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Original Article

Anti-biofilm Efficacy of Phenolic Rich Fraction from *Cinnamomum zeylanicum* Bark against *Pseudomonas aeruginosa*

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Abstract

Microbial biofilms augment the antibiotic the resistance of bacteria and poses challenges to treating chronic infections. *Pseudomonas aeruginosa,* an effective biofilm-forming bacterium, causes a variety of serious pathogenic manifestations including deadly lung, skin, and urinary tract infections. This study was carried out to investigate the anti-biofilm and anti-quorum sensing activity of phenolic compounds in *Cinnamomum zeylanicum* bark against P. aeruginosa. This study comprised the estimation of total phenolics in *C. zeylanicum* bark the methanolic extract, with column the chromatographic isolation of the phenolic-rich fraction from the methanolic extraction of the bark. GC-MS analysis, the minimum inhibitory concentration (MIC) and anti-biofilm activity against *P. aeruginosa* were studied. Phytochemical analysis illustrated the presence of 4.2% of condensed tannins (proanthocyanidins), a bioactive compound present in the methanol extract of the bark. Out of the 96 fractions that were collected from the column, fractions corresponding to 75 to 96 confirmed the presence of procyanidins. These fractions were pooled together to get phenolic-rich fraction of *C. zeylanicum* bark. The GC-MS spectrum with a peak at 12.23 retention time confirmed the presence of procyanidins in phenolic-rich fraction of bark. The MIC of phenolic rich fraction against *P. aeruginosa* was 12.5mg/ml. The efficacy in biofilm attenuation of phenolic-rich fraction of the bark at sub-MIC doses (1.56, 3.12, 6.25 mg/ml) was significant (P< 0.05) and was determined to 71± 8%, 55±7%, 33±4% respectively against *P. aeruginosa*, when compared to control. The inverse relation between sub-MIC doses and anti-biofilm efficacy revealed that the bioactive compounds triggered anti-biofilm activity without inducing the drug the resistance mechanism of the bacteria. The results showed procyanidin present in the *C. zeylanicum* bark has the efficacy to quench quorum sensing and inhibit biofilm formation in *P. aeruginosa*. Hence, procyanidins present in the *C. zeylanicum* bark may be used as a novel molecule in drug design to treating recalcitrant infectious diseases.

Keywords: *Anti-Biofilm, Bacterial the Motility, Phenolic Compounds, P. Aeruginosa, Quorum Sensing*

Introduction

Biofilms are complex aggregates of bacteria grouped together and covered in an extracellular polymeric matrix composed of proteins, nucleic acids, and polysaccharides. The bacterial group is structurally stabilized and protected by this polymeric matrix. Bacteria usually use the strategy of biofilm formation due to its implications to antibiotic the resistance and chronic infections. *P. aeruginosa* cells exhibit phenotypic heterogeneity with several subpopulations with each having its unique metabolic states and the resistance profiles to withstand antimicrobial treatments and the human immune system (Pang *et al*., 2019). Biofilm-associated diseases are chronic and have the potential to act as a reservoir for

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enduring infections, resulting in frequent episodes of infection even during antibiotic treatment because of their metabolic dormancy (Vestby *et al*., 2020). These characteristic features of biofilm developed by microbes are responsible for more than 80% of human clinical infections, including persistent pulmonary infections, cystic fibrosis (CF), burn wounds and otitis (Rather, Gupta & Mandal, 2021). Prolonged lung infections with *P. aeruginosa* are a leading cause of morbidity due to their great resilience to current medicines and capacity to evade host immune clearance (Garcia-Clemente *et al*., 2020). Due to its unique ability to change from a drug-tolerant, a planktonic to a more dangerous and treatment-resistant form with its biofilm, *P. aeruginosa* can cause additional serious infections (Thi, Wibowo & Rehm, 2020).

Quorum sensing (QS) plays a prominent role in controlling the biofilm formation, by regulation of virulence factors, biofilm maturation, and chronic infection development. In general, QS is a cell-density-dependent communication mechanism where bacteria can coordinate with their collective gene expression (Moreno-Gámez, Hochberg & van Doorn, 2023). The effectiveness of antimicrobial therapies is through QS signalling disruption that enhance the efficacy of antimicrobial treatments (Jiang *et al*., 2019). Due to significant clinical ramifications of *P. aeruginosa and its* biofilm action, innovative approaches to prevent and treat biofilm-associated infections are needed immediately. Proper anti-biofilm agents to interfere with the production of biofilms and make them more susceptible to antibiotics are needed (Shrestha *et al*., 2022). Understanding the molecular mechanisms involved in biofilm production and action is necessary for developing targeted therapies to combat these persistent infections (Zhao, Sun & Liu, 2023).

Research on the plant phenolics action of anti-biofilm have shown that these compounds have antibacterial properties aligning with anti-biofilm action to influence bacterial regulatory systems like quorum sensing or the other global regulators (Takó *et al*., 2020). Proanthocyanidins, the primary polyphenolic compounds present in *Capsicum zeylanicum* are usually oligomers and polymers with flavan-3-ol units. Procyanidins that consist of (epi)catechin are also proanthocyanidins (Gunawardena, Govindaraghavan, & Münch 2013). Matrix-Assisted Laser Desorption/Ionization Time-of-Flight/Time-of-Flight mass spectrometry (MALDI-TOF/TOF) was used to analyze proanthocyanidins components present in cinnamon which identified a combination of compounds such as (epi)gallocatechin, (epi)afzelechin, (epi)catechingallate and (epi)catechin. This successful combination produces a very heterogeneous mixture of procyanidins and prodelphinidin, thus attributing to the beneficial effects of cinnamon ingestion on health (Mateos-Martín *et al*., 2012).

The bark of Ceylon cinnamon (*Cinnamomum zeylanicum*) is a popular spice belongs to *The Lauraceae* family. Apart from its culinary applications, cinnamon has been used in traditional herbal medicine for various medical ailments. Cinnamon acts as antidiabetic, anti-inflammatory, antimicrobial and antioxidant. They also have hypotensive and cholesterol-lowering effects (Shoqairan *et al*., 2023). *C. zeylanicum* bark contains a rich content of phytochemical known as cinnamaldehyde that accounts for 60-80% of the essential oil extracted from the bark. Eugenol and coumarin are the other essential component present in cinnamon (Yanakiev, 2020). The antioxidant activity of cinnamon derived from its phenolic content that deteriorate the oxidative damage (Enogieru & Williams, 2024). Therefore, the use of plant-derived, new antibacterial and anti-biofilm to limit microbial growth and biofilm formation has drawn more focus to limit microbial growth and biofilm formation. This study aimed to ascertain the anti-biofilm and anti-quorum sensing effectiveness of a phenolic compound from the bark extract of *C. zeylanicum* against *P. aerogenosa*.

Material and Methods

The methanolic extract of C. zeylanicum bark

Fresh C. zeylanicum bark was gathered from Gandhi Market area in September 2023 in Tiruchirappalli, Tamilnadu and was authenticated by John Britto Rapinat Herbarium, Department of Botany, St Joseph's College, Tiruchirappalli. It was then shade-dried, powdered and extracted with methanol for 18 hours using a Soxhlet apparatus. The residue obtained was weighed and dissolved in Dimethylsulfoxide (DMSO) to obtain the desired concentration.

Preliminary phytochemical screening of methanol extract of C. zeylanicum bark

The methanolic bark extract of *C. zeylanicum* was analysed for preliminary phytochemical screening to identify the presence of alkaloids, flavonoids, saponin, tannin, condensed tannins etc (Sharma *et al*., 2016).

UV Absorption spectral analysis of methanol extract of C. zeylanicum bark

Components present in the methanolic bark extract of *C. zeylanicum* was analysed by UV-Vis spectrophotometer at 200 - 400 nm (Subramanian, Subbramaniyan, & Raj, 2013).

Estimation of total phenolic compounds in methanol extract of C. zeylanicum bark

Phenolic compounds present in the methanolic bark extract was determined by the Folin-Ciocalteu reagent. For this, 0.5 ml of the extract and 0.5 ml of The Folin-Ciocalteu reagent were taken in a test The tube. To it, 2 ml of 7.5% sodium carbonate solution was added after 5 minutes and was made up to 8 ml. After 2 hours, the absorbance was measured at 725 nm using the gallic acid standard (Molole, Gure & Abdissa *et al*., 2022).

Isolation of phenolic-rich fraction by column chromatography

To the methanolic bark extract of *C. zeylanicum*, equal amount of methanol was added for its application to the chromatographic column (2.5×30 cm). The column developed with mobile phase (eluant) of increasing polarity. A rota evaporator then successively concentrated the fractions collected. The concentrates were further analysed for the presence of procyanidins (Karamać, Kosińska & Chavan 2005).

Identification of phenolic compounds by thin-layer chromatography

Fractions collected was developed using precoated TLC plates, using three different solvent systems like Hexane, chloroform and toluene & ethyl acetate (1:1). The TLC plates were dipped in vanillin-HCl (10% vanillin in ethanol: concentrated HCl in 2:1 ratio) and heated to observe the red spots in daylight. With procyanidin B2 [(−)-Epicatechin-(4β→8) -(−)-epicatechin] as standard, the fractions with similar spots and Rf values were pooled together to get a phenolic rich fraction of *C. zeylanicum* bark (Di Lorenzo *et al*., 2016).

GC-MS analysis of phenolic-rich fraction of C. zeylanicum bark

The phenolic rich extract was analysed using gas chromatography at 35 - 400 m/z (Carvalho *et al*., 2022).

Determination of the minimum Inhibitory Concentration (MIC) using the microdilution method

The lowest concentration of drug to inhibit the visible growth of a microorganism after incubation is defined as *MIC. P. aeruginosa* MTCC 2488 strain were purchased from Microbial Type Culture Collection and Gene Bank, Chandigarh, India. MIC of phenolic rich fraction of *C. zeylanicum* bark was determined using microdilution method. In this method, the phenolic rich fraction of *C. zeylanicum* bark was diluted to form a series of concentrations from 3.12, 6.25, 12.5, 25, 50 and 100 mg/ml in sterile nutrient broth. The *P. aeruginosa* suspension of 10^5 -10^6 CFU /ml was added to the broth dilutions. The tube containing microorganism suspension served as positive control and the tube without microorganisms as a negative control. Incubation done at 37ºC, 24 hours for visible growth after mixing (Mogana *et al*., 2020).

Anti-biofilm activity of phenolic rich fraction of C. zeylanicum bark

P. aeruginosa strain in LB broth was prepared and diluted to 10% LB in water. About 50 µL broth was added into the 96-well plate containing 50 µL of three different sub-MIC doses (1.56, 3. 12, 6.25 mg/ml) of phenolic rich fraction of *C. zeylanicum* bark. After getting a final cell density of 5 X 10^5 $CFUmL^{-1}$, the plate was incubated for 20 h at 37 °C. Then, 200µl of 0.05 % aqueous crystal violet solution was added for staining. After bounding, 200 µl of 33 % acetic acid was added to release crystal violet stain and measured at 600 nm. The experiment was performed in triplicates. Mean **±** SD was calculated as shown below (Mogana *et al*., 2020).

$$\text{Biofilm inhibition \%} = \frac{\text{OD of untreated control - OD of treated sample}}{\text{OD of untreated control}} \times 100$$

The motility assays

For the motility assays, 5 µl aliquots of the overnight bacterial culture with or without treatment with phenolic rich fraction of *C. zeylanicum* extract at a sub-MIC doses were inoculated. Plates with different agarose concentration was used to simulate bacterial movement with tobramycin as a standard. Diameter of circular bacterial growth was measured after incubation at 30 °C for 24 h (Ye *et al*., 2022; Kamiya *et al*., 2019).

IBM SPSS Statistics for Windows, version 19 (IBM Corp., Armonk, N.Y., USA) was used to compare the variables with the chi-squared test and Student's *t*-test where values of $p < 0.05$ was statistically significant.

Results and Discussion

Phytochemical compounds in methanol extract of C. zeylanicum bark

Phytochemical screening is used to reveal the constituents of the plant extracts with the predominant bioactive compounds. Besides the phytochemical shown in Table 1, the methanolic bark extract of *C. zeylanicum* contains condensed tannins, which are categorised as proanthocyanidins.

Phytochemical screening results for bioactive substances, which can be utilized as beneficial medications. Comparing the phytochemicals present, phenolic and flavonoid compounds are the most important classes as phenolic compounds contain free hydrogen to exhibit their anti-biofilm property (Sun & Shahrajabian, 2023). In addition, phenolic nature of tannins is responsible to anti-biofilm action with inhibition of quorum sensing and induction of trans-glucosylase activity (Villanueva *et al*., 2023).

Table 1: Phytochemical analysis of methanol extract of *C. zeylanicum* bark

Test to Alkaloids	
(a) Dragendroff's reagent	+ (Positive)
(b) Hager's test	+ (Positive)
(c) Wagner's reagent	+ (Positive)
Test for Steroid Glycosides	
(a) Salkowski test	+ (Positive)
(b) Libermann Burchard test	+ (Positive)
Test for Tannins	
(a) Lead acetate	+ (Positive)
(b) With Phenazone	+ (Positive)
(c) With Gelatin	+ (Positive)
Test for Pseudo tannins	
Test for Catechin	
Test for Condensed and hydrolysable tannins	

Ferric chloride test	+ (Positive condensed tannins)
Test for Saponin	
Foam test	-(Negative)
Test for Phenolics	
Folin–Ciocalteau test	+ (Positive)
Test for Total Flavonoids	
(a) Ammonia	+ (Positive)
(b) Shinoda's test	+ (Positive)
(c) Alkaline reagent	+ (Positive)

UV absorption spectrum of methanol extract of C. zeylanicum Bark

UV spectrum of the methanol extract showed three distinctive peaks at 220, 278 and 300 nm. These absorption patterns indicate that the methanol extract is rich in alkaloids, flavonoids and phenolic compounds. In one research study, two proanthocyanidin (phenolic compound) from the phenolic extract of green tea and the isolated fractions elicited UV spectra with maxima at 279 nm (Karamać, Kosińska & Chavan 2005). Similar spectral data was measured for proanthocyanidin fractions of leguminous seeds at 279nm (Tsamo, Mohammed & Dakora, 2020). Based on the literature support, the peak at 278 nm revealed the abundance of phenolic compounds in methanol extract of *C. zeylanicum* bark as shown in Figure 1.

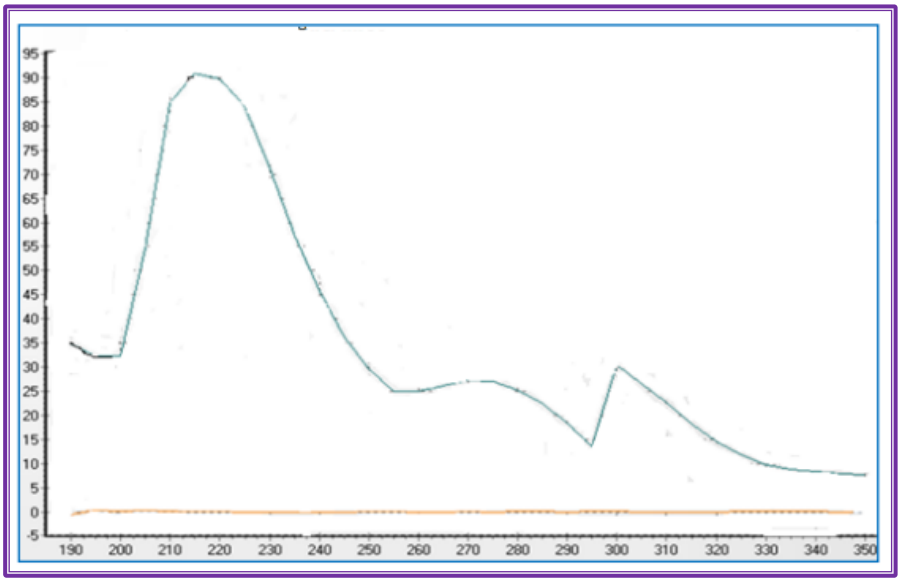

Figure 1: UV –Visible spectrum of phenolic content present in methanol extract of *C. zeylanicum* Bark

Isolation and identification of Phenolics in methanol extract of C. zeylanicum by chromatography

In total, TLC tested 96 fractions eluted from the column for the presence of procyanidins. The spots on the TLC plate were developed with vanillin-HCl. No spots for procyanidins were found in the fractions from 1 to 74, in all three solvent systems used. The spots obtained for elutions from 75 to 96 in toluene: ethyl acetate solvent system confirmed the presence of procyanidins as shown in Figure 2. Elutions with the same retardation factor was taken and the phenolic rich fraction was prepared. TLC for phenolic analysis of raw plant extracts is affordable and less time consuming since numerous detections can be done on a single TLC plate (Khoddami, Wilkes & Roberts, 2013). Phenolic compounds and flavonoids present in sage species (Sajewicz *et al*., 2012) and in *Baccharis trimera* extract were identified using TLC (De Oliveira *et al*., 2012).

Figure 2: TLC analysis of phenolic-rich fraction of *C. zeylanicum* bark

GC-MS analysis of phenolic-rich fraction of C. zeylanicum bark

The results of GC-MS analysis revealed that the phenolic rich fraction is primarily composed of five major compounds as shown in Table 2 and Figure 3. The peak at RT 12.23 value confirms the presence of procyanidins in the fraction. Due to the presence of these five major compounds, phenolic-rich fraction of *C. zeylanicum* bark can be used in various pharmaceutical applications.

Table 2: GC-MS Analysis of phenolic-rich fraction of *C. zeylanicum* bark

S.No	Compound Name	Retention Time (min)
1	Octadecanoic acid 9,10, -dichloro, methyl ester	11.38
2	Glycine n(3a,5a,7a,12a)24-oxo3,7,12, tris (trimethylsiyl)ocy) cholan-24-yl), methyl ester	11.66
3	(-)- Epicatechin-(4 β→8)-(-)-epicatechin	12.23
4	16-Octadecanoic acid, methyl ester	12.52
5	Dodecanoic acid ,2,3-bis (acetylox) propyl ester	12.65

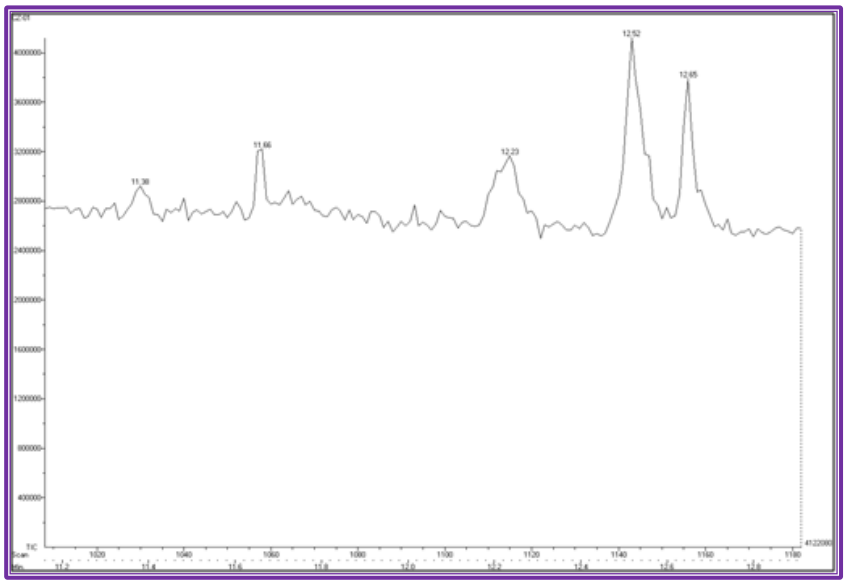

Figure 3: GC-MS spectrum of phenolic-rich fraction of *C. zeylanicum* bark

MIC of phenolic-rich fraction of C. zeylanicum against P. aeruginosa

MIC is a quantitative method to confirm the resistance of microorganisms to a given antimicrobial agent (Kowalska-Krochmal & Dudek-Wicher, 2021). To examine the efficacy of phenolic rich fraction of bark extract to inhibit biofilm formation, sub -MIC doses were used where the MIC against a planktonic form of *P. aeruginosa* was determined. The results revealed that all selected sub-MIC doses (1.56, 3.12, 6.25 mg/ml) of phenolic-rich fraction of *C. zeylanicum* bark exhibited significant ($P< 0.05$) biofilm attenuation when compared to untreated control as shown in Figure 4.

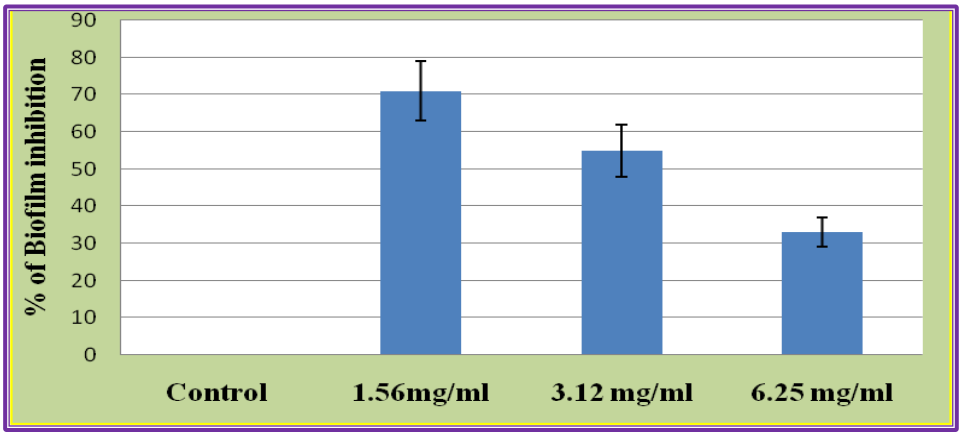

Figure 4: Anti-biofilm activity of phenolic rich fraction of *C. zeylanicum* bark on *P. aeruginosa*

It was also observed that the minimum dose (1.56 mg/ml) showed the highest (71±8%) attenuation of biofilm formation, and the highest dose (6.25 mg/ml) showed the minimum (33± 4 %) of attenuation of biofilm formation, while the intermediate dose (3.12 mg/ml) showed moderate (55± 7%) attenuation of biofilm formation when compared to untreated control. Hence, the result reports an inverse relationship between the sub-MIC doses of phenolic-rich fraction of *C. zeylanicum* bark and biofilm inhibition on *P. aeruginosa.* Therefore, suppression of biofilm in *P. aeruginosa* was efficient at sub- MIC doses of the bark extract without eliciting the resistance mechanism of the bacteria. Same results were shown when screened the clove herbal extract for inhibitory properties of quorum sensing on *P. aeruginosa*. It was reported that eugenol present in cloves at sub-inhibitory concentrations showed good inhibition of quorum sensing (Lou *et al*., 2019). When investigating the effect of Egyptian medicinal plant such as *Mangifera indica* on the QS signaling system of *P. aeruginosa,* methyl gallate compound extracted from *M. indica, showed* the most potent quorum sensing inhibition (Naga *et al*., 2023).

Effect of phenolic-rich fraction of C. zeylanicum bark extract on swimming, swarming and twitching the motility of P. aeruginosa

Attenuation of swimming, swarming and twitching the motility of *P. aeruginosa* by phenolic-rich fraction of *C. zeylanicum* bark at sub-MIC doses (1.56, 3.12, 6.25 mg/ml) was determined as shown in Table 3. It was observed that the lowest MIC (1.56 mg/ml) showed maximum and significant (P< 0.05) attenuation of all three kinds of motilities than the other two sub-MICs. Hence, the result reports an inverse relationship between the sub-MIC doses of phenolic-rich fraction of *C. zeylanicum* bark and the motility ability of P. aeruginosa, without inducing the resistance mechanism of the bacteria. Reduction in swimming, swarming and twitching diameter indicated the QS inhibitory effect of phenolic compounds present in *C. zeylanicum* bark against *P. aeruginosa.*

Table 3: Effect of Sub-MIC doses of phenolic-rich fraction of *C. zeylanicum* bark extract on swimming, swarming and twitching the motility of *P. aeruginosa*

The Motility	Control (Untreated)	6.25 mg/ml	3.12 mg/ml	1.56 mg/ml	Tobramycin (Standard)
Swimming the motility (cm)	3.2 ± 0.8*	1.9 ± 0.3	0.8± 0.2	0.3± 0.1*	0
Swarming the motility (cm)	3.0 ± 0.5*	1.6± 0.25	0.7± 0.1	0.4± 0.1*	negligible
Twitching the motility (cm)	2.1 ± 0.5*	1.7 ± 0.2	0.7± 0.2	0.4± 0.1*	negligible

*Note: *Significant difference between control and least MIC (P< 0.05)*

The area of the motility zones of *P. aeruginosa showed* significant reduction in the swimming, swarming and twitching the motility *when* treated with sub-MIC dose (1.56, 3.12, 6.25 mg/ml) of phenolic-rich fraction of *C. zeylanicum* bark as shown in Figure 5.

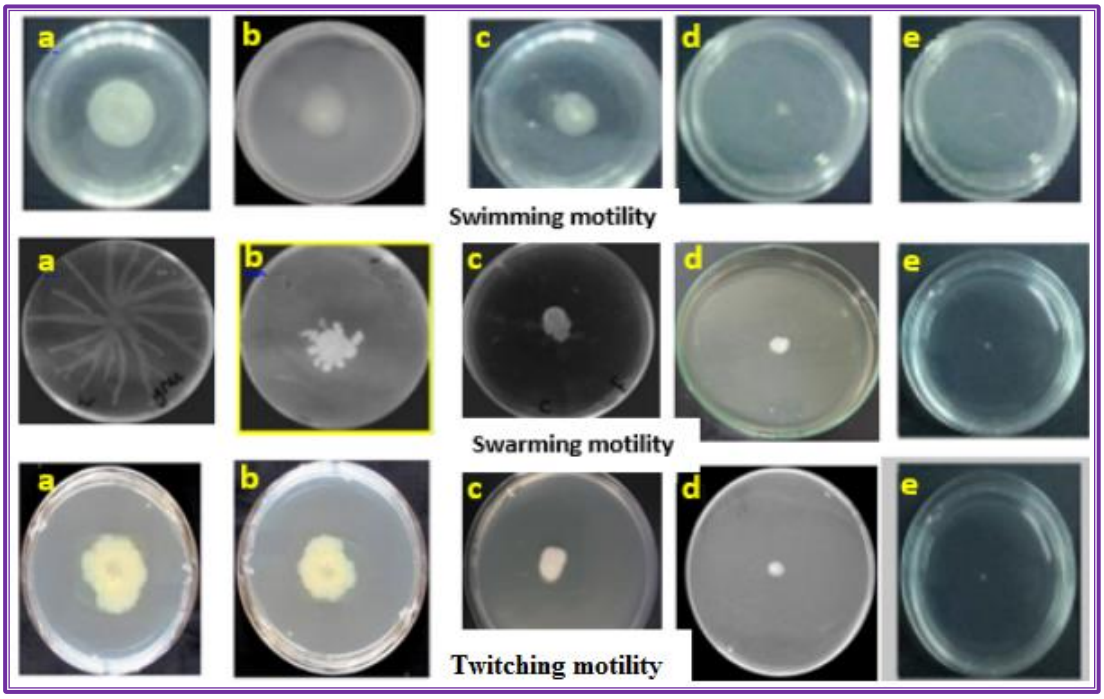

Figure 5: Inhibition of swimming, swarming and twitching the motility of *P. aeruginosa by* phenolic-rich fraction of *C. zeylanicum* bark at sub-MIC doses. a) negative control b) 1.56 mg/ml, c) 3.12 mg/ml and d) 6.25 mg/ml e) positive control

The in *vitro* formation of biofilm is initiated by the attachment of bacterial cells to a surface. Bacterial movement induced by polar flagella in aqueous environments (swimming the motility) aids this attachment (Zegadło *et al*., 2023). Bacteria then start to multiply and get entrapped in extracellular polymeric substances. The extension and retraction of type IV pili plays an important role in twitching the motility, which aids the bacteria to move within the biofilm community (Okaro *et al*., 2022). Hence twitching activity is crucial for the cell-to-cell interactions which are essential for microcolony synthesis and initiation of cell agglomeration (Casciaro *et al*., 2019). In addition to these two motilities, *P. aeruginosa* can swarm across the viscous medium. It is more complicated than the other two motilities which is characterised by a coordinated movement of cells across a viscous medium with the help of both polar flagella and type IV pili. Swarming the motility aids in colonization of surfaces and plays an important role in the early biofilm formation (Murray, Ledizet & Kazmierczak 2010). The suppression of swarming the motility of *P. aeruginosa* by a fatty acid (*anteiso*-C15:0) was analysed and reported that *anteiso*-C15:0 showed good inhibitory effect on swimming the motility and completely suppressed swarming the motility at a concentration of 10μgmL^{-1} (Oura *et al*., 2015). Our study showed a significant impact on twitching the motility. In contrast, other study showed that furanone inhibited biofilm-formation, in *P. aeruginosa* but had *a* negative impact on twitching and swarming the motility zone formation (Otton *et al*., 2017).

Conclusion

This research work explored the potential of *Cinnamomum zeylanicum* (Ceylon cinnamon) bark extract in fighting against *Pseudomonas aeruginosa* biofilms, which is an important culprit for the development of antibiotic the resistance and chronic infections. The study found that the extract, rich in condensed tannins (proanthocyanidins), effectively inhibits biofilm formation and quorum sensing, a process that helps bacteria communicate and resist antibiotics. The extract showed significant anti-biofilm activity at sub-MIC levels, reducing biofilm formation by 71%, 55%, and 33% at different concentrations. These findings suggest that procyanidins in Ceylon cinnamon bark could be used as lead molecules to develop new therapeutics that target biofilm formation and quorum sensing, potentially reducing antibiotic the resistance and improving treatment outcomes for chronic infections.

Conflicts of Interest

Authors did not disclose any conflicts of interest.

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