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Research Article

Antibiofilm activities of the floral extract of *Clitoria ternatea* against the biofilm of two nosocomial pathogens, namely *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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ABSTRACT

Biofilm, being an aggregate of microbial cells play a pivotal role in the development of chronic diseases by making the conventional antibiotics ineffective in killing the pathogenetic microbes. Such incomplete bactericidal activities of antibiotics on the other hand, gives rise to antibiotic resistance. In order to find out some alternative therapeutic approaches the flowers of *Clitoria ternatea*, an ethnobotanical plant with several medicinal attributes were tested for its antibiofilm activities both by *in vitro* as well as by *in silico* analyses in detail. Floral extract of *Clitoria ternatea* containing various bioactive compounds were found to 85% and 82% biofilm produced by *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively, whereas it was only 25% after antibiotic treatment. FTIR analysis indicated significant alterations in the active residues in the spectral regions of lipids, polysaccharide, nucleic acids and proteins after phytoextract treatment. Remarkable eradication of biofilm bound sessile cells of both *P. aeruginosa* and *S. aureus* was brought about by the treatment of petal extracts, as evident from the scanning electron micrographs. Docking interactions between biofilm forming proteins of *S. aureus* and *P. aeruginosa* and the bioactive compounds of *C. ternatea* clearly indicate the antibiofilm efficacies of the bioactive compounds present in the floral extract of *C. ternatea*. Hence the floral extract can be used as an herbal and nontoxic source of prospective drug to combat biofilm mediated diseases without generating the risk of antibiotic resistance.

Keywords:
Antibiofilm, *Clitoria ternatea*, Phytoextract, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

1. INTRODUCTION

Biofilm being an organized collection of microbial communities remaining enclosed in a matrix of self-secreted extracellular polymeric substance, is found to form on various biotic and abiotic surfaces[1]. It provides a secured abode for the participating microorganisms by protecting them from various antimicrobial agents. The biofilm bound sessile microorganisms are naturally found to be more resistant and recalcitrant than their planktonic counterparts.

It is the polymicrobial biofilm, which makes the management of long-lasting lesions extremely difficult. The key role behind the organization of such poly microbial biofilm, is found to be played by *Pseudomonas aeruginosa* and *Staphylococcus aureus*[2] where the former are mainly located at superficial layer, while the later remain present at deeper in the wound[3]. Hence the enfeeblement of such poly biofilm with the help of some alternative antimicrobial can be the only way to treat the chronic wound.

Since, such persister cells concealed within biofilm matrix are found to be the root cause of antimicrobial resistance (AMR), a rapidly increasing terror for health sector[4], scientists are doing extensive search to explore alternative source of antimicrobial and antibiofilm agent.

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Nature being a rich source of bioactive compounds, often showcase number of phytocompounds that can effectively be used as an alternative to the antibiotics.

Clitoria ternatea is a common plant, which is known for several medicinal attributes like antiarthritic, antioxidant, antipyretic, anti-inflammatory, anti-asthmatic activities. The blue flowers, being a rich source of flavonol glycosides and anthocyanins, apart from being used as a source of synthetic blue food colorants also can be applied for their antimicrobial and antioxidant properties[5]. The flower petal was found to show antibacterial and antibiofilm activities against *Streptococcus mutans*[6], and the anthocyanin rich fraction of the flower showed against *P. aeruginosa*[7].

But almost no report is available on the *in silico* and *in vitro* analyses of antibiofilm activities of the floral extract of *C. ternatae* against *S. aureus* and *P. aeruginosa*, the two key nosocomial microbes.

The present paper deals with the detailed *in vitro* studies on antibiofilm activities of floral extract of *C. ternatea* against *S. aureus* and *P. aeruginosa*, and confirmation of antibiofilm activities through *in silico* studies.

2. MATERIALS AND METHODS

2.1. Phytoextract preparation from *C. ternatea* flowers

The purplish-blue flowers of *C. ternatea* were accumulated from the temple wastes of West Bengal. The flowers were cleaned with triple distilled water followed by drying. Subsequently, the flowers were pulverized using mortar pestle in presence of 95% methanol of analytical standard and then incubated for 16-24 h at 28±2°C[8]. After this, the floral extract was strained with the help of a gauge cloth and centrifuged at 5,000 rpm for 10 minutes, after which the obtained supernatant was kept at 4°C for future use.

2.2. Analysis of the biologically active compounds from the floral extract of *C. ternatea* using GC-MS

Methanolic phytoextract of *C. ternatea* was chromatographically analysed by the use of gas chromatography (Model: Trace GC Ultra; MS model, POLARISQ, Thermo Scientific). The bioactive compounds obtained, were identified from the NIST library[9]. The experimental parameters of the GC MS analysis were: DB 5—MS Capillary Standard Non—Polar Column of dimension 30 mm and inner diameter of 0.25 mm with 0.25 µm thick film. The flow rate of carrier gas Helium (mobile phase) was fixed to 1 ml/min. In the portion of gas chromatography, the temperature program of the oven was set at 40°C, which was gradually increased to 200°C at a rate of 10°C per minute with an injection quantity of 1 µL. Samples suspended in methanol were run entirely at a rate of 50-650 m/z and the compounds produced were analysed with the help of Wiley Spectral Library search programs. All of the bioactive compounds' peak areas and retention times were obtained, and the relative percentage of each compound was estimated by comparing its average peak area to the overall area.

2.3. Growth of the microorganisms *P. aeruginosa* ATCC 10145 and *S. aureus* ATCC 23235

Biofilm producing strains of *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 were cultivated in different 100 ml Erlenmeyer flasks each containing 50 ml of Luria Bertani broth for 24 h at a pH of 7.4 and temperature 37°C. The formation of biofilm by the working strains of bacteria can be analyzed with the help of microplate assay technique. Growth of biofilm is dependent on the synergistic actions of salt and sugar concentrations[10]. The optimum concentration for biofilm formation was obtained by the addition of glucose at varying concentrations of NaCl (0.5-7%) and glucose (0.25-10%) within the culture medium. Each of the setups of the experiment was incubated at 37°C for a period of 72 h.

2.4. Determination of the antimicrobial activity of *C. ternatea* phytoextract

Antimicrobial action of *C. ternatea* phytoextract and conventional antibiotics were determined by analysis of the diameters (in mm) of the zones of inhibition found through the method of agar well diffusion. LB agar plates with the test strains of bacteria (*S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145) were treated with the phytoextract and standard antibiotic (ampicillin for *S. aureus* ATCC 23235 and doxycycline for *P. aeruginosa* ATCC 10145) in varying concentrations in the wells bored on the agar plates followed by their incubation at 37°C for 24 h. The plates were observed for zones of inhibition as per the guidelines of the National Committee for Clinical Laboratory Standards[11].

2.5. Minimum inhibitory concentration (MIC) determination

The MIC values of the phytoextract of *C. ternatea* were determined against *P. aeruginosa* ATCC 10145 and *S. aureus* ATCC 23235) by microdilution method[12]. 10 µL each of *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 were inoculated in each of the test tubes having 5 ml LB broth followed by phytoextract treatment at varying concentrations (10-50 µg/mL) apart from the test tubes used as control. These test tubes were incubated at 37°C for 24 h and the intensity of the growth of the bacterial strains was determined using a spectrophotometer at 660 nm[13].

2.6. Minimum biofilm eradication concentration (MBEC) determination

The MBEC values were calculated by the use of MTT assay[14]. In a 96-well plate, was added 100 µl of LB broth in each of the wells followed by inoculation with 2 µl of the test organisms (*S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145) and incubated at 37°C for 72 h for optimum biofilm formation. Subsequently, the LB broth was pipetted out after 72 h in order to eliminate the planktonic cells, and except for the control wells, 20 µl of phytoextract was applied to each well before incubating at 37°C for 24 hours. Afterward, the MTT reagent was supplemented in all the wells including the control wells and thoroughly mixed followed by incubation at 37°C for 4 h and then the absorbance was measured at 550 nm with the help of a Thermo Scientific Multiskan Sky ELISA plate reader.

2.7. Determination of the rate of reduction of biofilm formation by *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 after phytoextract treatment

The microtiter plate assay was being used for quantitative estimation of the reduction in formation of biofilm[15]. In 6-well plates, was added 5 ml of LB broth followed by the dropping of a single autoclaved coverslip in each of the wells and 10 µl of the test bacteria were inoculated in all the wells, which were incubated for 72 h at 37°C. Subsequently, 240 µl of phytoextract was added in every well except the control wells and again incubated at 37°C for 24 h. Post-incubation, the LB broth along with the planktonic cells were discarded and the coverslips containing the sessile biofilm cells were stained with 41 ml of 0.1% w/v crystal violet (CV) in acetic acid and kept for 1 minute. Followed by this, the extra CV was discarded and the marked biofilm cells were treated with 1 ml acetic acid and kept for 1 minute. After which, the absorbance of the acetic acid was spectrophotometrically measured at a wavelength of 540 nm. The percentage reduction in the formation of biofilm was calculated with the following formula:

$$\text{Percentage Biofilm Inhibition} = \frac{\text{OD of untreated control -OD of treated sample}}{\text{OD of untreated control}} \times 100 \quad (1)$$

2.8. Quorum Quenching activities of *C. ternatea* phytoextract against the biofilm of *P. aeruginosa* ATCC 10145

2.8.1. Determination of the reduction of N-Acyl homoserine lactone (AHL) content

To 5 ml LB broth in every test tube, was inoculated 10 µl of *P. aeruginosa* ATCC 10145 followed by incubation for a period of 72 h at a temperature of 37°C and treated with 240 µl of phytoextract and further incubated at 37°C for 24 h. Followed by incubation, 2 ml culture was centrifuged at 10,000 rpm for 15 minutes. The supernatant collected was sieved through a membrane filter of 0.22 µm diameter. The filtrate was shaken gently with diethyl ether for a period of 10 minutes for separation of the phases and was kept undisturbed. The upper immiscible extract of the AHL was concentrated for 24 h at 40°C. After AHL concentration, 40 µl of the upper layer of AHL was pipetted to a 96-well plate and supplemented with 50 µl of the mixture of 2 M hydroxylamine and 3.5 M NaOH at a ratio of 1:1. Followed by this, 90 µl of the mixture of ferric chloride (10% in 4 M HCl) and 95% ethanol at 1:1 ratio was further added and the absorbance was recorded with the help of Thermo Scientific Multiskan Sky ELISA plate reader at a wavelength of 520 nm[16].

2.8.2. Determination in the reduction of rhamnolipid content by drop collapse assay

The reduction in the content of rhamnolipid was determined with cetyltrimethylammonium bromide (CTAB) and methylene blue. 1.5% LB agar mixed with CTAB (0.2% w/v) and methylene blue (0.005% w/v) were poured in the plates. The phytoextract was spread on all the agar plates except for the control plate, followed by which, a 24 h grown culture of the test organism *P. aeruginosa* ATCC 10145 was dropped in the centre of the plate inside the bored well. All the plates were incubated at 37°C for 24 h. Subsequently, the plates were again incubated at ambient temperature again for 24 h and then the reduction in rhamnolipid content was analyzed by measuring the diameter of the dark blue halo in the vicinity of the well containing the microbe after 48 h[17].

2.8.3. Determination in the reduction of pyocyanin content

To test tubes comprising 5 ml LB broth, was inoculated 10 µl of *P. aeruginosa* ATCC 10145 and incubated at 37°C for 24 h. All the test tubes were treated with 240 µl of the phytoextract except the control tube and incubated again for 48 h at 37°C. After 48 h of incubation, the treated bacterial culture was centrifuged at 10,000 rpm for 15 minutes and to the supernatant was added 3 ml chloroform. The lower portion of the chloroform was re-extracted with 1 ml 0.2 N HCl and the absorbance was spectrophotometrically measured at 520 nm[18].

2.8.4. Determination in the reduction of elastase content

To test tubes having 5 ml LB broth, 10 µl *P. aeruginosa* ATCC 10145 was inoculated followed by incubation at 37°C for 72 h. To all the test tubes 240 µl of the phytoextract was added except in the control tubes and

further incubated for 48 h at 37°C. After 48 h of incubation, the bacterial culture was centrifuged at 10,000 rpm for 15 minutes. An aliquot of 100 µl supernatant was pipetted to 900 µl 0.2% w/v Elastin Congo Red (ECR) followed by an incubation at 37°C for 3 h. The insoluble portion of the ECR was discarded via centrifugation and the optical density of the supernatant was spectrophotometrically recorded at a wavelength of 495 nm[19].

2.9. Fourier Transformed Infrared Radiation analysis of the biofilm matrix in *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 after phytoextract treatment

After 72 hours of incubation at 37°C on chitin flakes acting as a surface for biofilm attachment in LB broth, *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 developed biofilms. Except for the control test tubes, the completely formed biofilms on chitin flakes were challenged with 240 l of the phytoextract and dried with the help of a hot air oven for 48 hours. The FTIR spectra were measured in the 450-4000 cm region[20] by the use of a PerkinElmer FT-IR spectrometer (Frontier).

2.10. Analysis in the reduction of biofilm formation by scanning electron micrographic studies

Biofilms were formed by *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 in LB broth using chitin flakes as adherence surface for 72 h at 37°C. Post-incubation, the fully grown biofilms were treated with 240 µl of the phytoextract except the control tubes and incubated at 37°C for another 24 h. After incubation, the LB broth was removed to discard the free-living cells and the chitin flakes were cleaned with NaCl (0.9% w/v) to remove any cell debris. The chitin flakes were thereafter dropped in 2.5% glutaraldehyde for a period of 20 minutes for fixation on the flakes trailed by recurrent dehydration with the help of upgraded ethanol and centrifuged at 6,000 rpm for a period of 5 minutes each time. The dried chitin flakes holding only the sessile biofilm cells were viewed with the help of a ZEISS EVOMA 10 scanning electron microscope at a magnification of 15,000 X[21].

2.11. *In silico* studies of the antibiofilm activity of the floral extract against *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145

The X-ray crystal structures of the proteins 3TIP and 3ZYB that are involved in the formation of biofilms in *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 respectively were obtained from Protein Data Bank (PDB) having a resolution of 1.5-2 Å. In order to determine the antibiofilm efficacy of the bioactive components of *C. ternatea* phytoextract, molecular docking was carried out[22]. The three-dimensional structures of 1,3,5 cycloheptatriene and 5-hydroxymethylfurfural were collected from PubChem. The docking interactions were performed using Autodock and the interaction energies were analyzed[23].

2.12. Statistical analysis

Each of the experiments were repeated thrice and the standard deviation is calculated as a measure of the amount of variation about their average. The results obtained, were demonstrated in the form of mean±SD (standard deviation) and *p* value less than 0.05 is considered as statistically significant.

2.13. Chemicals and reagents

Each of the reagents and the chemicals utilized for the experiments were of analytical standard and bought from SRL and HiMedia.

3. RESULTS AND DISCUSSIONS

3.1. Bioactive component identification from *C. ternatea* phytoextract

It was found that the phytoextract of *C. ternatea* flowers is composed of various chromogenic groups like phenolic, hydroxyl and carbonyl (Figure 1). Mass spectrum determination by GC-MS of the unknown bioactive components and subsequent comparison of these compounds with that of the database of National Institute-

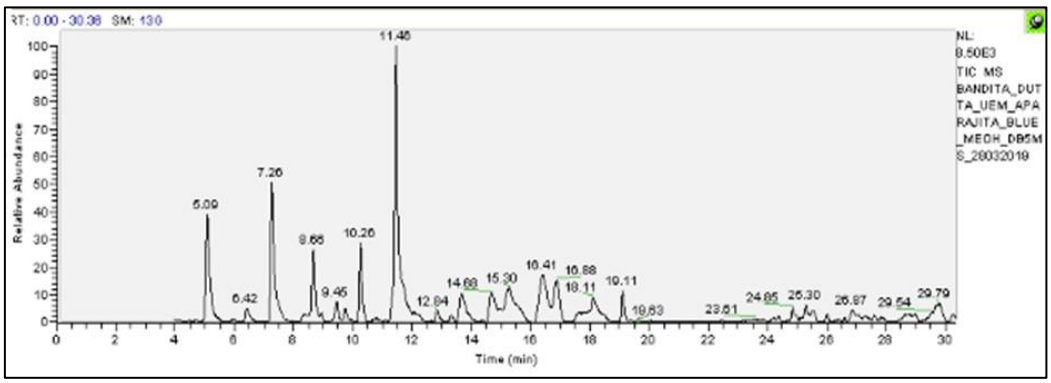

Figure1. GCMS chromatogram of the different bioactive compounds of *C. ternatea*.

Table 1. The bioactive compounds with antibiofilm activities present in the extract of CT petals (analysed by GC MS).

SL. No	R/T	Name of the identified compounds	Probability	Molecular Formula	MW	Peak Area%	Properties
1.	5.09	4-Pyridazinamine	35.42	$C_4H_5N_3$	96	7.87	Antibiofilm
2.	7.26	2-Furancarboxaldehyde-5-methyl	58.00	$C_6H_6O_2$	110	9.87	Antibiofilm
3.	8.66	1,3,5-cycloheptatriene	20.74	C_7H_8	92	5.03	Antibiofilm
4.	10.26	4-H-pyran-4-one	85.47	$C_6H_8O_4$	144	4.31	Antibiofilm
5.	11.46	5-Hydroxymethylfurfural	94.82	$C_6H_6O_3$	126	19.99	Antibiofilm
6.	16.41	α-D Galacto-pyranoside methyl	11.11	$C_7H_{14}O_6$	194	7.18	Antibiofilm

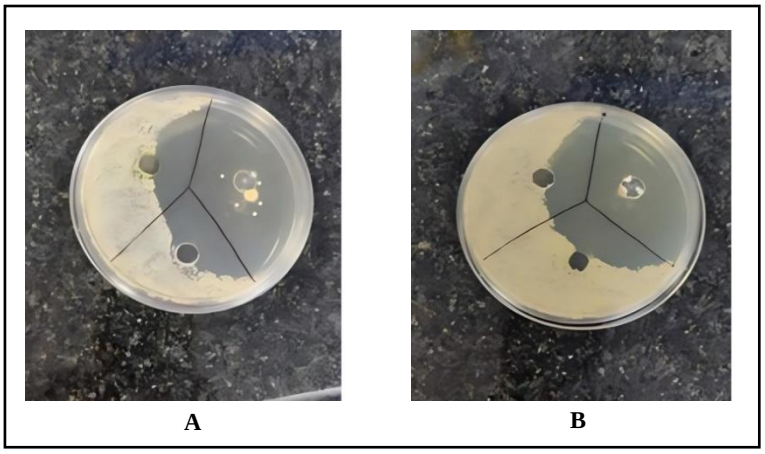

Figure 2. Antimicrobial activity of CT petal extract against (A) *S. aureus* ATCC 23235 (ZOI= 38±0.03 mm) and (B) *P. aeruginosa* ATCC 10145 (ZOI= 40±0.02 mm) against respective controls.

Standard and Technology (NIST) library confirmed the biochemical recognition of 6 bioactive components in *C. ternatea* flower phytoextract. The name of the components, their area of the peak, molecular formula and biological activity of the various compounds were analysed and mentioned in Table 1. The relative percentage content of each bioactive phytocompound was calculated by comparing the peak's average area to the total area. The extract was enriched in polyphenols like flavonols glycosides, quercetin, myricetin, kaempferol, phenolic acids, anthocyanins etc.

3.2. Antimicrobial activity and MIC value determination of the floral extract against *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145

Amongst the standard antibiotics and phytoextract, the phytoextract showed zones of inhibition of diameters 38±0.03 mm (Figure 2A) and 40±0.02 mm (Figure 2B) in *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 respectively. The respective control wells were also bored on the LB agar plates as well containing methanol and the standard antibiotics (ampicillin and doxycycline), which did not exhibit any remarkable antimicrobial action against *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 verifying that these test bacteria already developed resistance against methanol and the standard antibiotics. This confirms that the phytoextract possesses antimicrobial effects against *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145[24]. The phytoextract from the flowers of *C. ternatea* possessed an MIC value as low as 2 µg/ml (Figure 3A) and 20 µg/ ml (Figure 3B) against *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 proving that the phytoextract can show significant antimicrobial action at even a very minute concentration, much less that reported against other bacteria[7].

3.3. MBEC value and antibiofilm property determination of the floral extract of *C. ternatea* against *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145

The floral extract of *C. ternatea* demonstrated remarkable antibiofilm properties against *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 as evident from distinct colour change in 96-well plate after addition of MTT reagent. The MBEC value possessed by the phytoextract was found to be as low as 20 µg/ml (Figure 4A) and 200 µg/ml (Figure 4B) for *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145[25]. The CV assay aided in determining the percentage reduction in the formation of biofilms by the test organisms. The percentage reduction in biofilm formation in *S. aureus* ATCC 23235 was found to be 85±0.035% while that of ampicillin was 25±0.016%. (Figure 5A) On the other hand, the percentage reduction in formation of biofilm in *P. aeruginosa* ATCC 10145 was observed to be 82±0.037% and that of doxycycline was 25±0.029% (Figure 5B). The reduction in the percentage of biofilms was statistically significant ($p<0.05$)[26]. This remarkable decrease in biofilm formation is definitely through the inhibition of quorum sensing affecting

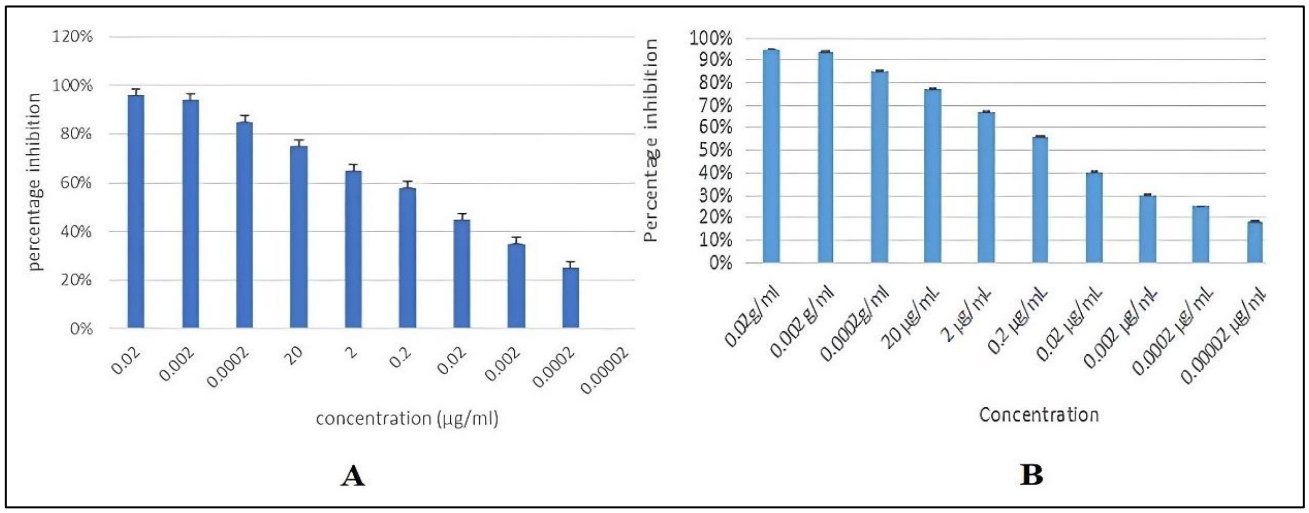

Figure 3. MIC graph with three replica each for (A) *S. aureus* ATCC 23235 (MIC= 2 µg/ml), for (B) *P. aeruginosa* ATCC 10145 (MIC= 20 µg/ml) (p<0.05).

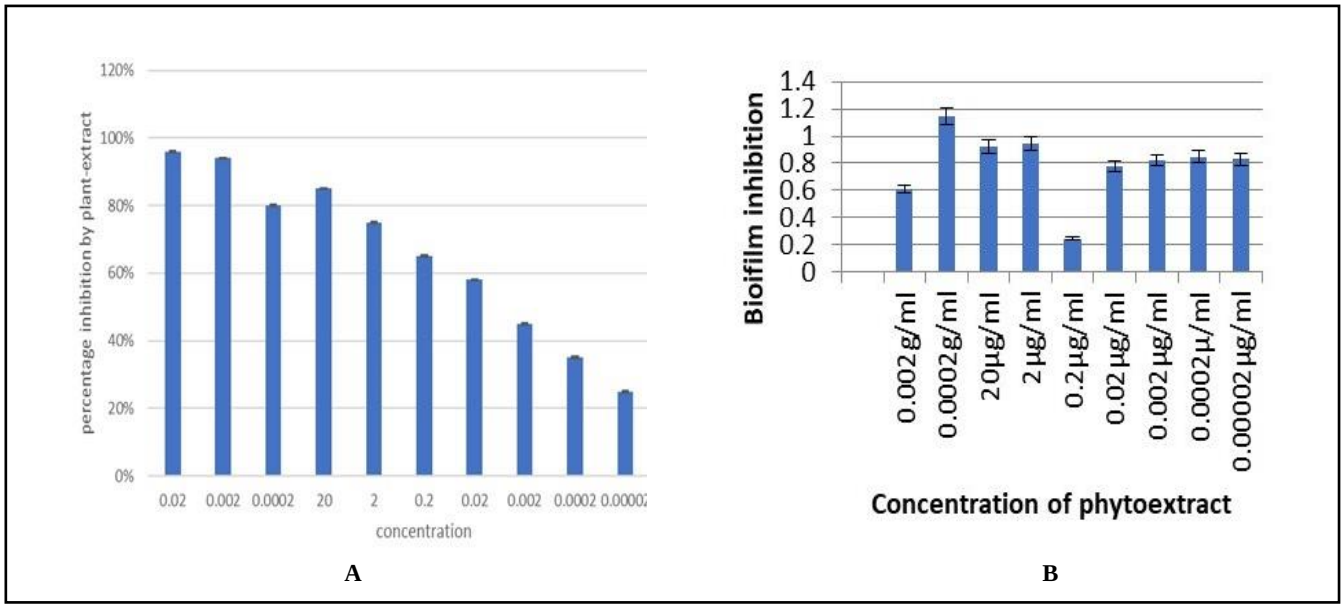

Figure 4. Assessment of MBEC of CT petal extract against the biofilm of (A) *S. aureus* ATCC 23235 (MBEC= 20 µg/ml) and (B) *P. aeruginosa* ATCC 10145 (MBEC= 200 µg/ml) (p<0.05).

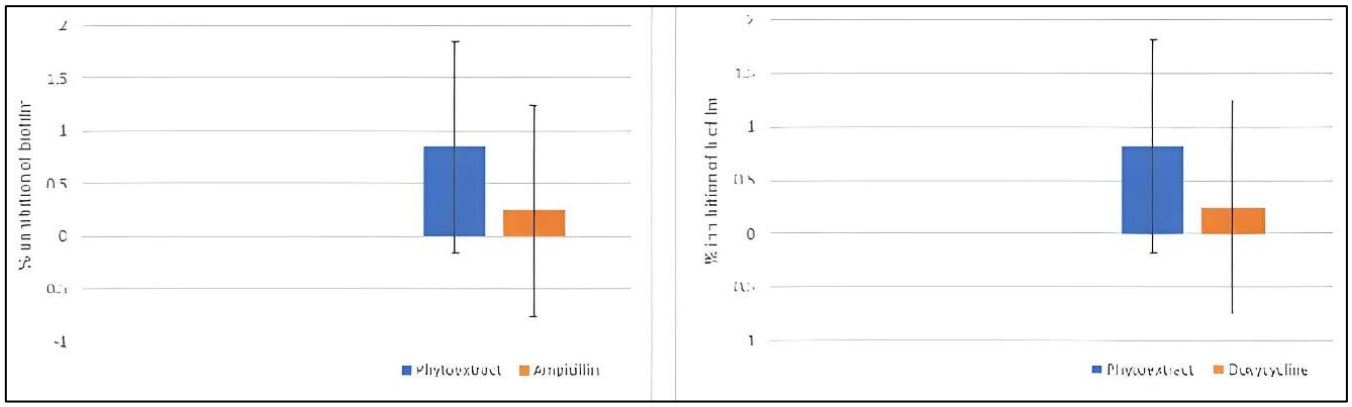

Figure 5. Percentage reduction in biofilm formation by (A) *S. aureus* ATCC 23235 (85±0.025%) and (B) *P. aeruginosa* ATCC 10145 (82± 0.037%) (p<0.05) derived from three replica.

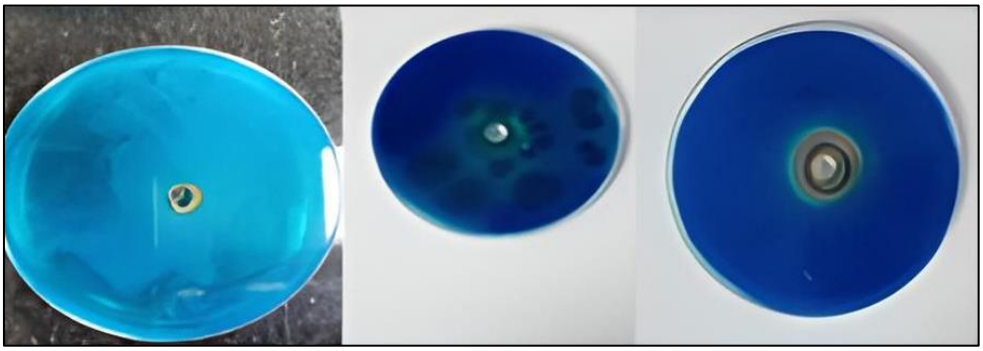

Figure 6. CTAB methylene blue LB agar plates (A) untreated control (B) treated with doxycycline and (C) treated with *C. ternatea* phytoextract.

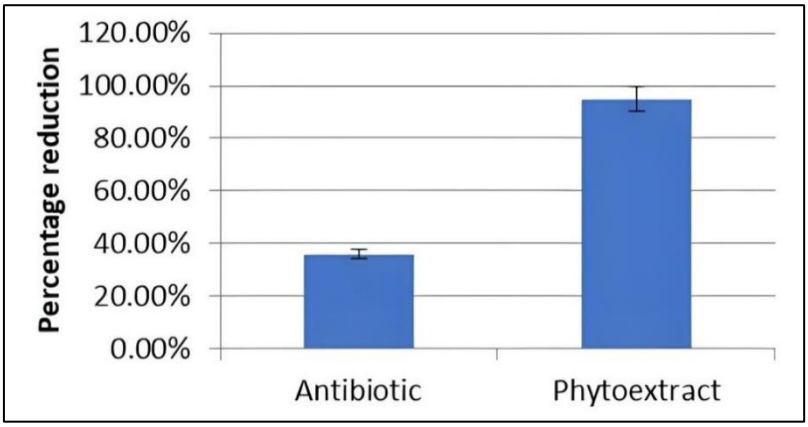

Figure 7. Percentage reduction of AHL content (82±0.16%) after treatment with doxycycline and phytoextract of *C. ternate* flowers.

different quorum-sensing signals and such signals are different in different bacteria. Antibiotic could not approach the undisrupted biofilm and kill the sessile bacteria.

3.4. Quorum quenching assays of *P. aeruginosa* ATCC 10145

The test bacterial strain of *P. aeruginosa* ATCC 10145 (Figure 6A) after being treated with the phytoextract of the flowers of *C. ternatea* displayed remarkable reduction in the quorum sensing actions. QS in *P. aeruginosa* ATCC 10145 is mediated by genes such as *rhl* and *las*. *LasB* and *LasA* also mediate the production of pyocyanin and elastase. *Rhl* genes mediate the rhamnolipid production thereby providing swarming motility to the test bacterial strain. It was observed that the swarming motility was drastically reduced in case of the phytoextract treatment (Figure 6C) in comparison to that of the doxycycline treated (Figure 6B) and this can be confirmed from the prominent dark blue halo present in the agar plate treated with the phytoextract. The percentage of AHL content, which is another QS protein that helps in cell-cell communication for effective biofilm formation, was significantly reduced to 82±0.16% in comparison to that of doxycycline was found to be quite negligible (Figure 7). Hence, AHL reduction prevents the primary step for biofilm formation by inhibiting the effective cell-cell communication. Las B encodes for elastase, which is a virulence factor rendering the biofilm virulent. The percentage reduction of LasB after being treated with the phytoextract of *C. ternatea* flowers was found to be 81±0.22% in comparison to that of doxycycline, which was not very remarkable (Figure 8). So, reduction of AHL content destroys the virulence of the biofilms. Pyocyanin increases reactive oxygen species (ROS) production thereby inducing oxidative stress and helping in formation of biofilms by *P. aeruginosa* ATCC 10145. The content of pyocyanin was also significantly reduced by phytoextract treatment to 83±0.2 % while percentage reduction of pyocyanin with doxycycline was found to be negligible (Figure 9). Thus, reduction in pyocyanin pro-duction will reduce oxidative stress among the bacterial cells thereby preventing the formation of biofilms[27].

3.5. FTIR analysis of the modification of the biofilm EPS matrices of *P. aeruginosa* ATCC 10145 and *S. aureus* ATCC 23235 after being challenged with the *C. ternatea* phytoextract

FT-IR was conducted in order to detect the alterations among the functional groups present in the matrices of the biofilms formed by *S. aureus* ATCC 23235 (Figure 10A) and *P. aeruginosa* ATCC 10145 (Figure 10B) after being treated with the floral extract of *C. ternatea*. Significant alterations within the spectral regions of lipids (3,000-2,800 cm^{-1}), polysaccharides (890-1,175 cm^{-1}), nucleic acids (1,300-900 cm^{-1}) and proteins (1,700-1,500 cm^{-1}) were observed in the FT-IR spectrometric analyses.

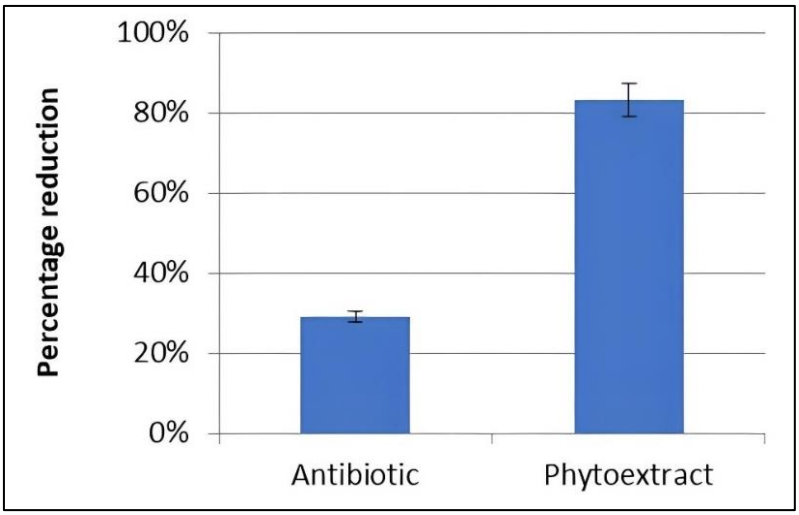

Figure 8. Percentage reduction of LasB (81±0.22%) after treatment with doxycycline and phytoextract of *C. ternate* flowers.

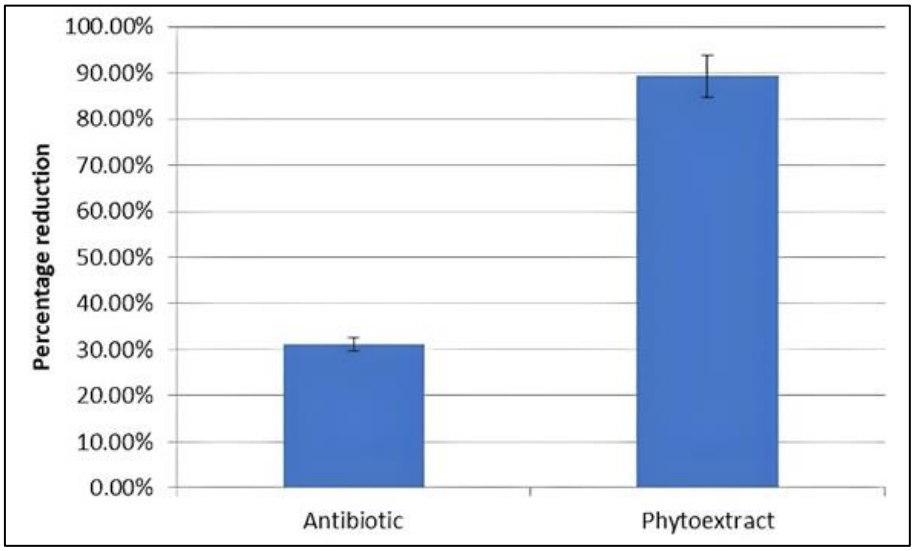

Figure 9. Percentage reduction in the pyocyanin content (83±0.2%) after treatment with doxycycline and *C. ternatea* floral extract.

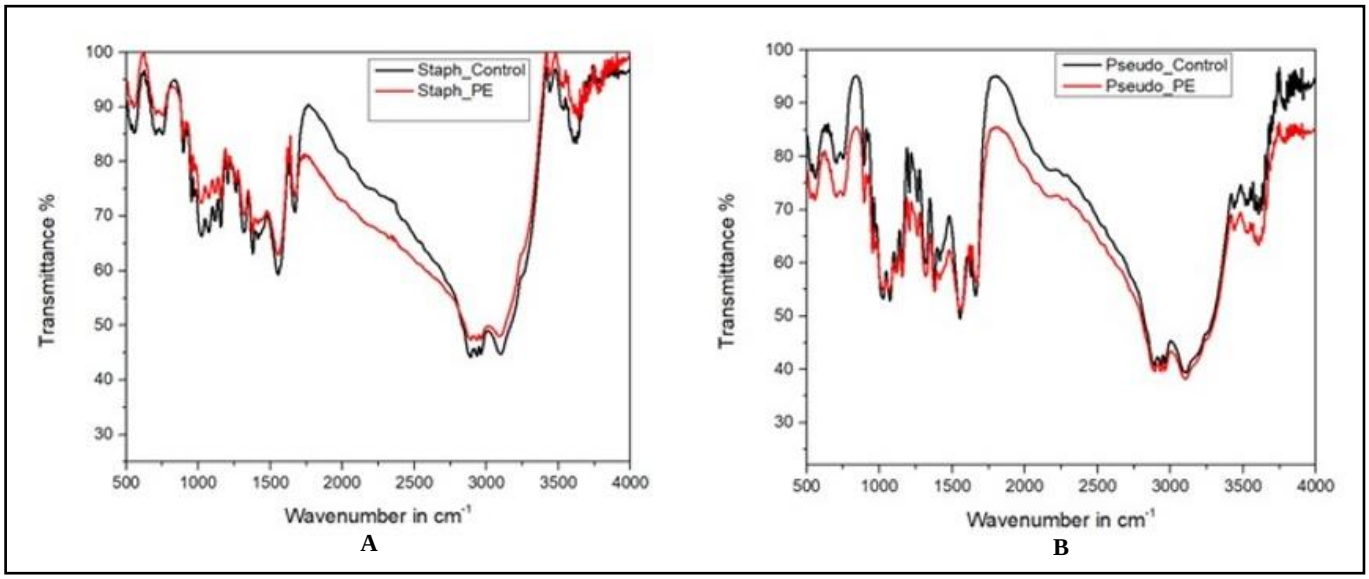

Figure 10. Relative FT-IR spectral analyses displaying the acitivity of *C. ternatea* phytoextract treatment on (A) *S. aureus* ATCC 23235 and (B) *P. aeruginosa* ATCC 10145.

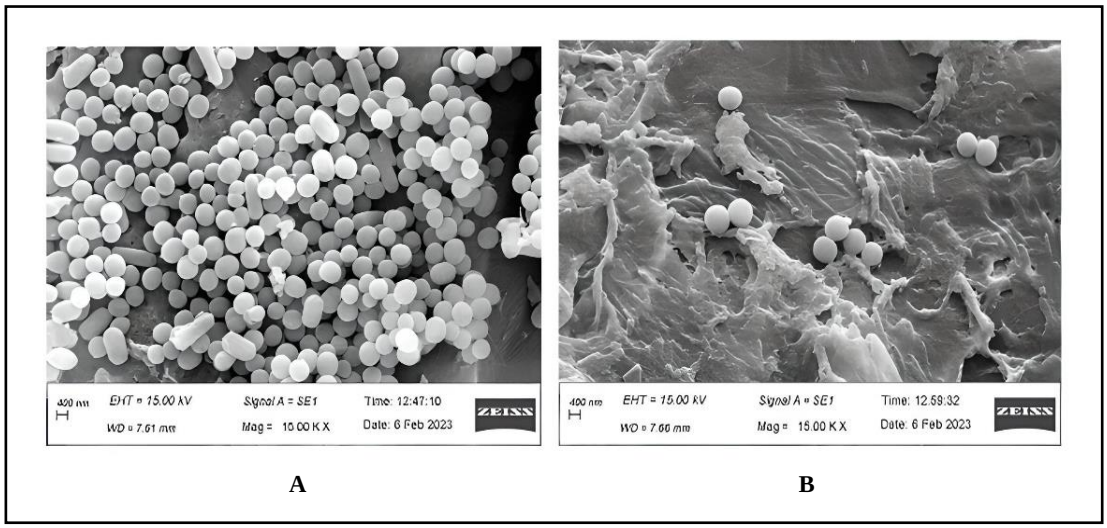

Figure 11. Scanning electron micrograph of *S. aureus* ATCC 23235 (A) before treatment and (B) after treatment with floral extract of *C. ternatea*.

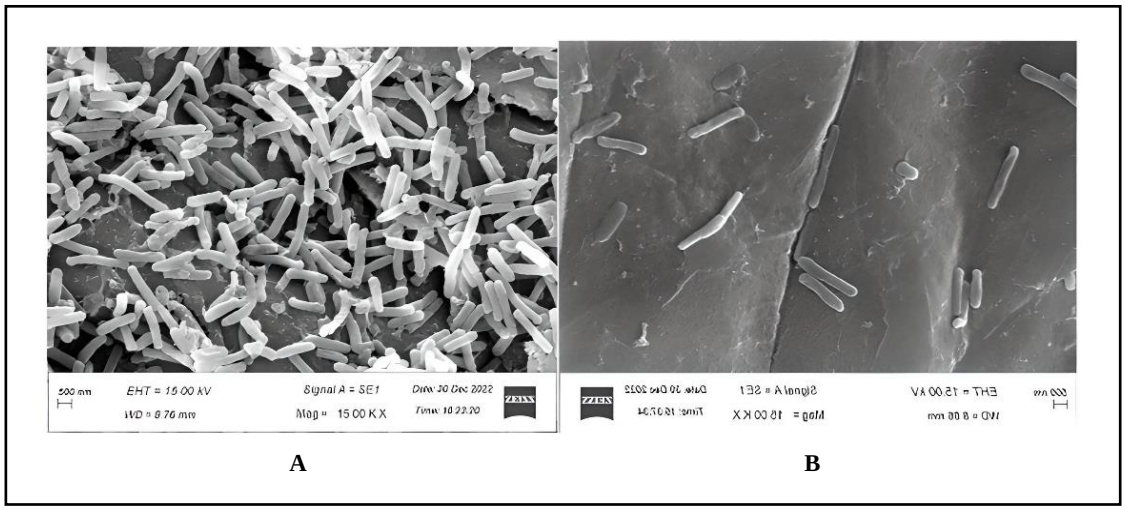

Figure 12. Scanning electron micrograph of *P. aeruginosa* ATCC 10145 (A) before treatment and (B) after treatment with floral extract of *C. ternatea*.

The *C. ternatea* phytoextract induced the most significant decrease in shape modifications, intensities of the peaks and wavelength shifts as compared to the untreated control samples of bacteria. This indicates that the floral extract of *C. ternatea* can directly interact and lessen the concentration of different compounds present in the EPS matrices of the biofilms in *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 like lipids, nucleic acids and polysaccharides as confirmed by the decrease in peak intensities.

3.6. Photomicrographic analyses of the biofilm forming cells in *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 on treatment with phytoextract of *C. ternatea*

The floral extract of *C. ternatea* was observed to significantly reduce the bacterial sessile cell count thereby clearing away the dense layer of biofilms formed by *S. aureus* ATCC 23235 (Figure 11) and *P. aeruginosa* ATCC 10145 (Figure 12) as compared to that of the untreated control samples.

3.7. *In silico* analysis of the interactions among the biofilm forming proteins of *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 and the bioactive compounds of *C. ternatea* flowers

In silico analyses were performed to study the molecular interactions between the biofilm forming proteins of *S. aureus* ATCC 23235 (Figure 13) and *P. aeruginosa* ATCC 10145 (Figure 14) with 1,3,5-cycloheptatriene and 5-hydroxymethylfurfural, which showed that these 2 biologically active compounds could significantly bind with the biofilm forming proteins in *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 thereby validating the *in vitro* analysis, which showed the significant biofilm inhibition after being treated with the floral extract of *C. ternatea*. Table 2 shows the binding energies of the various bioactive compounds with that of the biofilm forming proteins of the test bacteria. Lower the binding energy, better is the interaction and so, 3ZYB, the biofilm forming protein of *P. aeruginosa* ATCC 10145 showed the best interaction with 1,3,5-cycloheptatriene with an

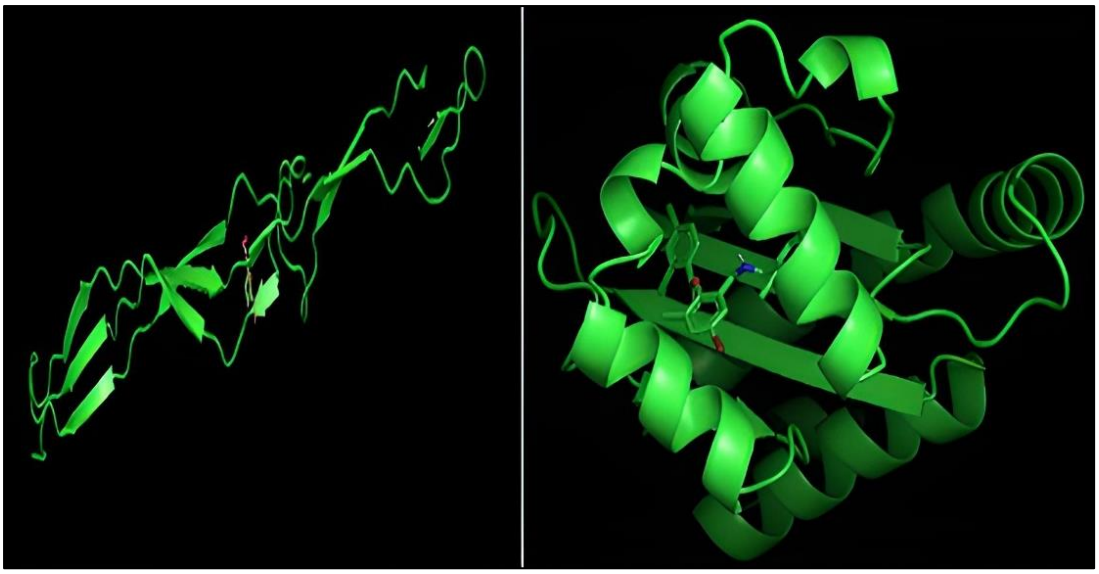

Figure 13. Effect of (A)5-hydroxymethylfurfural and (B) 1,3,5-cycloheptatriene on 3TIP protein of *S. aureus* ATCC 23235.

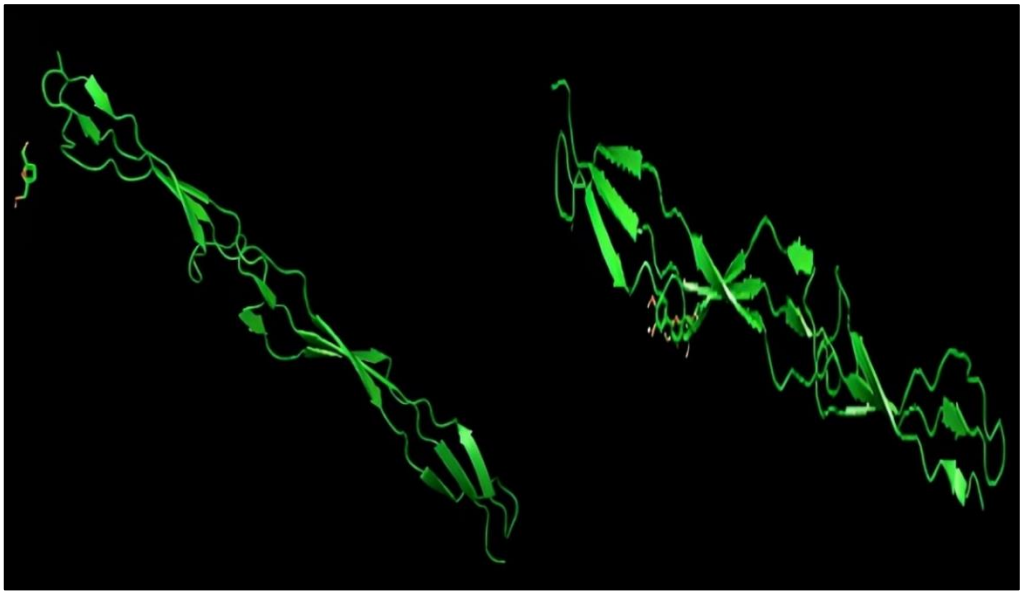

Figure 14. Effect of (A)5-hydroxymethylfurfural and (B) 1,3,5-cycloheptatriene on 3ZYB protein of *P. aeruginosa* ATCC 10145.

Table 2. Docking interactions between biofilm forming proteins of *S. aureus* ATCC 23235 and *P. aeruginosa* ATCC 10145 and the bioactive compounds of *C. ternatea*

Docked complex	Binding/ Interaction energy (kJ/mol)
3TIP +5-hydroxymethylfurfural	-3.56
3TIP+1,3,5-cycloheptatriene	-5.35
3ZYB+5-hydroxymethylfurfural	-4.69
3ZYB+1,3,5-cycloheptatriene	-5.67

interaction energy as low as -5.67 kJ/mol. This result indicates that the compound 1,3,5-cycloheptatriene is mainly responsible for bringing about biofilm inhibition in *P. aeruginosa* ATCC 10145. On the other hand, 1,3,5-cycloheptatriene binds with an interaction energy of as low as -5.35kJ/mol, which again confirms that this bio-active compound is mostly bringing about the inhibition of the formation of biofilm by *S. aureus* ATCC 23235.

4. CONCLUSION

Clitoria ternatea is a plant with several medicinal attributes that can be effectively used to remove the sessile and planktonic forms of pathogenic bacteria like *S. aureus* and *P. aeruginosa.* The antimicrobial and anti-biofilm activities of the floral extract were remarkably higher than that of conventional antibiotic. This efficacy was also showcased by the scanning micrographs. The biofilm eradication in *P. aeruginosa* was brought about

through hindering of quorum sensing mechanism. The *in-silico* study clearly indicates the 1,3,5-cycloheptatriene is mainly responsible for bringing about biofilm inhibition both in *S. aureus* and *P. aeruginosa.*

The effective eradication of biofilm from two major pathogenic bacteria indicates that the phytoextract may be applied to remove biofilm produced by other pathogenic strains of these bacteria, isolated from various clinical conditions and also to combat other ESKAPE strains in future and the effectivity of this phytoextract may be further enhanced by using it as a nanoconjugate.

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REFERENCES

1. Sharma S, Mohler J, Mahajan SD, Schwartz SA, Bruggemann L, Aalinkeel R. Microbial biofilm: A review on formation, infection, antibiotic resistance, control measures, and innovative treatment. Microorganisms. 2023;11(6):1614.
2. Serra R, Grande R, Butrico L, Rossi A, Settimio UF, Caroleo B, et al. Chronic wound infections: The role of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Expert Rev Anti Infect Ther. 2015;13(5):605-13.
3. Pouget C, Dunyach-Remy C, Magnan C, Pantel A, Sotto A, Lavigne JP. Polymicrobial biofilm organization of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in a chronic wound environment. Int J Mol Sci. 2022;23(18):10761.
4. Hall CW, Mah TF. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS Microbiol Rev. 2017;41(3):276-301.
5. Vidana Gamage GC, Lim YY, Choo WS. Anthocyanins from *Clitoria ternatea* flower: Biosynthesis, extraction, stability, antioxidant activity, and applications. Front Plant Sci. 2021;12:792303.
6. Yanti Y, Setiawan T, Lay BW. Antibacterial, antibiofilm and quorum sensing inhibitory activities of *Clitoria ternatea* anthocyanin against *Streptococcus mutans*. Int J Infect Dis. 2018;73:143-4.
7. Jeyaraj EJ, Nathan S, Lim YY, Choo WS. Antibiofilm properties of *Clitoria ternatea* flower anthocyanin-rich fraction towards *Pseudomonas aeruginosa*. Access Microbiol. 2022;4(4):000343.
8. Quave CL, Estévez-Carmona M, Compadre CM, Hobby G, Hendrickson H, Beenken KE, et al. Ellagic acid derivatives from *Rubus ulmifolius* inhibit *Staphylococcus* aureus biofilm formation and improve response to antibiotics. PloS one. 2012;7(1):e28737.
9. Patra JK, Kim ES, Oh K, Kim HJ, Kim Y, Baek KH. Antibacterial effect of crude extract and metabolites of *Phytolacca americana* on pathogens responsible for periodontal inflammatory diseases and dental caries. BMC Complement Altern Med. 2014; 14(1):343.
10. Ghosh S, Mondol S, Lahiri D, Nag M, Sarkar T, Pati S, et al. Biogenic silver nanoparticles (AgNPs) from *Tinospora cordifolia* leaves: An effective antibiofilm agent against *Staphylococcus aureus* ATCC 23235. Front Chem. 2023;11:1118454.
11. Reller LB, Weinstein M, Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: A review of general principles and contemporary practices. Clin Infect Dis. 2009;49(11):1749-55.
12. Lahiri D, Nag M, Dutta B, Dash S, Ghosh S, Ray RR. Synergistic effect of quercetin with allicin from the ethanolic extract of *Allium cepa* as a potent anti-quorum sensing and anti-biofilm agent against oral biofilm. In: Ramkrishna D, Sengupta S, Dey Bandyopadhyay S, Ghosh A, editors. Advances in bioprocess engineering and technology. Lecture Notes in Bioengineering. Singapore: Springer; 2021. p. 69-81.
13. Lahiri D, Nag M, Dutta B, Mukherjee I, Ghosh S, Dey A, et al. Catechin as the most efficient bioactive compound from *Azadirachta indica* with antibiofilm and anti-quorum sensing activities against dental biofilm: An *in vitro* and *in silico* study. Appl Biochem Biotechnol. 2021;193:1617-30.
14. Ghosh S, Lahiri D, Nag M, Dey A, Sarkar T, Biswas R, et al. Analysis of antibiofilm activities of bioactive compounds from Honeyweed (*Leonurus sibiricus*) against *P. aeruginosa*: An *in vitro* and *in silico* approach. Appl Biochem Biotechnol. 2023; 195(9):5312-28.
15. Baishya R, Bhattacharya A, Mukherjee M, Lahiri D, Banerjee S. Establishment of a simple reproducible model for antibiotic sensitivity pattern study of biofilm forming *Staphylococcus* aureus. Mater Today: Proc. 2016;3(10):3461-6.
16. Yang YH, Lee TH, Kim JH, Kim EJ, Joo HS, Lee CS, et al. High-throughput detection method of quorum-sensing molecules by colorimetry and its applications. Anal Biochem. 2006;356(2):297-9.
17. Caiazza NC, Shanks RM, O'toole GA. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. J. Bacteriol. 2005;187(21):7351-61.
18. Datta S, Jana D, Maity TR, Samanta A, Banerjee R. *Piper betle* leaf extract affects the quorum sensing and hence virulence of *Pseudomonas aeruginosa* PAO1. 3 Biotech. 2016;6:18.
19. Lahiri D, Nag M, Dutta B, Sarkar T, Ray RR. Artificial neural network and response surface methodology-mediated optimization of bacteriocin production by *Rhizobium leguminosarum*. Iran J Sci Technol Trans A Sci. 2021;45:1509-17.
20. Pati S, Chatterji A, Dash BP, Raveen Nelson B, Sarkar T, Shahimi S, et al. Structural characterization and antioxidant potential of chitosan by γ-irradiation from the carapace of horseshoe crab. Polymers. 2020;12(10):2361.
21. Lahiri D, Nag M, Dutta B, Dey S, Mukherjee D, Joshi SJ, et al. Antibiofilm and anti-quorum sensing activities of eugenol and linalool from *Ocimum tenuiflorum* against *Pseudomonas aeruginosa* biofilm. J Appl Microbiol. 2021;131(6):2821-37.
22. Sarkar T, Bharadwaj KK, Salauddin M, Pati S, Chakraborty R. Phytochemical characterization, antioxidant, anti-inflammatory, anti-diabetic properties, molecular docking, pharmacokinetic profiling, and network pharmacology analysis of the major phytoconstituents of raw and differently dried *Mangifera indica* (himsagar cultivar): An *in vitro* and *in silico* investigations. App Biochem Biotechnol. 2021:1-38.
23. Wang Y, Xiao J, Suzek TO, Zhang J, Wang J, Bryant SH. PubChem: A public information system for analyzing bioactivities of small molecules. Nucleic Acids Res. 2009;37(suppl_2):W623-33.
24. Ouyang J, Sun F, Feng W, Sun Y, Qiu X, Xiong L, et al. Quercetin is an effective inhibitor of quorum sensing, biofilm formation and virulence factors in *Pseudomonas aeruginosa*. J Appl Microbiol.

2016;120(4):966-74.
25. Yuan YG, Peng QL, Gurunathan S. Effects of silver nanoparticles on multiple drug-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from mastitis-infected goats: An alternative approach for antimicrobial therapy. Int J Mol Sci. 2017;18 (3):569.
26. Radji M, Agustama RA, Elya B, Tjampakasari CR. Antimicrobial activity of green tea extract against isolates of methicillin-resistant *Staphylococcus aureus* and multi-drug resistant *Pseudomonas aeruginosa*. Asian Pac J Trop Biomed. 2013;3(8):663-7.
27. Silva LN, Da Hora GC, Soares TA, Bojer MS, Ingmer H, Macedo AJ, et al. Myricetin protects *Galleria mellonella* against *Staphylococcus aureus* infection and inhibits multiple virulence factors. Sci Rep. 2017;7(1):2823.