RESEARCH ARTICLE

Evaluation of anti-quorum sensing potential of *Averrhoa bilimbi* (Kamias) against *Pseudomonas aeruginosa* ATCC 27853

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ABSTRACT

Background & Objective: Many opportunistic and nosocomial pathogens like *Pseudomonas aeruginosa* are very reliant on a bacterium-to-bacterium communication system called quorum sensing (QS). Without the aforementioned process, gene expressions associated with virulence factors will not be produced. In this study, the sub-inhibitory concentrations (sub-MICs) of methanolic leaf extract and obtained fractions from *Averrhoa bilimbi* (kamias) were screened for ability to inhibit quorum sensing-controlled phenotypes of *P. aeruginosa* ATCC 27853.

Methodology: *A. bilimbi* crude extract was fractionated through liquid-liquid extraction, producing four (4) fractions: hexane fraction, dichloromethane (DCM) fraction, ethyl acetate (EtOAc) fraction, and water (H2O) fraction. Among the sub-MICs obtained from resazurin-based fluorimetric microtiter assay, only 50 µg/mL was utilized in evaluating the anti-QS properties of crude extract and fractions against *P. aeruginosa* ATCC 27853's virulence factors: swarming motility and production of pyocyanin.

Results: In the swarming motility assay, hexane fraction (9.39 mm ± 0.67) and DCM fraction (10.82 mm ± 0.95) displayed restriction in the treated *P. aeruginosa* ATCC 27853 swarms against the control (16.20 mm ± 2.55). In the anti-pyocyanin production assay, hexane fraction exhibited an inhibition of 42.66 % ± 12.94. TLC analysis and phytochemical screening revealed that hexane fraction contains steroids, terpenes, triterpenes, and glycolipids; and DCM fraction contains cardiac glycosides, triterpenoids, terpenes, triterpenes, steroids, alkaloids, and glycolipids.

Conclusion: Hexane and DCM fractions obtained from A. bilimbi significantly inhibited swarming of *P. aeruginosa* ATCC 27853 while none of the extracts were able to significantly inhibit pyocyanin formation of *P. aeruginosa* ATCC 27853.

Keywords: *Pseudomonas aeruginosa, Averrhoa bilimbi, kamias, quorum sensing, swarming motility, pyocyanin*

Introduction

One of the dilemmas faced by healthcare practitioners is the management and treatment of infectious diseases instigated by pathogenic Gram-negative rod, *Pseudomonas aeruginosa* [1]. In a 2018 healthcare forum entitled "Fighting Multi-Drug Resistant Infections," *P. aeruginosa* was stressed by WHO as a 'critical priority pathogen' because it is considered as one of the most significant nosocomial pathogens which often demonstrates resistance against commercially available antibiotics [2].

Recently, many antimicrobial studies gave emphasis on attenuating bacterial infections through developing drugs that avert pathogenicity. Quorum sensing (QS) is a cell-to-cell communication process that allows bacteria to share information about cell density and adjust gene expression accordingly. Once it is disrupted, bacterial pathogenicity will be inhibited. Development of quorum sensing inhibitors (QSI) is a promising novel approach to the management and treatment of bacterial infections since resistance is less likely to develop. These QSIs are able to mitigate bacteria's pathogenicity without generating new forms of resistant strains [3].

Over the years, plant-derived antibacterial drugs have been developed. In the Philippines, there are a lot of widely

cultivated plants that had been used traditionally to treat ailments that were suggestive of microbial infections. In this study, the methanolic leaf extract of *Averrhoa bilimbi* (kamias) will be screened for ability to inhibit quorum sensing.

A. bilimbi is mainly cultivated in different tropical and sub-tropical countries like Philippines for homeopathic, medicinal, and nutritional purposes. The leaves of *A. bilimbi* are usually prepared as decoction or infusion. These preparations are used as an antibacterial, antiscorbutic, astringent, postpartum protective medicine, antipyretic, proctitis, and antidiabetic agent [4]. Leaves extracts of *A. bilimbi* were found to contain alkaloids, tannins, saponins, flavonoids, cardiac glycosides, glycosides, triterpenes, phenols, and carbohydrates [5,6]. The ethanolic leaf extract of *A. bilimbi* was demonstrated to have substantial antimicrobial activity against six pathogenic microorganisms namely: two Gram-positive bacteria (*Bacillus cereus* and *Bacillus megaterium*), two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), and two fungi (*Aspergillus ochraceous* and *Cryptococcus neoformans*) [7]. Contemplating its vast availability, wide array of phytochemicals, and relevant ethnopharmacological use as antibacterial, it is a suitable candidate in evaluating its potential anti-quorum sensing activity against *P. aeruginosa*. This study aimed to evaluate the effect of crude extract and fractions of *A. bilimbi* against QS-controlled phenotypes of *P. aeruginosa* ATCC 27853.

Methodology

P. aeruginosa is a Biosafety Level 2 (BSL-2) pathogen, hence, compliance with the latest biosafety protocols developed by The Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH) is imperative [8]. Prior to the implementation of the study, a certificate from the Institutional Biosafety and Biosecurity Committee (IBBC) was obtained.

Collection, Authentication, Extraction, and Fractionation of Plant Materials

One kilogram of *A. bilimbi* leaves was collected at Brgy. Parada, Sta. Maria, Bulacan (14.8051° N, 121.0032° E). Sample plant parts were submitted to the Institute of Biology, University of the Philippines Diliman for plant sample authentication. The fresh leaves were comminuted using a household homogenizer or blender. The ruptured leaves were macerated with methanol, technical grade for 48 hours. After filtration, the resulting liquid extracts were pooled together. The solvent was removed under reduced pressure in Buchi Rotavapor R-114 at 40°C. The obtained concentrate was freeze-dried through Lyoquest-85 standard freeze dryer. The lyophilized crude extract was utilized on TLC analysis and microbiological assays.

About 200 mL of distilled water was added to 800 mL of *A. bilimbi* methanolic crude extract to acquire a ratio of 4:1 methanol-water. The resulting liquid was initially partitioned with equivalent amount of n-hexane [9]. The hexane layer was then dried with anhydrous sodium sulfate and the solvent was removed *in vacuo* at 40°C. This was labeled as the hexane fraction. On the other hand, the aqueous methanol portion was concentrated *in vacuo* at 40°C. The resulting concentrate was partitioned against dichloromethane (DCM). The DCM layer was dried with anhydrous sodium sulfate, prior to solvent evaporation *in vacuo* at 40°C, resulting to the DCM fraction. The aqueous layer was partitioned with equivalent amount of ethyl acetate (EtOAc). Removal of the solvent from the EtOAc layer *in vacuo* at 40°C result to the EtOAc fraction. The remaining aqueous layer was lyophilized at Norberto R. Agcaoili Memorial Tissue Bank, Department of Orthopedics, University of the Philippines – Philippine General Hospital (UP-PGH).The hexane, DCM, and EtOAc fractions were stored in a desiccator, while the lyophilized crude and water extracts were stored at 4°C in a freezer prior to use. These fractions were all subjected to TLC, determination of sub-MICs, anti-pyocanin production, and swarming motility assays. Only the methanolic crude extract and active fractions that showed significant anti-pyocyanin and anti-swarming activity were subjected to phytochemical screening.

Thin Layer Chromatography (TLC) Analysis

A volume of 10 µL of the methanolic crude extract together with the obtained fractions from liquid-liquid partitioning was loaded on a silica gel F254 TLC plate using a CAMAG TLC Automatic Applicator. The TLC plates were developed using the solvent system: toluene: ethyl acetate: methanol: glacial acetic acid, 7.5: 1.5: 0.8: 0.2 [10]. Visualization of TLC plates were done under UV 254 nm and UV 366 nm with the aid of CAMAG TLC Visualizer. To further detect important plant metabolites, ferric chloride-methanol reagent and vanillin-sulfuric acid reagent were utilized as TLC visualization reagents.

Bacterial Strains and Culture Media

For this study, the strain of P.aeruginosa ATCC 27853 was obtained from Department of Medical Microbiology,

College of Public Health, University of the Philippines Manila (UPM). The cultivation medium was Brain Heart Infusion (BHI) agar slant. For microbiological assays, three or four isolated colonies were inoculated in 5 mL of BHI broth and were incubated without agitation for 24 hours at 37 °C. The cultures were diluted with fresh medium to approximate the density of 0.5 McFarland standard, which represents an estimated concentration of 1.5 × 108 CFU/mL [11]. The dilution was performed using the Wickerham card. The 0.5 McFarland standard was prepared by adding 9.95 mL of 1% sulfuric acid with 0.05 mL 1% barium chloride.

Preparation of Stock Solutions

About 10 mg of lyophilized A. bilimbi crude extract, hexane fraction, DCM fraction, EtOAc fraction, and water fraction were dissolved in 100 µL of 100% DMSO. The samples were mixed with a FINEPCR vortex mixer for about 5 minutes to ensure complete dissolution to the given solvent. The resulting solutions were diluted to 10 mL using ultrapure water to obtain a stock solution of 1000 µg/mL in 1% DMSO. The solutions were then filtered using 0.22 µm nylon membrane MS ® sterile syringe filter inside the biosafety cabinet. To obtain a concentration of 500 µg/mL in 0.5% DMSO, 500 µL from the stock solution was diluted to 1 mL using ultrapure water. Two-fold serial dilution was performed to all samples to give the final actual working concentration.

Approximately 67.50 mg of resazurin (alamarBlue) was dissolved in 10 mL ultrapure water resulting to a concentration of 0.675%. A sterile FINEPCR vortex mixer was utilized to mix the solution for 15 minutes to ensure homogeneity. The preparation was performed in the dark, and was then kept in a 10 mL Eppendorf tube covered with aluminum foil to protect it from light.

Determination of Sub-MICs via Resazurin-based Fluorimetric Microtiter Assay

Sub-MICs were determined using the method as described by Teh *et al.* (2017) and Clinical and Laboratory Standards Institute (2007) [12,13]. Serial two-fold dilutions of methanolic crude extract and fractions were prepared with 0.5% dimethyl sulfoxide (DMSO), resulting to a concentration range from 100 to 25 µg/mL. All ten wells in a horizontal row were filled with 95 µL sterile BHI broth and 95 µL crude/fractions/0.5% DMSO. Then, 5 µL of an overnight grown *P. aeruginosa* ATCC 27853 bacterial suspension (diluted using 0.5 McFarland standard) was incorporated to each well and mixed thoroughly. The assay was performed in triplicates. Uninoculated wells containing sterile BHI broth and crude extract/fractions/ 0.5% DMSO were used as blank and were subtracted from the original wells to remove possible interference in the fluorescence of each mixture. Growth control comprised of 0.5% DMSO and bacterial suspension. After incubation for 24 hours at 37 °C, 5 µL of 0.675% resazurin was added to all wells and incubated for another 4 hours.

Changes of color were observed and recorded. A concentration that did not show substantial bacterial growth inhibition (changed from blue to pink) was utilized in virulence factor assays. The fluorescence of the mixtures was quantified at 525 nm excitation and 595 nm emission using CLARIOstar® BMG Labtech microplate reader through MARS Data Analysis software. The percentage inhibition of the samples was then computed using the equation as follows. Both negative and test controls were blank corrected before using the below-mentioned formula.

$$\%\ inhibition = \frac{F\ negative\ control - F\ test\ control}{F\ negative\ control}\ x\ 100$$

Phenotypic Evaluation of Quorum Sensing Inhibition Activity via Virulence Factor Assays

Swarming Motility Assay

The swarming motility of *P. aeruginosa* ATCC 27853 was investigated using a 10 mL pre-warmed swarming agar containing 0.8% nutrient broth, 0.5% glucose, and 0.5% agar [14]. The agar was overlaid with 9 mL of agar augmented with 1 mL of test extracts or 0.5% DMSO at a final concentration of the chosen sub-MIC. An equivalent amount of 0.5% DMSO without the test extracts was incorporated to the agar medium that served as a solvent control. The agar media were air-dried for 5-10 minutes. A 24-hour grown *P. aeruginosa* ATCC 27853 bacterial cells were carefully inoculated using an inoculating needle at the midpoint of each agar plate. Agar plates were incubated for 24 hours at 37 °C. Constrained swarming motility of *P. aeruginosa* ATCC 27853 indicated by limited spreading will be inferred as anti-quorum sensing activities of the plant extract/fractions. The assay was performed in triplicates. The diameter of the swarms on the interface between the agar medium and petri dish was observed and was measured using a digital Vernier caliper in millimeters (mm) [15].

Anti-pyocyanin Production Assay

Quantification of pyocyanin from *P. aeruginosa* ATCC 27853 was performed using the protocol from Krishnan, T.

et al. (2012) with slight modification [16]. A 48-hour culture of *P. aeruginosa* ATCC 27853 was diluted with sterile BHI broth to approximate the density of 0.5 McFarland standard (OD 600 nm = 0.10). Concisely, 250 µL of plant extracts (crude extract and fractions of *A. bilimbi*) were combined with 2.25 mL diluted *P. aeruginosa* ATCC 27853 bacterial suspension. Sterile BHI broth treated similarly was used as blank, while 2.25 mL diluted *P. aeruginosa* ATCC 27853 bacterial suspension with 250 µL of 0.5% DMSO was utilized as control. The mixtures were incubated overnight at 37 °C. Pyocyanin was extracted by incorporating 1.5 mL of dichloromethane to the mixtures. Mixtures were mixed robustly with the aid of sterile FINEPCR vortex mixer. The DCM layer of each mixture was mixed with 0.5 mL of 0.2 M HCl. After centrifugation via Heraeus Megafuge 8R centrifuge for 15 minutes, 28 °C, 4,000 rpm, the OD absorbance of the HCl layer was quantified at 520 nm using CLARIOstar® BMG Labtech microplate reader. Percentage inhibition of extracted pyocyanin more than 50% were inferred as anti-quorum sensing activity and were then undergone to statistical analysis to determine significance.

The percentage inhibition of extracted pyocyanin was computed using the equation as follows:

$$\% \, inhibition = \frac{(OD\ negative - OD\ test)}{(OD\ negative - OD\ blank)}\ x\ 100$$

Statistical Analysis

The obtained data from the triplicate trials of microbiological assays were presented as mean ± standard error of the mean (SEM) and percentage inhibition (% inhibition) through Microsoft Office 2016. Quorum sensing inhibition activities of *A. bilimbi* crude extract and fractions in *P. aeruginosa* ATCC 27853 is evident when restriction of swarming motility was observed against the control; and pyocyanin production was diminished in the treated cultures at OD520nm in contrast to the control. Significance in the swarms' zone diameter measurements and pyocyanin absorbance measurements were determined using Student's t-test with a 0.05 level of significance. The aforementioned statistical treatment was done with the help of a Microsoft Excel add-in for data analysis, Real Statistics Resource Pack.

Results

TLC fingerprint of A. bilimbi crude extract and obtained fractions

TLC is one of the primary chromatographic technique to identify plant-derived secondary metabolites in plant extracts. In this study, the crude methanolic extract along with solvent fractions were incorporated on a silica gel F254 TLC plate. Keller-Kiliani test and Liebermann-Burchard test were only conducted to the active fractions due to their limited solubility. Figure 1 shows TLC chromatograms of A. bilimbi crude extract and fractions under different visualization methods.

To further identify phytochemical constituents, A. bilimbi crude extract and active fractions (hexane and DCM) were loaded to silica gel F254 TLC plate using the same solvent system and were treated with different TLC spray reagents (see Figure 2).

Recipe of spray reagents, treatments, and interpretations were adapted from Natural Products Isolation [17] and Practice of Thin Layer Chromatography, 3rd Edition [18].

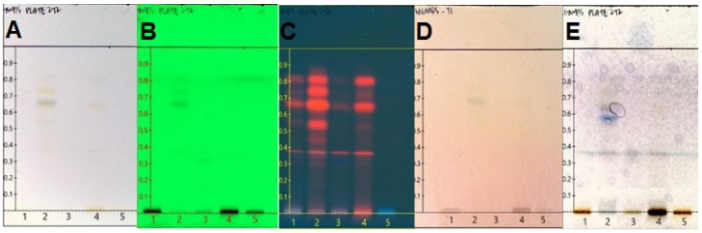

Figure 1. *TLC results of crude methanolic extract and solvent fractions of A. bilimbi leaves.*
(1) crude extract, (2) hexane fraction, (3) DCM fraction, (4) EtOAc fraction, and (5) water fraction using the solvent system toluene: ethyl acetate: methanol: glacial acetic acid (7.5: 1.5: 0.8: 0.2), visualized under (A) visible light, (B) UV-254 nm, and (C) UV-366 nm, (D) after derivatization using ferric chloride-methanol reagent, and (E) after derivatization using vanillin-sulfuric acid reagent

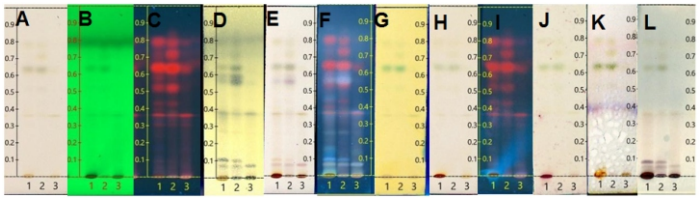

Figure 2. *TLC Chromatograms of A. bilimbi using different visualizing agents.*
(1) crude extract, (2) hexane fraction, and (3) DCM fraction using the solvent system toluene: ethyl acetate: methanol: glacial acetic acid (7.5: 1.5: 0.8: 0.2), visualized under (A) visible light, (B) UV-254 nm, (C) UV-366 nm, after derivatization using: (D) phosphomolybdic acid, (E) Tin (IV) chloride-vis, (F) Tin (IV) chloride-UV 365nm, (G) Dragendorff's reagent, (H) Borntrager reagent-vis, (I) Borntrager reagent-UV 365nm, (J) Ninhydrin reagent, (K) Ehrlich reagent, and (L) Bial's reagent

Table 1 sums up the results and interpretations of the phytochemical screening tests for *A. bilimbi* crude extract and active fractions.

Determination of Sub-MICs via Resazurin-based Fluorimetric Microtiter Assay

In determining the sub-MICs of the crude extract along with the fractions using resazurin-based fluorimetric microtiter assay, all sterility control wells persisted as blue color after a 24-hour incubation followed by a 4-hour incubation with 0.675 % resazurin. On the contrary, inoculated wells underwent color transition from blue to pink coloration. In the aforementioned assay, viable *P. aeruginosa* bacterial cells irreversibly reduced the blue-colored weakly fluorescent dye, resazurin into a pink-red gradient highly fluorescent compound, resorufin. Even though the color transition can be perceived by naked eyes, fluorescence of all wells were quantified at 525 nm excitation and 595 nm emission using a microplate reader. The generated fluorescence signal of each well is proportional to the number of living bacterial cells in the sample. After observing that none of the concentrations even reached the 50% inhibition of bacterial growth, all the concentrations used can be considered sub-MICs. Therefore, all the extracts and fractions were still subjected to anti-pyocyanin production assay and swarming motility inhibition.

Phenotypic Evaluation of Quorum Sensing Inhibition Activity via Virulence Factor Assays

Swarming Motility Assay

Swarming is a QS-controlled phenotype that involves a coordinated group movement across semi-solid surface. P. aeruginosa requires a functional flagellum and yields rhamnolipids in order to demonstrate this kind of motility [19]. This phenomenon is highly reliant on the surface's moisture, density of the bacterial cells and nutrients from the agar media [20]. Among the three sub-MICs identified from the resazurin-based fluorimetric microtiter assay, 50 µg/mL was chosen as the final concentration of each test control in the swarming agar medium.

Two (2) out of the six (5) treatments namely, hexane fraction (9.39 mm ± 0.67) and DCM fraction (10.82 mm ±

Table 1. *Phytochemical constituents of A. bilimbi crude extract and active fractions.*

Phytochemical Test/Spray Reagent	Observation		
	A. bilimbi crude extract	Hexane fraction	DCM fraction
1. Cardiac Glycosides *-Keller-Kiliani Test*		(-)	(+)
2. Steroids *-Liebermann-Burchard Test*		(+)	(-)
3. Triterpenoids **-***Liebermann-Burchard Test*		(-)	(+)
4. Terpenes *-Phosphomolybdic acid spray reagent* *-Vanillin-sulfuric acid reagent*	(+) (+)	(+) (+)	(+) (+)
5. Triterpenes, sterols, steroids, phenols, and polyphenols *-Tin (IV) chloride* *-Ferric chloride-methanol (polyphenols)*	(+) (+)	(+) (+)	(+) (-)
6. Alkaloids *-Dragendorff's reagent*	(+)	(-)	(+)
7. Anthraquinone Glycosides *-Borntrager reagent*	(+)	(-)	(-)
8. Amino acids and amines *-Ninhydrin reagent*	(+)	(-)	(-)
9. Amines, indoles, ergot alkaloids *-Ehrlich reagent*	(+)	(-)	(+)
10. Glycolipids *-Bial's reagent*	(+)	(+)	(+)

Legend: (+) presence, (-) absence

0.95) demonstrated restriction in the diameter of the swarms, while the remaining three (3) treatments exhibited unrestrictedness in the swarms' diameter. Figure 3 shows the mean diameter (in millimeters) of the treated P. aeruginosa ATCC 27853 swarms. The obtained data were analyzed using Student's t-test with a 0.05 level of significance. Among the two active fractions, only the hexane fraction (9.39 mm ± 0.67) differed significantly from the control (16.20 mm ± 2.55). The mean diameter of the swarms treated with DCM was less than the mean diameter of the control, but the difference was not big enough to be statistically significant.

Anti-pyocyanin Production Assay

Pyocyanin is a blue redox-active compound capable of killing and attenuating other microorganisms that might compete with the growth of *P. aeruginosa*. It has been previously isolated in large quantities from patients with *P. aeruginosa* – induced cystic fibrosis. Nevertheless, different cellular pathways can be influenced by pyocyanin [21]. Among the three sub-MICs identified from the resazurin-based fluorimetric microtiter assay, 50 µg/mL was chosen as the final concentration of each test control in the treated *P. aeruginosa* bacterial suspension.

The obtained data were evaluated using Student's t-test with a 0.05 level of significance. Hexane fraction exhibited an inhibition of 42.66 % ± 12.94. This extract can be subjected to further purification to determine if there are compounds present capable of inhibiting pyocyanin formation. On the other hand, crude extract did not show inhibition against pyocyanin. In conclusion, none of the extracts were able to significantly inhibit pyocyanin formation of *P. aeruginosa* ATCC 27853.

Discussion

Determination of Sub-MICs via Resazurin-based Fluorimetric Microtiter Assay

All concentrations of each sample exhibited very low percentage inhibition and did not even reach 15% which is quite far from the 50% mark. In fact, no inhibition can be observed in hexane fraction (25 µg/mL) and water fraction (25 µg/mL and 50 µg/mL). Hence, ruling out the potential antibacterial-induced inhibition of phenotypic virulence characteristics of *P. aeruginosa* ATCC 27853.

Phenotypic Evaluation of Quorum Sensing Inhibition Activity via Virulence Factor Assays

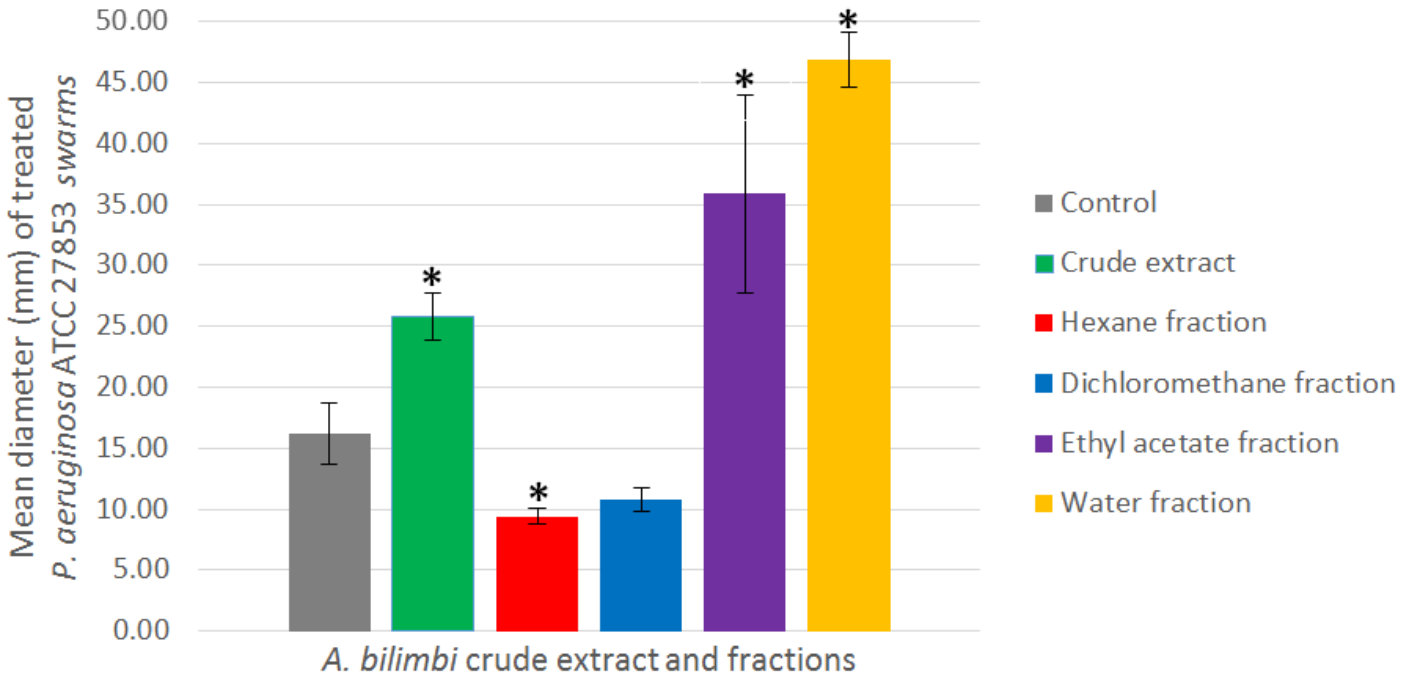

Figure 3. *Mean diameter (mm) of treated P. aeruginosa ATCC 27853 swarms. Error bars represent mean (n = 3) ± SEM. Asterisk (*) indicates significant difference (α = 0.05) between control and treatment.*

Swarming Motility Assay

In this assay, bull's eye pattern of swarming was observed using *P. aeruginosa* ATCC 27853. It is characterized as cyclical and regular waves [22]. Kohler *et al.* (2000) inferred that in order for *P. aeruginosa* to swarm, cell-to-cell signalling, and both flagella and type IV pili, are necessary [23]. They determined that rhlA mutant was incapable to swarm, hence production of a biosurfactant known as rhamnolipid is prerequisite for swarming motility of *P. aeruginosa*. Rhamnolipids aid *P. aeruginosa* to withstand surface tension and permit flagellum-based thrust of *P. aeruginosa* over semisolid surfaces. Identified phytochemicals in the active fractions may have interrupted QS or deregulated the biosynthetic pathway of rhamnolipids leading to swarming restrictions. Phytochemical constituents such as alkaloids (*e.g.* caffeine and 7-fluoro indole) and triterpenes (*e.g.* tormentic acid, 23-hydroxycorosolic acids, and analogs of oleanolic acid) efficiently impeded the swarming motility of *P. aeruginosa* [24,25,26]. The active fractions may have also interferred QS-linked gene expressions responsible for the production of rhamnosyl-transferases (rhamnolipids): rhlAB [15]. On the other hand, inactive extract and fractions may have benefited the swarming phenomenon by inducing the production of rhamnolipids. Ajoene analogs [27], allicin [28], cucurmin [29], zingerone [30], and eugenol [31] are known phytochemicals that can effectively attenuate rhamnolipid formation.

Anti-pyocyanin Production Assay

Polyphenolic compounds (e.g. ellagic acid, quercetin, and catechin) are able to prevent formation of pyocyanin produced by *P. aeruginosa* [32-34]. Little to no inhibition of extracted pyocyanin were observed due to possible interferences in the absorbance readings. Aside from pyocyanin, DCM potentially extracted plant pigments from *A. bilimbi* extracts which hindered absorbance readings. Other extraction method and blank solutions containing sterile BHI broth and plant extracts can be employed to remove potential interferences coming from extracted plant pigments in the absorbance readings.

Conclusion and Recommendation

This study evaluated the anti-quorum sensing activity of *Averrhoa bilimbi* (kamias) methanolic leaf extract and obtained fractions namely hexane, dichloromethane, ethyl acetate, and aqueous fractions, against the bio-monitor strain, *Pseudomonas aeruginosa* ATCC 27853. After observing that none of the concentrations even reached the 50% inhibition of bacterial growth, all the concentrations used can be considered sub-MICs. Therefore, all the extracts and fractions were still subjected to anti-pyocyanin production assay and swarming motility inhibition. Hexane and DCM fractions possess promising inhibitory activity against swarming motility. Identified phytochemicals in the active fractions may have interrupted QS or deregulated the biosynthetic pathway of rhamnolipids, leading to swarming restrictions. In conclusion, none of the extracts were able to significantly inhibit pyocyanin formation of *P. aeruginosa* ATCC 27853. TLC analysis and phytochemical screening tests revealed that *A. bilimbi* methanolic crude extract contains terpenes, triterpenes, steroids, alkaloids, anthraquinone glycosides, amino acids, and glycolipids; hexane fraction contains steroids, terpenes, triterpenes, and glycolipids; and DCM fraction contains cardiac glycosides, triterpenoids, terpenes, triterpenes, steroids, alkaloids, and glycolipids. These identified phytochemicals could possibly interfere the QS-controlled phenotypes of *P. aeruginosa* ATCC 27853.

For future endeavors of this study, it is suggested that the *A. bilimbi* active fractions be further studied with different sub-inhibitory concentrations to determine if there will be a dose-dependent inhibition to a certain virulence factor like swarming motility. Also, *A. bilimbi* fractions can be further purified through chromatographic techniques that will separate the bioactive from non-bioactive compounds so these can be fully identified and characterized. Other virulence factor assays can be utilized, such as rhamnolipid plate assay since rhamnolipids are known to be the surfactants associated *P. aeruginosa*'s swarming motility, biofilm formation assay since *P. aeruginosa* infection is a highly persistent nosocomial infection because of its ability to form biofilm. Biofilm formation can be also observed with the aid of scanning electron microscopy and atomic force microscopy [35]. Furthermore, it is recommended to utilize other strains of *P. aeruginosa* e.g. PAO1 since every strain may have distinct virulence characteristics. For an instance, pyocyanin can be produced solely by not all but approximately 90-95 % of different strains of *P. aeruginosa* [36]. In quantifying pyocyanin, other extraction method and blank solutions containing sterile BHI broth and plant extracts can be employed to remove potential interferences coming from extracted plant pigments in the absorbance readings.

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