative (non-biofilm producers) when they produced pink or red-orange colonies on the Congo red agar (Freeman et al., 1989).

2.4 Plant Collection

The fruit of Phyllanthus emblica was collected from Namakkal district, Tamilnadu, and was shade dried, powdered, and extracted in a Soxhlet apparatus successively with ethanol and chloroform solvents. The extracts were dissolved in DMSO and stored at 4 oC for phytochemical screening and antimicrobial analysis.

2.5 Phytochemical Screening of Bark Extract

The presence of various phytochemical compounds in the fruit of Phyllanthus emblica was confirmed by using the methods of Solomon et al. (2013).

2.6 Determination of the Antibacterial Activity of Plant Extract

This test was carried out according to the method of Jahir and Saurabh (2011). The plates were inoculated with freshly prepared overnight inoculums that were swabbed over the entire surface of each MHA medium, rotating the plate 60 degrees after each application by using a sterile cotton swab, to ensure the complete spread of the tested microbes on the surface of the plate. Inoculums had 108 CFU/mL of bacteria. On the agar plates, a borer was used to make a 6 mm (about 0.24 in)-diameter well. Different concentrations of fruit extract (ethanol) were filled in wells with the help of a micropipette. Ampicillin (10µg/ml) was used as a positive control, and 100 l of DMSO was added as a negative control. The plates were incubated at 37°C for 24 hours for bacterial isolates. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition (in mm).

2.7 GCMS analysis

The secondary metabolite compounds were observed with GC-MS analysis. The relative percentage amount of each part was calculated by comparing its average peak area to the total area. Turbo Mass was used to handle mass spectra and chromatograms (Deepakumari and Chitra, 2022).

2.8 Molecular docking analysis

2.8.1 Protein Model Preparation

The three-dimensional structure of bacterial cellulose synthase BcsB with the polyalanine BcsA model from Escherichia coli was downloaded from the RCSB PDB database. The downloaded protein model was subjected to energy minimization using SPDB Viewer Software. Furthermore, the model structure was refined using the Galaxy web refinement server (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE). The prepared protein model was subjected to a docking study.

2.8.2 Ligand Preparation

The compounds acetanilide, isoquinoline, and sitosterol were selected for in silico anti-biofilm activity by a molecular docking study. The selected ligands were energy-minimized using the Galaxy web refinement server (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE). The energy-minimised ligand was saved in PDB format using the Open Babel web server (http://www.cheminfo.org/Chemistry/Cheminformatics/FormatConverter/index.html). Finally, the structure-refined ligand compound was docked against the drug target Bacterial Cellulose Synthase BcsB using the polyalanine BcsA model from Escherichia coli.

2.8.3 Molecular Docking

In docking studies, the compounds' structures are retrieved from PubChem databases. The chosen ligands were docked to the drug target Bacterial Cellulose Synthase BcsB using the polyalanine BcsA model from Escherichia coli.Vinamycin is loaded with macromolecules in Autodock, and water is removed. Then hydrogen polar bonds are added manually and add charges. Then macromolecule files are saved as PDBQT. Ligand files are fed into the programme and saved as PDBQT files. Grid boxes are formed in the centre grid and automatically calculated by the MGL tool. Then a configuration file was made by using all the details correctly. Command prompts are run using data. After the file was generated, it was split using Vina Split. Then it is analysed using BIOVIA Discovery Studio software used for protein and ligand interactions and publication-quality images (Beg et al., 2020). After protein and ligand docking analysis, interactions of protein-ligand sites (amino acids) and minimum binding energy were interpreted.

2.8.4 Molecular Visualization

The best docked poses of the protein-ligand complex were visualised using BIOVIA Discovery Studio open-source software. The hydrogen bond interaction, hydrophobic interaction, and pi-pi interaction of the protein-ligand complex were further analyzed. The amino acids shown to interact with docked proteins were tabulated in Table No. 3.

3. Result and Discussion

A total of 26 bacterial isolates of 10 genera were observed from 5 shrimp samples. Among the genera, *Vibrio sp.* and *Aeromonas sp.* were predominant, *while S. aureus* and Pseudomonas sp. had the lowest percentage of isolates. The species-level identification of bacterial isolates and percentage of maximum identity were presented in Fig. Kusumaningrum and Zainuri (2015) observed the wide range of bacterial species in Penaeus monodon. Vibrio sp. is mostly blamed for diseases in cultivated shrimp that result in significant output losses. Vibriosis is a major problem on shrimp farms. Cai *et al*. (2006) reported that 1.0 x 106 CFU/ml *V.*

parahaemolyticus concentration is enough to cause 90% mortality of abolone post larvae with a LD50 value less than 1.0 x 103 CFU/ml (Haldar *et al*., 2007; Uma *et al*., 2008).

The presence of coliforms shows inadequate sanitation measures, improper handling practices, etc. Nilla *et al*. (2012) found contamination from the water source and poor sanitation and sanitary conditions on the processing premises. The use of poor-quality water and improper storage conditions may contribute to a high incidence of microorganisms (Hossain *et al*., 2012). Farmers indiscriminately use hormones, antibiotics, disinfectants, and other chemicals in fish feed and culture water to protect their crops from disease and reduce crop losses. The development of resistant bacteria is a result of the continuous use of antibiotics and chemicals (Watts et al. 2017).

Presently, the highest antibiotic resistance was observed against E. coli (67%) and was followed by *Vibrio* sp (57.5%); the lowest resistance was observed against a single isolate of *S. aureus* (40%) (Fig. 2). In 2012, Hossain *et al.* also observed the highest antibiotic resistance isolates of *E.* coli in shrimp samples. Latterly, in Bangladesh, Talukder *et al*. (2021) also observed the highest antibiotic resistance of E. coli in *Penaeus monodon.* In terms of antibiotic resistance, ampicillin was the most resistant to 88.4% of isolates, followed by tetracycline (61.5%). Among the 26 isolates tested, 10 were resistant to more than 5 types of antibiotics; most of the isolates were resistant to the beta-lactamase group of antibiotics (Fig. 3). Tricia *et al.* (2006) observed the highest drug resistance of *E. coli* isolates against ampicillin, and later Hossain *et al.* (2012) also observed the highest drug resistance against ampicillin. The main reason for pathogenic bacteria to become resistant to antibiotics may be the use of ampicillin in shrimp farming (Lim Mui Hua and Kasing Apun, 2013).

Multidrug-resistant isolates are not easily eradicated because they may be robust to biofilm formation. During the development of biofilm, bacteria are encased in an extracellular matrix made up of proteins, polysaccharides, nucleic acids, and lipids. This matrix offers defence against a range of stresses, including immune cell attack and antimicrobial exposure. The alarming prevalence of strains that create biofilms poses a substantial challenge for clinicians who treat and care for hospitalised patients (Verderosa et al., 2019; Bassetti et al., 2018). In the present study, 80.7% of isolates were biofilm producers, among which *E. coli* and *Aeromonas sp.* were highly predominant isolates.

To address this problem, scientists and researchers have begun to focus on medicinal plants as viable remedies for reducing illness recurrence in aquaculture. Herbs act as agents in aquaculture to control or reduce pathogen infections and prevent economic losses caused by pathogens by reducing the survival of organisms during disease management (Lim Mui Hua and Kasing Apun, 2013). Most herbs work as anti-pathogens and antibiotics because they boost the immune system, prevent disease, or help aquaculture grow fish that are resistant to disease.

The results of phytochemical screening of a fruit extract of *Phyllanthus emblica* showed the presence of various phytochemicals (Table 1). Two solvents, such as ethanol and chloroform, showed positive results for the presence of alkaloids, terpenoids, and sterols. In addition, the ethanol extracts showed the presence of carbohydrates, flavonoids, sterols, and quinones. Similarly, Mohamed *et al.* (2022) observed the flavonoids and terpenoids in a solvent extract of a fruit extract of *Phyllanthus emblica*. These constituents play a vital role in the treatment of different diseases.

There is a lot of evidence of various plants being used for aquaculture in this way. This study was designed to test whether *Phyllanthus emblica* extract has anti-bacterial properties against biofilm-producing shrimp pathogens. Among the ten bacterial genera, E. coli and P. aeruginosa were highly inhibited, with inhibition zones ranging from 16 mm to 26 mm, while Salmonella spp. had the lowest inhibitory activity. The extracts exhibited bacterial growth inhibitory activity in a dose-dependent manner. All genera were inhibited with a 7.5 mg concentration of plant extract, and a minimum concentration of 2.5 mg inhibited five genera. In this study, the effects of plant extracts on gramme-negative and positive bacteria were indistinguishable (Table 1).

Amla extract is used by Hannan et al., 2019 to control the shrimp pathogen Vibrio alginolyticus.In 2021, authors in India showed that amla extract was active against shrimp pathogens. Gandhi et al. (2020) revealed that *Phyllanthus emblica* shows good antimicrobial activity against bacteria and fungi isolates. Although several studies have demonstrated the antimicrobial activity of amla extract, this is the first to demonstrate the killing of biofilm-forming bacteria that infect shrimp.

The present study was undertaken to find out the bioactive compounds present in the ethanolic extract of *Phyllanthus emblica* by using gas chromatography and mass spectrometry. Numerous antimicrobial compounds from phytochemicals were observed. Acetanilide, sitosterol, and isoquinoline were all detected. Additionally, the fatty acid compounds octadecanoic acid and hexadecenoic acid are also found in the extract. Previously, the sterol compound Sitosterol was observed in *Phyllanthus spp. through the* GCMS method (Sparzak *et al*., 2009), and a recent study by Alawode et al., 2021, determined the antimicrobial and antioxidant

activity of Sitosterol. The alkaloid compound isoquinoline showed antimicrobial potential against bacteria, fungi, protozoa, and viruses (Kim et al., 2002).

Nowadays, there are a wide variety of biomechanical modelling approaches that address a wide range of problems in structural biology, such as drug design. Utilizing computer programming, bioinformatics tools like molecular docking were used to investigate the interactions between molecules (such as proteins and peptides) and forecast their binding patterns and interactions at the molecular or atomic level. They have been extensively used as theoretical simulation techniques in drug discovery research and for virtual screening investigations intended to discover novel active biomolecules, such as bioactive peptides.

In this study, docking analysis was done using the chemical compounds found in GCMS analysis. Compounds such as acetanilide, sitosterol, and isoquinoline were combined with *E. coli* having the cellulose synthase protein BcsA, which was encoded by the bcsABZC operon in *E. coli*. Bacterial extracellular polysaccharides, such as cellulose and alginate, are a vital component of biofilms, which are multicellular, usually sessile, aggregates of bacteria. Biofilms exhibit a greater resistance to antimicrobial treatments compared with isolated bacteria and thus are a particular concern for human health (Omadjela *et al*., 2013). So, in this study, the molecular docking method was used to look at how virulence bacteria break the cellulose protein that makes biofilms.

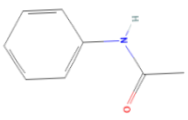
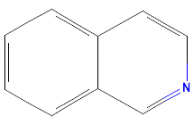
The compound sitosterol has potent antibiofilm activity, as proved by molecular docking, which revealed a significant binding energy and interaction (-8.4 Kcal/mol) between it and a key biofilm-forming protein. Based on these results, it seems like sitosterol could be used as an antibiofilm agent to stop E. coli infections.

"In conclusion, Vibriosis continues to be a significant challenge for shrimp farmers." The widespread use of chemicals to control infections has led to the emergence of drug-resistant bacteria. However, the results of the study suggest that bioremediation using Phyllanthus emblica extract is a promising alternative for controlling shrimp pathogens. The phytochemicals present in the extract show strong antibacterial and antibiofilm properties against key shrimp pathogens, such as E. coli and P. aeruginosa. The finding that sitosterol has potent antibiofilm activity highlights the potential of natural compounds for controlling bacterial infections in aquaculture. These findings lay the foundation for further research on the development of sustainable, plant-based solutions for the management of Vibriosis in shrimp farming.

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	

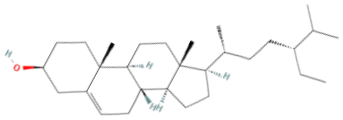
3.	Sitosterol	

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

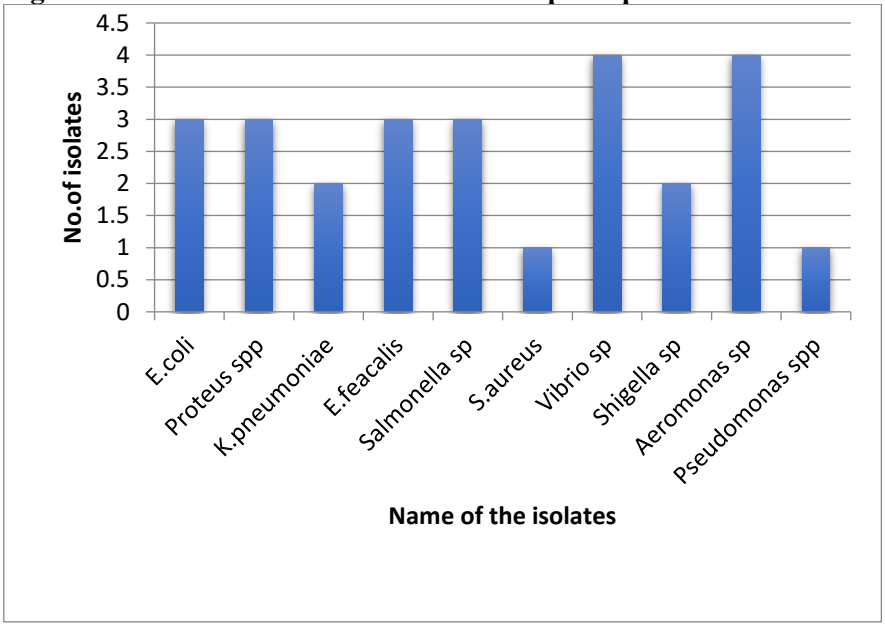


Fig.2 Isolation of antibiotic resistance bacterial isolates on shrimp samples

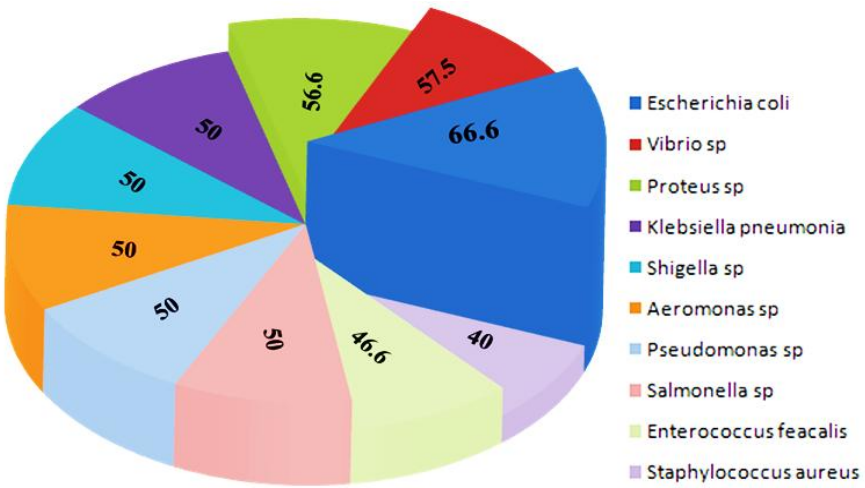


Fig.3 Percentage of antibiotic resistance on shrimp isolates

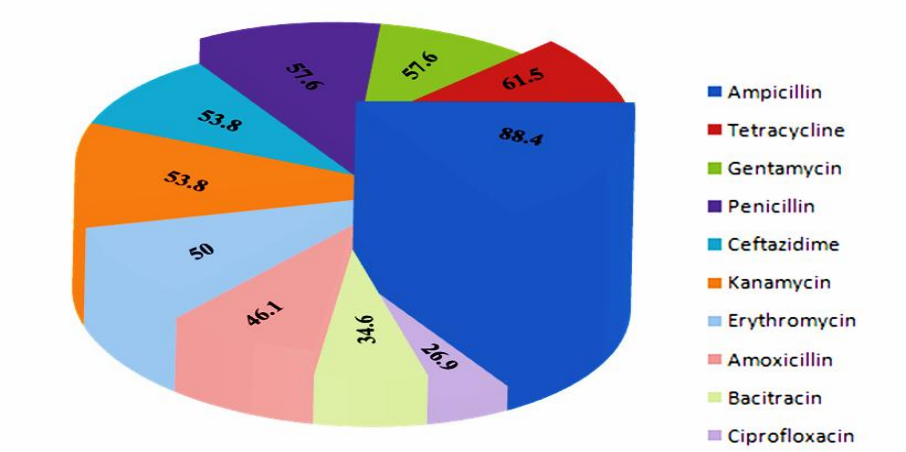
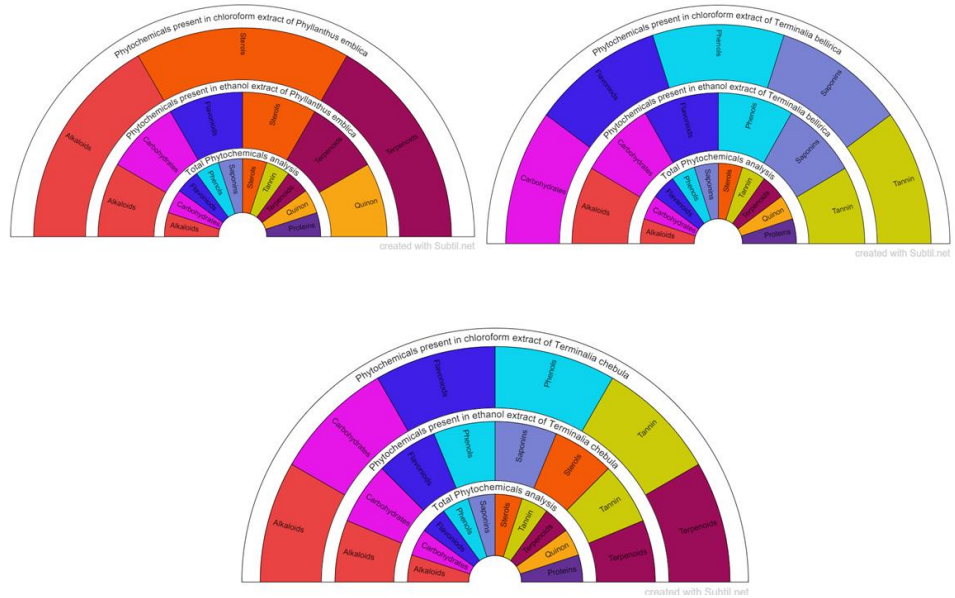


Fig.4 Assessment of preliminary phytochemicals on various plant extracts



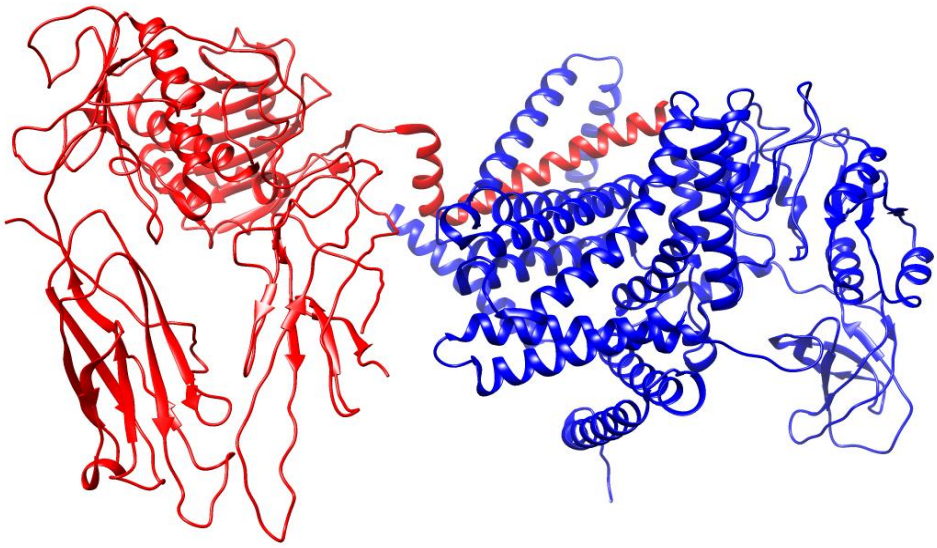


Figure No.1: Three Dimensional Structure of Bacterial cellulose synthase BcsB with polyalanine BcsA model from *Escherichia coli.* The model showed two chains. Chain A (Blue) and Chain B (Red). The model was visualized using UCSF Chimera software.

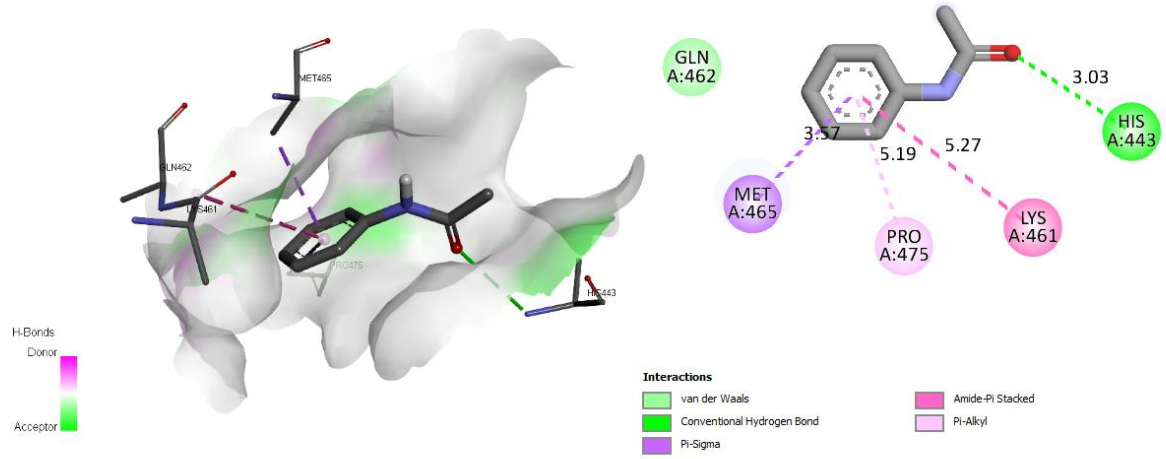


Figure No.2: Bacterial cellulose synthase BcsB with polyalanine BcsA model from *Escherichia coli* in Complex with Acetanilide.

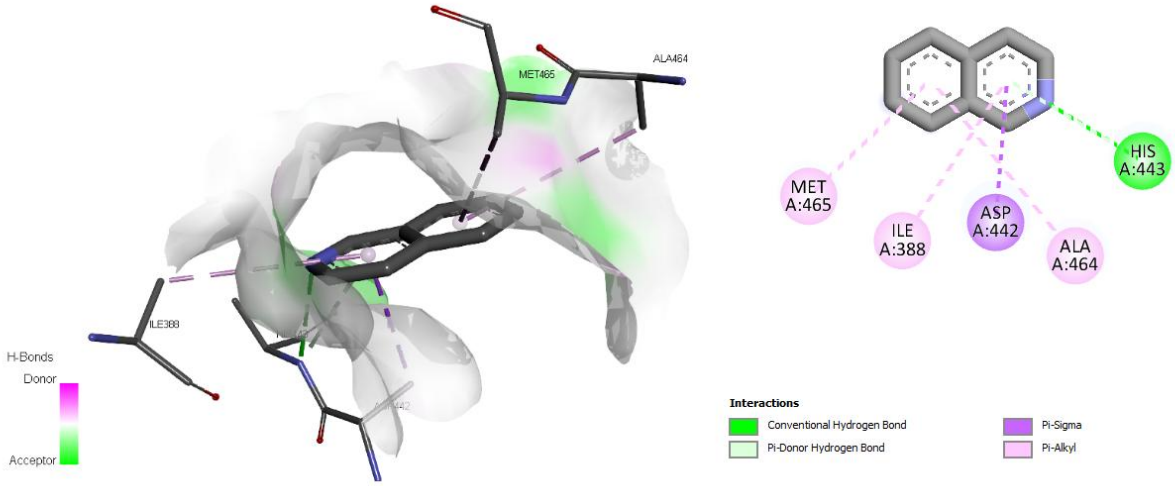


Figure No.3: Bacterial cellulose synthase BcsB with polyalanine BcsA model from *Escherichia coli* in Complex with Isoquinoline.

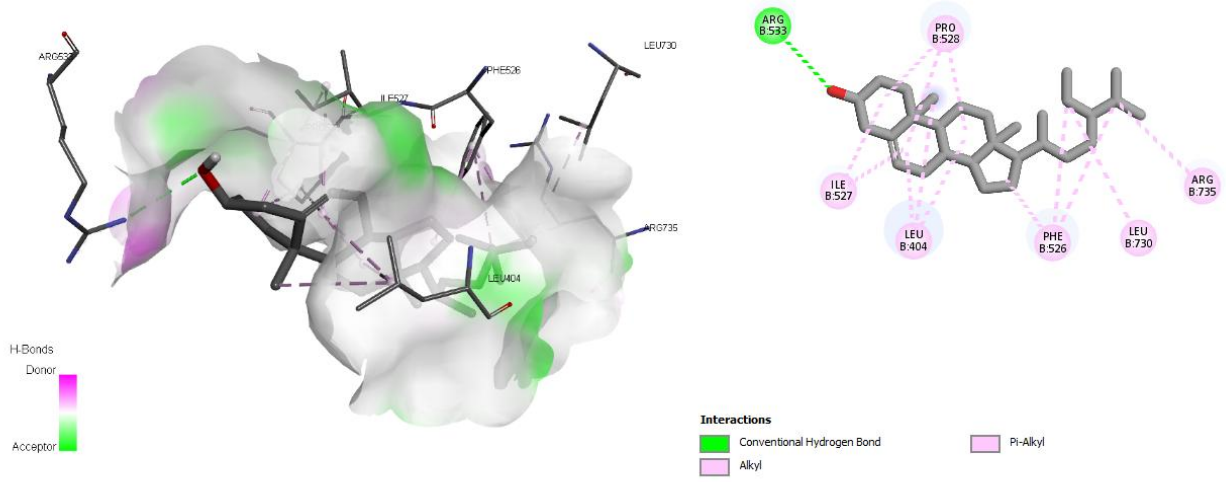


Figure No.4: Bacterial cellulose synthase BcsB with polyalanine BcsA model from *Escherichia coli* in Complex with Sitosterol.

Declaration
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