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Antibiofilm properties of *Clitoria ternatea* flower anthocyanin-rich fraction towards *Pseudomonas aeruginosa*

Ethel Jeyaseela Jeyaraj[1,2]†, Sheila Nathan[3]†, Yau Yan Lim[1]†,‡ and Wee Sim Choo[1,*,†]

Abstract

In Asia, *Clitoria ternatea* flowers are commonly used as a traditional medicinal herb and as a food colourant. Their bioactive compounds have anti-inflammatory, anti-microbial and anti-biofilm activities. *Pseudomonas aeruginosa* is one of the major pathogens that cause biofilm-associated infections resulting in an increase in antimicrobial resistance. Hence, the aim of this study was to investigate if the anti-biofilm properties of the anthocyanin-rich fraction of *C. ternatea* flowers were effective against *P. aeruginosa*. The effect of the anthocyanin-rich fraction of *C. ternatea* flowers on *P. aeruginosa* biofilms formed on a polystyrene surface was determined using the crystal violet assay and scanning electron microscopy (SEM). The anthocyanin-rich fraction reduced biofilm formation by four *P. aeruginosa* strains with a minimum biofilm inhibitory concentration value ranging between 0.625 and 5.0 mg ml^{-1}. We further show that the biofilm-inhibiting activity of *C. ternatea* flowers is not due to the flavonols but is instead attributed to the anthocyanins, which had significant biofilm inhibitory activity (64.0±1.1%) at 24 h in a time–response study. The anthocyanin-rich fraction also significantly reduced bacterial attachment on the polystyrene by 1.1 log c.f.u. cm^{-2} surface based on SEM analysis. Hence, anthocyanins from *C. ternatea* flowers have potential as an agent to decrease the risk of biofilm-associated infections.

INTRODUCTION

Antibiotics have been used for the treatment of bacterial diseases since the early 20th century [1]. Antibiotics are incorporated in animal feed to promote growth, and to treat and prevent disease while antibiotic-plant-based sprays and solutions are used to combat plant infections [2]. An increase in the use of antibiotics in clinical practice as well as in agriculture and poultry farming has not only led to the emergence of antibiotic resistance but also to environmental antibiotic pollution (e.g. antibiotics released from human waste streams and waste from livestock farming into water, manure and soil). This calls for stricter management of antibiotic usage to contain the development of resistant microorganisms [3]. Antibiotic resistance limits the effectiveness of current drugs and, significantly, causes treatment failure of bacterial infections [4–6]. Treatment of infections becomes a challenge when resistance at both the cellular and community levels occurs in parallel [7]. Biofilms are aggregates of microbial cells (single or multiple bacterial species) encased in a self-produced matrix mainly composed of polysaccharides, proteins, nucleic acids, lipids, extracellular DNA and water [8, 9] that are attached to biotic (living tissues) or abiotic surfaces (e.g. medical implants) [10]. Biofilm infections can be divided into device- and non-device-related infections. Non-device-related infections occur in human tissues, causing various chronic diseases such as cystic fibrosis pneumonia, periodontitis, endocarditis and chronic wound infections [11, 12]. Device-related infections involve medical implants and catheters such as intravenous and urinary catheters, joint prostheses, cardiovascular and biliary stents, contact lenses, pacemakers and breast implants [11, 12].

Pseudomonas aeruginosa is an opportunistic pathogen responsible for severe nosocomial infections and has developed resistance to multiple antimicrobial agents. *P. aeruginosa* infections pose a high risk to immunocompromised patients where infections

can cause severe respiratory problems [13, 14]. *P. aeruginosa* has been associated with patients with chronic or inherited lung diseases such as bronchiectasis and cystic fibrosis. These individuals are highly susceptible to persistent pulmonary infection, ventilator-associated pneumonia, catheter-related bloodstream infections as well as chronic wounds where biofilm formation benefits *P. aeruginosa* in terms of maintaining persistent infections, ability to evade the immune system and development of antimicrobial resistance [13]. In a clinical situation, infections caused by biofilm-forming pathogens require aggressive treatment with a combination of antibiotics, which is challenging as infections may recur or persist even after the recommended treatment protocol due to the presence of biofilms [15, 16]. When the problem is device-related, there is a requirement to remove and replace the device, which requires surgery, increased costs, risks and complications [16]. Quorum sensing is an important regulator of biofilm formation. Bacteria use this intercellular signalling system in the communication and regulation of gene expression through the release of autoinducers into the environment. These autoinducers may serve as important targets for the repression of biofilm formation [14]. The *las* and *rhl* quorum sensing systems have been linked to biofilm formation, maturation and virulence of *P. aeruginosa* [17, 18].

Numerous studies have focused on identifying antibiofilm agents from natural products and synthetic agents that are non-biocidal, as such molecules should not lead to the development of drug resistance [19]. *Clitoria ternatea* flowers, commonly known as butterfly pea flowers, have been traditionally used to treat various ailments and as a food colourant. *C. ternatea* flowers, being rich in anthocyanins and flavonols, have been reported to possess various bioactive properties including antioxidant, antibacterial, anti-diabetic and anti-inflammatory activities [20–22]. The extract has antibacterial activity against, for example, *Escherichia coli*, *Klebsiella pneumoniae* and *P. aeruginosa* [20, 23]. However, the mode of action of the extract for antibacterial and anti-biofilm activities remains unclear. Thus, this study was undertaken to explore the anti-biofilm properties of the anthocyanin-rich fraction of *C. ternatea* flowers towards *P. aeruginosa* at non-biocidal concentrations.

METHODS

Preparation and extraction of samples

Freshly harvested *C. ternatea* cv. Double Blue flowers were obtained from a horticulture nursery in Subang Jaya, Malaysia. Only the petals of fresh *C. ternatea* flowers were used in the extraction process. The petals were cut into small pieces (approximately 0.5×0.3 cm) before usage. Ethanol extracts of the fresh flower material (10 g) were prepared by immersing the flowers in 200 ml of 50% ethanol with constant shaking for 3 h at room temperature (25 °C) while aqueous extracts were prepared from flowers in 200 ml distilled water subjected to constant shaking in a 50 °C water bath for 1 h. Extracts were then vacuum-filtered. The solution was concentrated under vacuum at 45 °C using a rotary evaporator followed by freeze-drying at −80 °C. The freeze-dried extracts were kept at −80 °C until further analysis.

Semi-purification of crude extract by Amberlite XAD-16 column chromatography

The anthocyanin-rich fraction was prepared according to a previous study [24]. The freeze-dried extract of *C. ternatea* flowers (5 g) was dissolved in distilled water (100 ml), adjusted to pH 2 and partitioned with ethyl acetate (100 ml) to facilitate the removal of flavonols. The aqueous fraction (containing anthocyanins) were collected and again partitioned with ethyl acetate twice more. The ethyl acetate fractions (containing flavonols) were pooled together and kept separately. The aqueous and ethyl acetate fractions were concentrated under vacuum at 37 °C in a rotary evaporator. The anthocyanin fraction was subjected to further purification using Amberlite XAD-16 column chromatography. Briefly, one litre of purified water was used to rinse the column followed by activation with 0.5 l of 2% aqueous sodium hydroxide solution. Acidified water (one litre) was used to wash the column to pH 3. The concentrated sample (10 ml) of the anthocyanin fraction was loaded onto the column, which was then rinsed with 0.3 l of acidified water (pH 3) at a flow rate of 10 ml min⁻¹ to remove phenolic acids. Anthocyanins were then eluted with acidified methanol [95:5, methanol–acidified water (pH 2), v/v]. The methanol fraction (containing anthocyanins) and ethyl acetate fraction (containing flavonols) were subjected to freeze-drying at −80 °C. The freeze-dried extracts were kept at −80 °C until further analysis.

Antibiofilm and biofilm disruption assay

The antibiofilm activity of the *C. ternatea* flower anthocyanin-rich fraction obtained from partial purification of the crude ethanol extract was tested against laboratory control strains obtained from the American Type Culture Collection (ATCC) of *P. aeruginosa* (ATCC 9027, ATCC 27853, ATCC 10145 and ATCC BAA-47). Biofilm inhibitory activity was evaluated using a microtitre plate assay [25]. Bacteria were grown in 10 ml of Brain Heart Infusion (BHI) broth at 37 °C for 18 h. The overnight bacterial suspension was adjusted to 0.5 McFarland standard to obtain an optical density reading at 625 nm (OD_{625}) of 0.1, which is about 1×10^8 c.f.u. ml⁻¹. The suspension was then diluted 1:100 in BHI broth containing 1% glucose. The anthocyanin-rich fraction was sterilized using a polyethersulfone Millipore filter (0.22 µm). The diluted bacterial suspension (100 µl) was added to a 96-well plate after which 100 µl of the anthocyanin-rich fraction was added to obtain a final concentration of 0.313–5.0 mg ml⁻¹. The plate was incubated at 37 °C for 24 h. After incubation, the contents of the wells were aspirated and wells were rinsed with water gently, three times. The wells were heat fixed at 60 °C for 1 h followed by

Table 1. List of primers used for real-time PCR analysis

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
lasI	AGGTGTTCTTGAGCATGTAGG	CGAGATGGAGATCGATGGTTATG
lasR	GATGAAGGCGTTCTCGTAGTC	GCTCAAGTGGGAAATTGGAATG
rhlI	TTCATCGAGAAGCTGGGTTG	GCTCCTGGCGATGATGTAG
rhlR	GGCTTCGATTACTACGCCTATG	TTCTGCATCTGGTAGCGTTC
algC	CAACTGGTCCAGCAGTTCTC	GGTGTAGTTCAGCTTCCAGTTC
pelC	GATCTGCAACTGGTCGATGA	GTTCTTGTACTGCCACTCCTC
lasB	GATCGGCTACGACATCAAGAA	GCACGTCGATACCGTTGTA
pslD	CGATCGTTGTTGACGGTGTA	TTCGTGCCCAAGTCGATG
lecB	ACGGCGTCGTTGTAATCAT	AAAGTGCAGATCCAGGTCAG
proC	CAGGCCGGGCAGTTGCTGTC	GGTCAGGCGCGAGGCTGTCT
rpoD	ACTTCTTGGCGATGGAGATG	TCGCCGAGATCAAGGAAATC

staining with 0.2 ml of 0.1% crystal violet for 10 min. The wells were rinsed with water and air-dried. Lastly, 200 µl of 95% ethanol was added and absorbance was measured at 570 nm. The negative control was a bacterial suspension with BHI broth (no treatment) while the positive control was a bacterial suspension with the addition of 100% sodium hypochlorite, a disinfectant with effective biofilm-eliminating properties [26]. The strains were classified into the following groups based on biofilm formation: OD_{570} <2=weak biofilm producer, 2≤OD_{570}≤4=moderate biofilm producer, OD_{570} >4=strong biofilm producer [27]. As for the biofilm disruption assay, the wells were inoculated with bacterial suspension alone and incubated for 24 h at 37 °C. The wells were washed three times with PBS before the addition of extracts with the same concentration range as above, and further incubated for 24 h. After 24 h, the solution in the wells was removed and wells were rinsed with water three times before being subjected to the crystal violet assay as described above.

Growth curve (pour-plate method)

The effect of the anthocyanin-rich fraction (5 mg ml^{-1}) on *P. aeruginosa* ATCC 9027 growth was evaluated using the standard pour-plate method [28]. Serial dilutions (1:10) at 10^3 to 10^6 of treated bacteria culture was performed and 100 µl of individual diluted bacteria culture was dispensed onto the bottom of Petri dishes. Then 18 ml of molten Mueller-Hinton agar (45 °C) was poured and mixed with the sample by gently swirling the plate. The agar was allowed to thoroughly solidify before inverting the plates for incubation at 37 °C for 24 h. The number of *P. aeruginosa* (ATCC 9027) colonies was recorded from plates with 30–300 colonies as colony-forming units (c.f.u.). The microbial data were then transformed to logarithm of the number of c.f.u. (log c.f.u. ml^{-1}) [29].

Attachment assay

The attachment assay was carried out on *P. aeruginosa* ATCC 9027 to determine if the anthocyanin-rich fraction (5.0 mg ml^{-1}) could reduce or inhibit bacterial attachment onto hydrophobic (polystyrene) and hydrophilic (glass) surfaces [25]. Polystyrene surfaces were obtained by cutting Petri dishes into 7 cm in length by 2 cm in width. The polystyrene strips were sterilized in 10% sodium hypochlorite and then immersed in 70% ethanol followed by distilled water. Glass slides were sterilized by immersion in 100% acetone followed by 70% ethanol and rinsed with distilled water. Bacteria were grown in BHI broth at 37 °C for 18 h. The bacterial cell culture was centrifuged at 4000 *g* (10 min at 4 °C) and the pellet was washed gently with PBS and resuspended to achieve an optical density reading of 1±0.2 at 550 nm. The bacterial suspension (15 ml) was added to 15 ml of he anthocyanin-rich fraction (5 mg ml^{-1}) or 15 ml of BHI broth (negative control) in a Petri dish with a glass or polystyrene strip and incubated at 37 °C for 1 h. After incubation, the glass or polystyrene strips were gently rinsed three times with PBS followed by staining with 0.1% (w/v) crystal violet. The attached cells were counted using a light microscope (BX51) equipped with NIS-Elements software (Olympus) at 1000× magnification with immersion oil and a total of 30 fields of view. The number of attached cells was expressed as log c.f.u. cm^{-2}.

Scanning electron microscopy (SEM) analysis

The biofilms of *P. aeruginosa* ATCC 9027 were allowed to grow on polystyrene squares (1×1 cm) placed in 24-well polystyrene plates supplemented with or without anthocyanin-rich fraction of *C. ternatea* flowers (5 mg ml^{-1}) and incubated for 24 h at 37 °C. The cells were fixed for 2 h using 2.5% glutaraldehyde before washing with PBS. The samples were dehydrated with
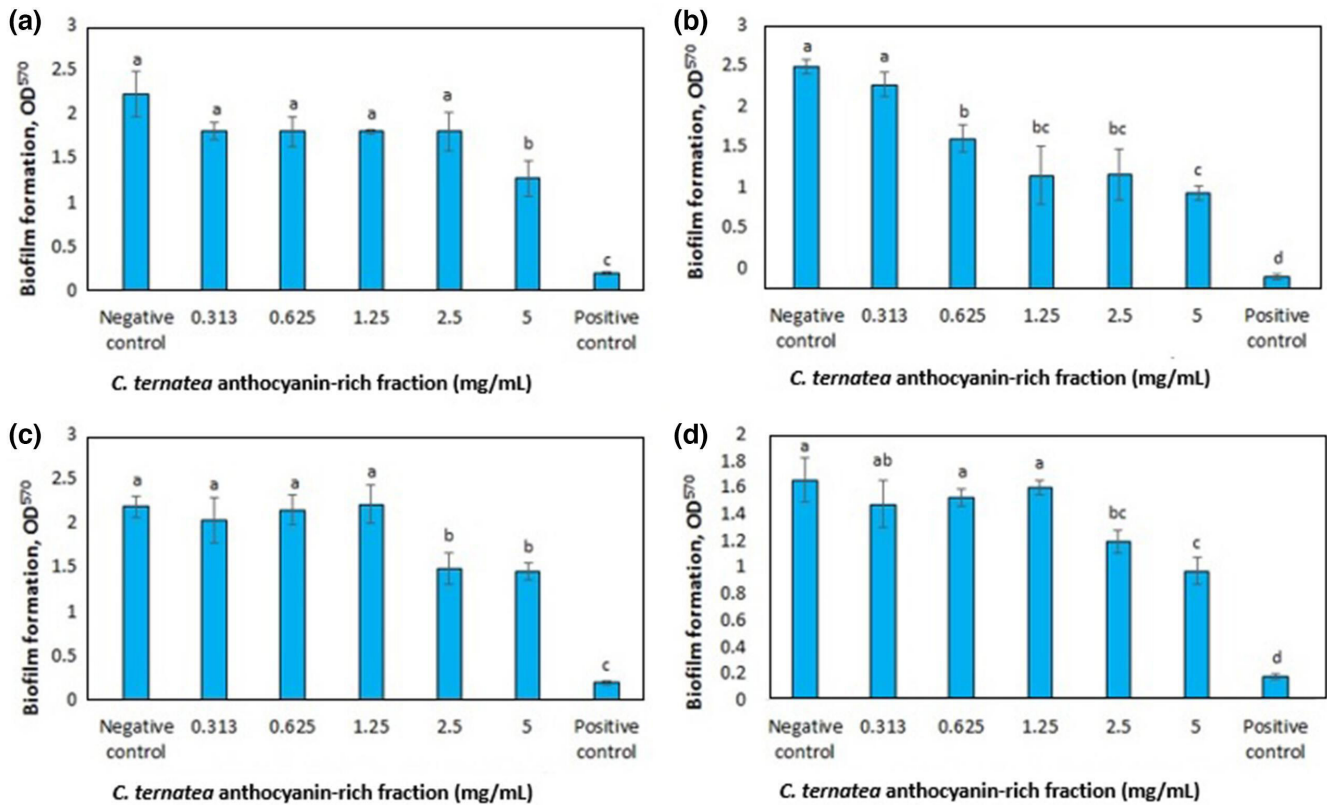
Fig. 1. Effect of the *C. ternatea* anthocyanin-rich fraction on biofilm formation at 24 h of *P. aeruginosa* (a) ATCC 10145, (b) ATCC 9027, (c) ATCC 27853 and (d) ATCC BAA-47. Different letters indicate significant differences in biofilm formation at $P<0.05$, as analysed by one-way ANOVA. Bacterial suspension without treatment was the negative control while bacterial suspension treated with sodium hypochlorite was used as the positive control.

increasing concentrations (50, 60, 70, 80, 90 and 100 %) of ethanol sequentially for 10 min each time. The samples were kept in a desiccator containing silica gel. The surface of the samples was gold-sputtered using a sputter coater (Q150RS; Quorum) for 10 min prior to SEM examination. The effects of the extract on bacterial biofilm formation on a polystyrene surface was examined using an Hitachi S3400N-II scanning electron microscope by observing bacterial cell morphology as well as cell numbers and biofilm cell density.

Quantitative real-rime PCR (RT-qPCR)

P. aeruginosa ATCC 9027 (1×10^6 c.f.u. ml^{-1}) was treated with and without anthocyanin-rich fraction of *C. ternatea* flowers (5 mg ml^{-1}) in a 24-well polystyrene plate and incubated for 24 h at 37 °C. After incubation, cells were washed with sterile PBS three times and collected after 10 min of centrifugation (4000 *g* at 4 °C). The extraction of total RNA from treated and untreated groups was done using TRIzol Reagent (Invitrogen) followed by cDNA synthesis using the Maxima First Strand

Table 2. Minimum biofilm inhibitory concentration (MBIC) of *C. ternatea* anthocyanin-rich fraction against *P. aeruginosa* strains

Bacterial strain	Biofilm production*	MBIC (mg ml^{-1})	Biofilm inhibition (%) at 5 mg ml^{-1}
P. aeruginosa ATCC 10145	Moderate	5.0	42.8±7.4[ab]
P. aeruginosa ATCC 9027	Moderate	0.625	57.0±3.3[b]
P. aeruginosa ATCC 27853	Moderate	2.5	33.6 ± 4.2[a]
P. aeruginosa ATCC BAA-47	Weak	2.5	41.4±6.4[ab]

MBIC values were means of three biological replicates. Values for biofilm inhibition (%) are expressed as mean±SD. Different letters indicate significant difference in biofilm inhibition among different bacterial strains at $P<0.05$, as analysed by one-way ANOVA.
*Weak: OD$_{570}$ <2; Moderate: 2≤OD$_{570}$≤4; Strong: OD$_{570}$ >4 [25].
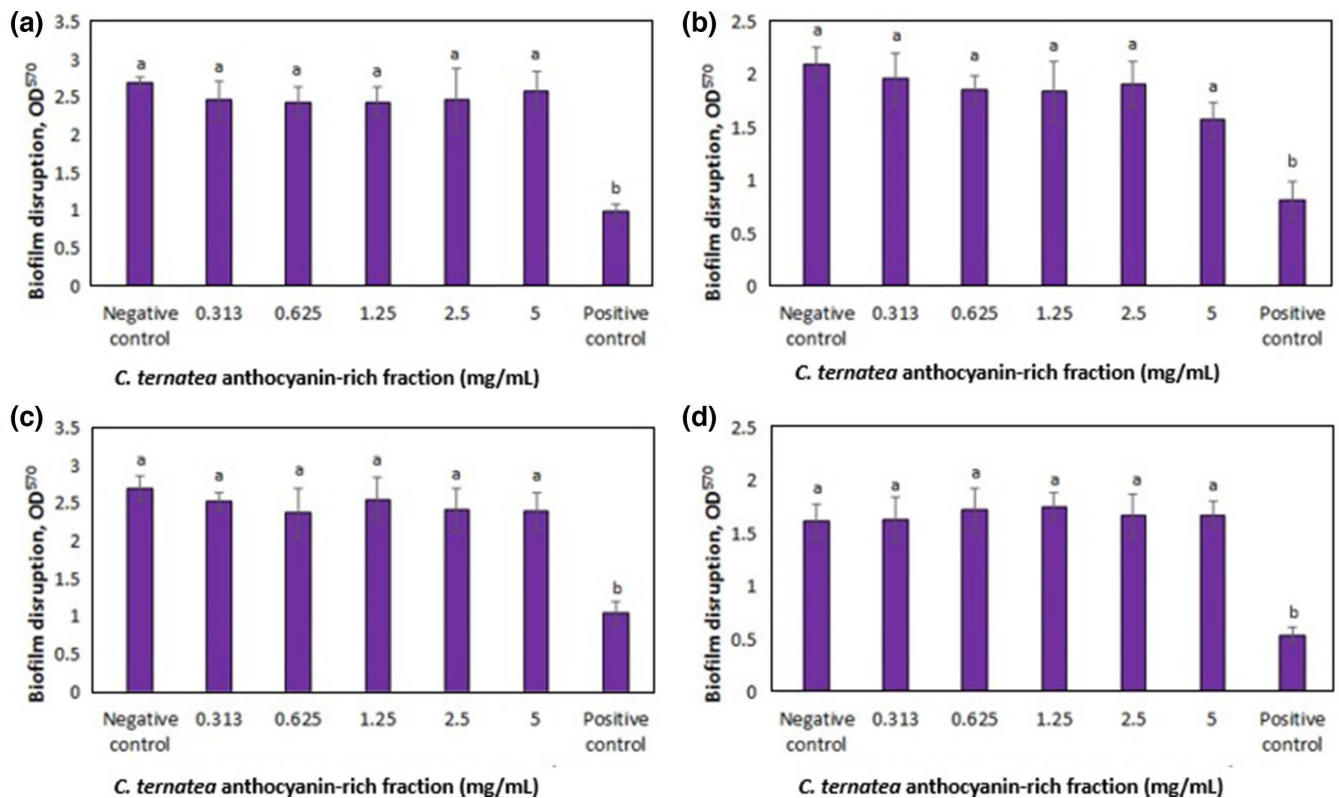
Fig. 2. Effect of the *C. ternatea* anthocyanin-rich fraction on biofilm disruption at 24 h of *P. aeruginosa* (a) ATCC 10145, (b) ATCC 9027, (c) ATCC 27853 and (d) ATCC BAA-47. Different letters indicate significant differences in biofilm disruption at $P<0.05$, as analysed by one-way ANOVA. Bacterial suspension without treatment was the negative control while bacterial suspension treated with sodium hypochlorite was used as the positive control.

cDNA Synthesis Kit (Thermo Scientific) according to the protocol stated by the manufacturer. RT-qPCR was performed with the 2×Brilliant III Ultra-fast SYBR Green qPCR Kit (Agilent) using the primer sets described in Table 1. RT-qPCR (Quant Studio 5 Real-Time PCR system; Applied Biosystems) was performed under the following set of temperatures: 50 °C for 2 min, followed by initial denaturation for 3 min at 95 °C. Consecutively, 40 PCR cycles were performed at 95 °C for 5 s, 64 °C for 10 s and 72 °C for 10 s. The genes *proC* and *rpoD* were used as internal controls. The critical threshold cycle (C_T) value was determined followed by calculation and determination of the fold change. The change-in-threshold was determined using the $2^{-\Delta\Delta CT}$ method to calculate the relative expression of the treated group compared to the control (untreated) group [30].

Statistical analysis

All experiments were carried out in triplicate. The results were expressed as the mean value ±SD. The data obtained were analysed using either an independent *t*-test, one-way ANOVA followed by post-hoc Tukey's test or two-way ANOVA followed by post-hoc Bonferroni test and significance was set at $P<0.05$ using the SPSS 23 software (IBM).

RESULTS AND DISCUSSION

Antibiofilm activity of *C. ternatea* flower water extract, ethanol extract and anthocyanin-rich fraction

The biofilm inhibitory activity of *C. ternatea* flower crude water and ethanol extracts and anthocyanin-rich fraction was investigated by quantifying biofilm formation by four *P. aeruginosa* strains when treated with extracts at a concentration range of 0.313–5 mg ml^{-1}. The crude water and solvent extracts did not display inhibition of biofilm formation over the range of concentrations tested (data not shown). The anthocyanin-rich fraction inhibited biofilm formation with increasing concentrations (Fig. 1) as observed in the reduction of absorbance readings. A lower absorbance reading (OD_{570nm}) indicates lower biomass of biofilm as stained by crystal violet. The minimum biofilm inhibitory concentration (MBIC) was determined when there was a significant difference between the treated group at the lowest concentration and the negative control.

The MBIC of the *C. ternatea* anthocyanin-rich fraction towards the *P. aeruginosa* strains is presented in Table 2. The strains were classified as strong, moderate or weak biofilm producers based on the OD values according to an established protocol,
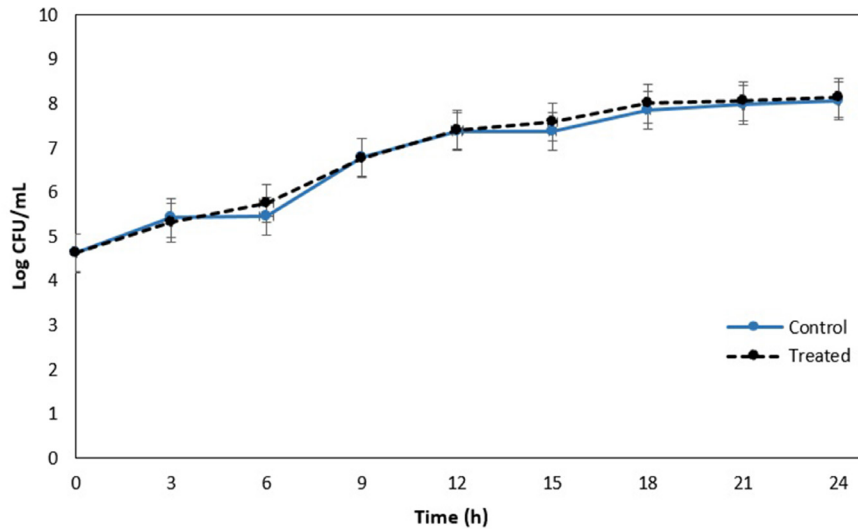
Fig. 3. Impact of the *C. ternatea* anthocyanin-rich fraction (5 mg ml^{-1}) on bacterial growth of *P. aeruginosa* ATCC 9027 over 24 h. There was no significant difference in bacterial growth over the respective time points ($P>0.05$) as analysed by an independent *t*-test.

which also enables investigation of the influence of treatment on the strains [27]. The MBIC values among the four strains were compared, with *P. aeruginosa* ATCC 9027 having the lowest value (0.625 mg ml^{-1}) and *P. aeruginosa* ATCC 10145 the highest (5 mg ml^{-1}). As the MBIC for *P. aeruginosa* ATCC 10145 was 5 mg ml^{-1}, biofilm inhibition was assessed for all strains treated with 5 mg ml^{-1} *C. ternatea* anthocyanin-rich fraction. The difference in OD at 5 mg ml^{-1} divided by the OD of the negative control was used to calculate the percentage of inhibition of biofilm. At 5 mg ml^{-1}, the biofilm inhibition of the anthocyanin-rich fraction against *P. aeruginosa* ATCC 9027 (57.0%) was higher compared to *P. aeruginosa* ATCC 27853 (33.6%) but not higher than the biofilm inhibition of *P. aeruginosa* ATCC 10145 and *P. aeruginosa* ATCC BAA-47. The MBIC value for *P. aeruginosa* ATCC 9027 treated with the anthocyanin-rich fraction was lower compared to *P. aeruginosa* ATCC 10145 and *P. aeruginosa* ATCC BAA-47, but there were no significant differences in biofilm inhibition at 5 mg ml^{-1} among the strains. The MBIC value of the anthocyanin-rich fraction against *P. aeruginosa* strains was comparable to other natural compounds such as cinnamon leaf essential oil which caused cellular shrinkage, cell wall damage and decreased
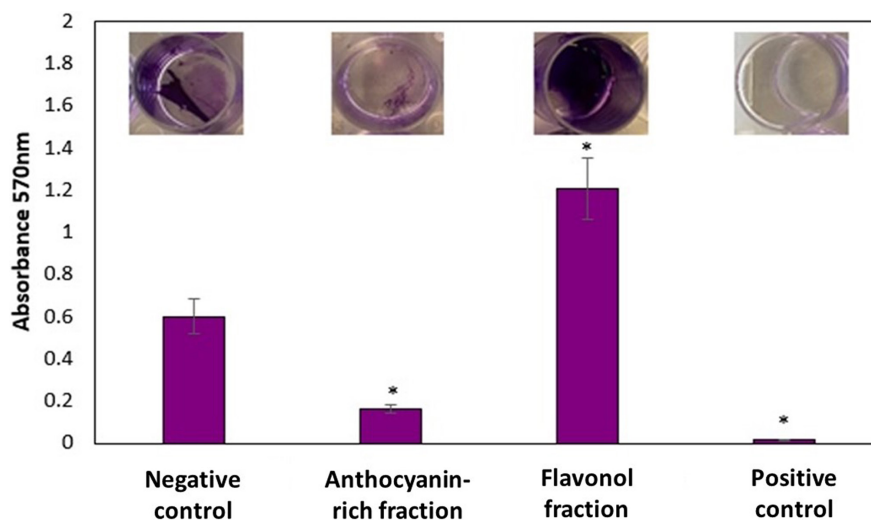
Fig. 4. Biofilm formation by *P. aeruginosa* ATCC 9027 treated with the *C. ternatea* anthocyanin-rich fraction and flavonol fraction at 5 mg ml^{-1} (24 h) and the corresponding biofilm biomass stained with crystal violet (0.1%). Significant differences relative to the negative control are marked (*$P<0.05$), as analysed by one-way ANOVA. The negative control was bacterial suspension with BHI broth (no treatment) while the positive control was bacterial suspension with the addition of sodium hypochlorite.
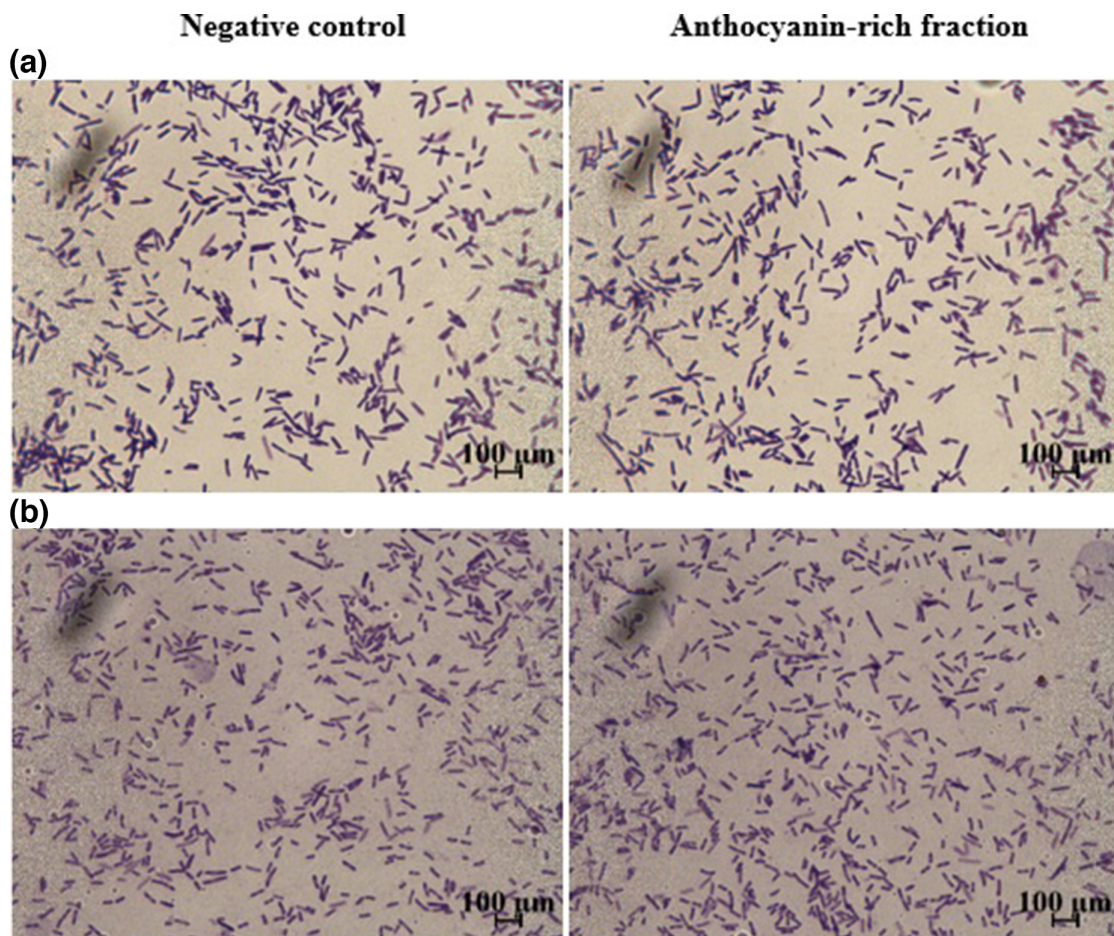
Table 3. Effect of *C. ternatea* anthocyanin-rich fraction (5 mg ml⁻¹) on the attachment of *P. aeruginosa* (ATCC 9027) to polystyrene and glass surfaces

| Bacterial strain	No. of *P. aeruginosa* cells attached			
	Polystyrene (log c.f.u. cm−2)		Glass (log c.f.u. cm−2)	
	Negative control	Anthocyanin-rich fraction	Negative control	Anthocyanin-rich fraction
P. aeruginosa (ATCC 9027)	7.03 ± 0.17*	7.12 ± 0.13*	7.25 ± 0.09*	7.19 ± 0.11*

*Same letters indicate no significant difference of the bacterial strain within the same surface at *P*>0.05, as analysed by two-way ANOVA.

biofilm density [31], and betacyanins from red pitahaya and red spinach which had antibiofilm activity through reduction of bacterial hydrophobicity and attachment to the surface without affecting bacterial cell viability [25].

The biofilm inhibition activity of *C. ternatea* flowers against *P. aeruginosa* is probably due to the anthocyanins since the effect was not observed in crude water or solvent extracts. The lack of antibiofilm activity in the crude extracts was probably due to the lower concentration of anthocyanins. A previous study reported that flower juice of *C. ternatea* had anti-biofilm activity against *P. gingivalis* [32]. However, the effective concentration was not reported as the fresh juice was tested at different percentages (v/v) of the juice. The flower juice inhibited biofilm formation at all concentrations tested compared to the control (untreated group), in which 100% *C. ternatea* flower juice was found to have the best biofilm inhibitory effect (89%) at 24 h. Another study found that *C. ternatea* flower extract at 0.06 mg ml⁻¹ significantly prevented *S. mutans* biofilm formation and eradicated the established biofilm compared to *S. mutans* treated with tetracycline [23]. However, the mechanism of action in eliciting this activity against *S. mutans* is not known. The study was extended and reported that the extract inhibited violacein

Fig. 5. Micrographs (1000× magnification; bars, 100 µm) of crystal violet- (0.1%) stained *P. aeruginosa* (ATCC 9027) attached to (a) polystyrene and (b) glass surfaces after 1 h in the presence or absence of *C. ternatea* anthocyanin-rich fraction (5 mg ml⁻¹).
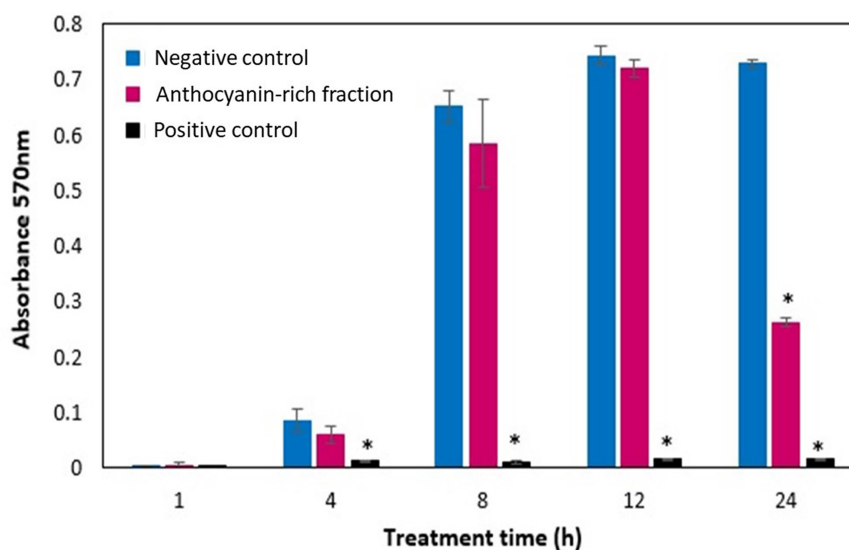
Fig. 6. Biofilm formation of *P. aeruginosa* (ATCC 9027) treated with *C. ternatea* anthocyanin-rich fraction (5 mg ml⁻¹) at 1, 4, 8, 12 and 24 h. Significant differences from the negative control group at respective time intervals are marked (*$P<0.05$), as analysed by one-way ANOVA. The negative control was bacterial suspension with BHI broth (no treatment) while the positive control was bacterial suspension with the addition of sodium hypochlorite.

produced by *Chromobacterium violaceum* bacteria, routinely used as a model for quorum sensing [33]. To the best of our knowledge, this is the first report on the anti-biofilm activity of the *C. ternatea* flower anthocyanin-rich fraction towards *P. aeruginosa* strains. *P. aeruginosa* has been listed as one of the ESKAPE pathogens responsible for a majority of nosocomial infections and capable of 'escaping' the biocidal action of antimicrobial agents [34, 35].

The ability of the *C. ternatea* anthocyanin-rich fraction to disrupt pre-formed biofilms was also determined (Fig. 2). The anthocyanin-rich fraction was not able to disrupt pre-formed biofilms against all *P. aeruginosa* strains tested. This suggests that the *C. ternatea* anthocyanins may inhibit bacterial cell attachment to the surface but are unable to act on pre-formed biofilms. Further investigations are needed to understand the possible mechanism of action for the antibiofilm activity of the anthocyanin-rich fraction as attachment is the initial step required for biofilm formation followed by bacterial cell proliferation, matrix production and detachment [36].

Effect of the anthocyanin-rich fraction on biofilm formation and bacterial viability

P. aeruginosa ATCC 9027 was selected for further studies as it had the lowest MBIC value compared to the other strains. A concentration of the anthocyanin-rich fraction of 5 mg ml⁻¹ was chosen for further investigation as this produced the highest inhibitory effect on biofilm formation. To determine if the reduction in biofilm formation was a true effect of the anthocyanin-rich fraction and not a result of toxic activity towards bacterial viability, *P. aeruginosa* (ATCC 9027) growth in the presence of 5 mg ml⁻¹ anthocyanin-rich fraction was determined every 3 h over a 24 h period. We observed that viability was not affected relative to the negative control (Fig. 3). Bacteria will develop drug resistance towards drugs that affect microbial cell viability, thus limiting the application of a drug [25]. Subsequent experiments on the antibiofilm potential and mechanism of action were carried out with the anthocyanin-rich fraction at 5 mg ml⁻¹ since this concentration did not affect bacterial viability.

The antibiofilm activity of the *C. ternatea* flowers may be attributed to the presence of flavonols and or anthocyanins. The crude extracts of *C. ternatea* flowers did not demonstrate any antibiofilm activity over the same concentration range tested with the anthocyanin-rich fraction (0.313–5 mg ml⁻¹). Therefore, the potential antibiofilm activity of the flavonol fraction was determined. Prior to column chromatography to obtain the anthocyanin fraction, the crude solvent extract of *C. ternatea* flowers was partitioned with ethyl acetate to obtain the flavonol fraction. The antibiofilm activity of the flavonol fraction was compared to the anthocyanin-rich fraction at 5 mg ml⁻¹ at 24 h. The flavonol fraction did not possess antibiofilm activity, but enhanced biofilm formation compared to the negative control seen as an increase in absorbance readings and biofilm biomass was observed by crystal violet staining (Fig. 4). Many studies have reported on the antibiofilm activity of plant-derived anthocyanin or flavonol fractions individually, towards various bacterial strains. However, the effect or the potency of these fractions may differ based on the content, composition or structural variation of the compounds. One study found the flavonoid-rich fraction (obtained through ethyl acetate partitioning) of *Centella asiatica* leaves had antibiofilm activity
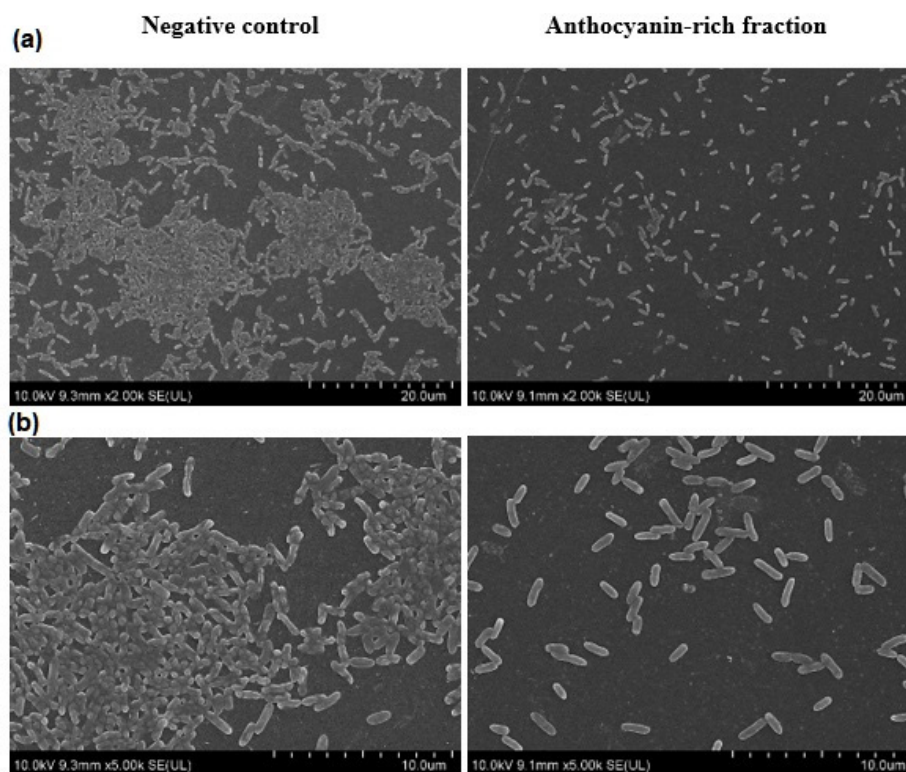
Fig. 7. SEM images of biofilm formation. Biofilm formation by *P. aeruginosa* (ATCC 9027) treated with and without *C. ternatea* anthocyanin-rich fraction (5 mg ml^{-1}) at (a) 2000× (bar, 20 µm) and (b) 5000× (bar, 10 µm) magnification at 24 h.

against *P. aeruginosa* (PAO1) [37]. The fraction was mainly composed of kaempferol, quercetin, apigenin, rutin and naringin [38] and it is believed that all the constituents acted synergistically in inhibiting biofilm formation. Although the flavonols of *C. ternatea* flowers consist mostly of various derivatives of kaempferol and quercetin [21], they differ in the structural/functional groups that, together, may have acted antagonistically to enhance biofilm formation. Thus, we believe that the antibiofilm effect of *C. ternatea* flowers is due to the anthocyanins. Further isolation of individual anthocyanins from the anthocyanin-rich fraction could identify the active molecule responsible for the antibiofilm activity.

Effect of the anthocyanin–rich fraction on bacterial attachment to hydrophilic and hydrophobic surfaces

Bacterial cell attachment to the surface is the initial step required for biofilm formation followed by bacterial cell proliferation, matrix production and detachment [36]. Hydrophobic bacteria would have a greater ability to attach to hydrophobic surfaces such as polystyrene, compared to hydrophilic surfaces [25]. Various outer membrane proteins are involved in the cell surface hydrophobicity and adhesion of *P. aeruginosa* [39]. *P. aeruginosa* (ATCC 9027) was evaluated for its ability to adhere to a hydrophobic (polystyrene) and hydrophilic (glass) surface treated with *C. ternatea* anthocyanin-rich fraction for 1 h (Table 3 and Fig. 5). Prior to the application of the anthocyanin-rich fraction, the number of *P. aeruginosa* attached to the polystyrene and glass surface was 7.03 and 7.25 log c.f.u. cm^{-2}, respectively (Table 3). The anthocyanin-rich fraction did not reduce the attachment of bacteria to the polystyrene or glass surface when compared to the negative control.

Biofilm inhibitory activity of the *C. ternatea* flower anthocyanin-rich fraction was evaluated against *P. aeruginosa* (ATCC 9027) in a time–response manner at 1, 4, 8, 12 and 24 h at 5 mg ml^{-1} (Fig. 6), helping to understand biofilm formation and

Table 4. Effect of *C. ternatea* anthocyanin-rich fraction on the number of bacterial cells on SEM images of *P. aeruginosa* (ATCC 9027)

Bacterial strain	Bacterial cell no. (log c.f.u. cm^{-2})	
	Negative control	Anthocyanin-rich fraction
P. aeruginosa (ATCC 9027)	6.14±0.06[a]	5.13±0.07[b]

Different letters indicate significant difference in bacterial cell number at $P<0.05$, as analysed by an independent *t*-test.
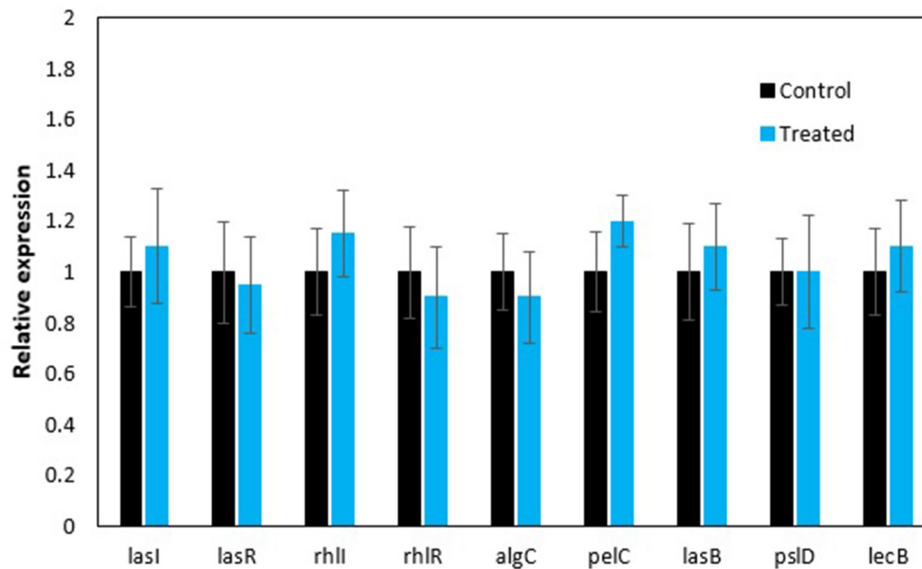
Fig. 8. RT–qPCR results of gene expression of *P. aeruginosa* (ATCC 9027) treated with *C. ternatea* anthocyanin-rich fraction for 24 h. Expression of genes in both the control and experimental groups was normalized to *proC* and *rpoD* as the reference genes. The control group expression level is designated as 1, so values >1 indicate up-regulation and values <1 indicate down-regulation. There was no significant difference between the control and treated group at $P<0.05$, as analysed by an independent *t*-test.

maturation as well as the effect of the fraction at different stages. The biofilm of *P. aeruginosa* (ATCC 9027) reached maturation around 8–12 h. The anthocyanin-rich fraction was found to have no effect against the biofilm during the maturation phase. However, the anti-biofilm effect of the fraction was observed at 24 h, which is during the stationary phase of the bacteria (Figs. 3 and 6). The results obtained suggest the anthocyanin-rich fraction does not exert its anti-biofilm effect through inhibition of the initial phase of bacterial cell attachment but possibly through a different mode of action that occurs later during the stationary phase of bacterial growth which may directly or indirectly affect biofilm formation.

P. aeruginosa (ATCC 9027) was treated with *C. ternatea* anthocyanin-rich fraction and visualized by SEM (Fig. 7). The reduction in biofilm formation of *P. aeruginosa* (ATCC 9027) observed using the crystal violet assay after 24 h (Fig. 6) was supported by the SEM images (Fig. 7). The SEM images demonstrate that the bacterial cells treated with the anthocyanin-rich fraction appear to be intact and have a lower cell density compared to the negative (untreated) control. The number of *P. aeruginosa* (ATCC 9027) bacterial cells was significantly reduced ($P<0.05$) after treatment with the *C. ternatea* anthocyanin-rich fraction (Table 4). *P. aeruginosa* (ATCC 9027) showed a 1.01 log reduction after treatment with the *C. ternatea* anthocyanin-rich fraction compared to the negative control. In the industry setting, effective cleaning should achieve at least a 1 log order of microorganism removal from surfaces [40]. The reduction of bacterial cell number (log c.f.u. cm^{-2}) using the anthocyanin-rich fraction is similar to treatment with betacyanin, a natural biofilm-inhibiting agent. Betacyanin possessed biofilm inhibition activity against *S. aureus* and *P. aeruginosa*, and was able to reduce 1.1–1.2 log c.f.u. cm^{-2} of bacteria attached to a surface [25].

Effect of anthocyanin-rich fraction on *P. aeruginosa* (ATCC 9027) gene expression

A substance may have exerted its antibiofilm activity either through alterations in gene expression, interference in quorum sensing pathways, inhibition of adhesion or alteration of metabolic pathways [40, 41]. The antibiofilm effect of the anthocyanin-rich fraction against *P. aeruginosa* (ATCC 9027) was therefore investigated by using gene expression studies. *P. aeruginosa* biofilm formation is known to be regulated by the quorum sensing system, two-component regulatory system GacS/GacA and RetS/LadS pathway, and c-di-GMP-dependent polysaccharide biosynthesis [42]. Real-time PCR-based quantification of the *lasR lasI*, *rhlR* and *rhlI* genes was performed to assess the effect of the anthocyanin-rich fraction on gene regulation in the quorum-sensing pathway as well as on the *algC*, *lecB*, *pslD* and *pelC* genes involved in biofilm formation, structure and stability and *lasB* gene involved in the cytotoxicity and virulence of *P. aeruginosa*. The housekeeping genes were *proC* and *rpoD* (Table 1).

There was no significant reduction in fold change of gene expression at the mRNA level in bacteria treated with the anthocyanin-rich fraction (Fig. 8). There are many other genes which could have been regulated that resulted in the observed inhibition of biofilm formation. One study found plant-derived ursolic acid with antibiofilm activity against *Escherichia coli* at non-toxic concentrations and inhibited biofilms without interfering with quorum sensing-related genes. Results from DNA microarrays

found that ursolic acid induced genes involved in chemotaxis and mobility (*cheA*, *tap*, *tar* and *motAB*), heat shock response (*hslSTV* and *mopAB*), cysteine synthesis (*cysK*), sulphur metabolism (*cysD*), as well as several genes with unknown functions [43].

The findings obtained in the current study suggest the possibility of other genes or pathways are likely to be responsible for the antibiofilm activity. The samples for gene expression studies were collected at 24 h because the anti-biofilm effect was not observed at earlier time points tested. It is also possible that the gene expression may have occurred before 24 h, and thus the effect was not seen at 24 h. Further studies would be needed and are recommended such as utilization of DNA microarrays to measure the expression levels of large numbers of genes simultaneously, which may assist in the elucidation of the genes that may responsible for the antibiofilm effect.

In conclusion, the anthocyanin-rich fraction of *C. ternatea* flowers has significant biofilm inhibitory activity towards *P. aeruginosa* and also reduces bacterial attachment to polystyrene surfaces. The compound can be potentially developed as an anti-biofilm agent to treat antibiofilm-related infections of *P. aeruginosa* or used in a clinical setting to prevent biofilm formation on surgical and non-surgical apparatuses. Determining the mode of action of the bioactive fraction together with *in vivo* studies would assist in improving the effectiveness of this anthocyanin-rich fraction in biofilm inhibition. In addition, the study could be extended to investigate the anti-biofilm activity of the *C. ternatea* flower anthocyanin-rich fraction towards Gram-positive bacteria such as *Staphylococcus aureus*.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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