Microbiology Research Journal International

22(1): 1-10, 2017; Article no.MRJI.37450
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Anti-Quorum Sensing Activity of *Tetracera scandens* and *Aleurites moluccana* Leaf Extracts against *Chromobacterium violaceum*

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Authors' contributions

This work was carried out in collaboration between both authors. Author JPMDG designed the study, conducted literature searches, performed the assays and statistical analyses, and wrote the first draft of the manuscript. Author LVP polished the study design, supervised the conduct of assays, and reviewed the manuscript. Both authors agreed on the interpretation of the results, and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2017/37450
Editor(s):
(1) Essam Hussein Abdel-Shakour, Professor, Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Cairo, Egypt.
Reviewers:
(1) Esraa Ashraf Ahmed ElHawary, Ain Shams University, Egypt.
(2) C. D. Dayanand, Sri Devaraj Urs academy of Higher Education and Research, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/21848

Original Research Article

Received 17th October 2017
Accepted 7th November 2017
Published 10th November 2017

ABSTRACT

Aims: To test the efficacy of *Tetracera scandens* (L.) Merr. and *Aleurites moluccana* (L.) Wiild. methanolic leaf extracts on the inhibition of quorum sensing-dependent pigmentation of *Chromobacterium violaceum*.
Study design: *In-vitro* quorum sensing inhibition design was conducted.
Place and Duration of Study: The study was conducted at the Biology Laboratory, College of Science, Pamantasan ng Lungsod ng Maynila between November 2015 to May 2016.
Methodology: The leaf extracts were obtained through soaking in methanol and subsequent rotary evaporation. Qualitative screening of the anti-quorum sensing activities of the extracts were done through Agar Well Diffusion Assay. The Minimum Quorum Sensing Inhibition Concentration was

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determined through two-fold serial dilution and then wells were streaked on Mueller-Hinton Agar plates to identify the Minimum Bactericidal Concentration. Disc Diffusion Assay was done to quantitatively measure the anti-quorum sensing activities of the leaf extracts.

Results: The crude methanolic leaf extracts of *T. scandens* and *A. moluccana* were able to inhibit quorum-sensing on *C. violaceum*. The anti-QS activities of both plants were comparable at their Minimum Quorum Sensing Inhibitory Concentrations (P>.05), however, weaker activities were observed against the positive control (P = 0.001). The two extracts were partially active in inhibiting quorum sensing but were inactive in growth inhibition.

Conclusion: This study revealed that *T. scandens* and *A. moluccana* exhibit anti-quorum sensing activities against *C. violaceum* and these may be due to their phytochemical constituents, including flavonoids, terpenoids, and phenolics, which were previously proven to exhibit anti-quorum sensing activity. Extensive studies on the isolation of these compounds which may be responsible for their activities may be required to further improve the efficacy and harness the potential of these plants on quorum sensing inhibition.

Keywords: Quorum sensing; quorum sensing inhibition; Tetracera scandens; malakatmon; Aleurites moluccana; lumbang; Chromobacterium violaceum.

1. INTRODUCTION

Quorum sensing (QS) is a form of density-dependent bacterial cell-to-cell communication mediated by signalling molecules called autoinducers (AIs) and receptors. In Gram-positive bacteria, quorum sensing is mediated by autoinducing peptides whereas in Gram-negative bacteria, acyl homoserine lactones (AHLs) are employed [1]. At High Cell Density (HCD), more AHLs are produced and accumulate into the external environment that leads to autoinducers binding to its receptors when a critical threshold concentration is attained [2]. LuxI synthase is responsible for the synthesis of AHL and a LuxR protein is an AHL receptor. LuxI synthase is an enzyme that utilizes S-adenosylmethionine (SAM) and acyl-acyl carrier protein (acyl-ACP) as its substrates wherein the LuxI synthase binds a specific acyl-ACP with SAM by an amide bond [3]. The LuxR protein in the cytoplasm acts as the receptor by binding with the AHL on its N-terminus and with the DNA on its C-terminus to initiate the transcription by the Ribonucleic Acid (RNA) polymerase. The transcription results to the production of specific proteins responsible for its expression of its virulence and other bacterial physiological mechanisms such as swarming motility in *Pseudomonas aeruginosa*, luminescence in *Vibrio fischeri*, biofilm formation in *Escherichia coli* and pigment production in *Chromobacterium violaceum* [4,5].

Chromobacterium violaceum is a gram-negative bacteria commonly found in water and soil [6]. Its quorum sensing system involving CviI/CviR controls its virulence as evident with the inhibition of quorum-sensing mediated killing of *C. violaceum* of *Caenorhabditis elegans* [7]. A phenotypic trait controlled by quorum sensing expressed by *C. violaceum* is the production of a purple pigment called violacein which is synthesized when the C6-AHL autoinducer forms a complex with CviR receptor bound with the DNA that results to the expression of vioABCDE genes (Fig. 1) [6]. Violacein is capable of inhibiting the growth of *Staphylococcus aureus*, and altering the feeding and morphology of amoeba [8].

The pressure due to the indiscriminate utilization of antimicrobial substances resulted to the emergence of multi-drug resistant bacteria, which has incapacitated several antibiotics used for the treatment of infectious diseases. Therefore, a strategy to combat this problem is to suppress its capability to utilize bacterial mechanisms [1]. An example of this is the inhibition of cell-to-cell communication through the utilization of natural quorum sensing inhibitors (QSIs) possessed by other organisms such as fungi, plants and animals. These QSIs disrupts the signalling pathways by targeting the autoinducers or the receptors to prevent the activation of transcription [9]. The first substance to be observed to exhibit quorum sensing inhibition is the halogenated furanones extracted from a red macroalga, *Delisea pulchra*, that interferes with the reception of the AHL by the LuxR protein due to its structural similarity with AHLs [10,5].

Several plant extracts have also been used to inhibit quorum sensing. The mechanism rely on compounds such as those which are capable of non-competitive inhibition by binding on a site in the substrate other than the AHL binding site.

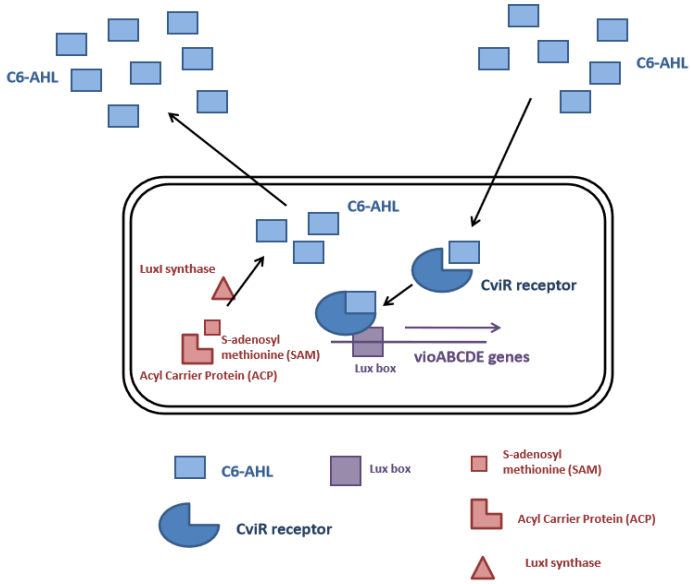

Fig. 1. Quorum sensing in *Chromobacterium violaceum*

This results to the alteration of the structure of the substrate that in turn prevents the formation of autoinducer-receptor complex therefore inhibiting quorum sensing. Flavonoids-rich *Psidium guajava and Centella asiatica* [11,12] and tannin-rich *Terminalia catappa* [13] are some of the plants previously proven to exhibit anti-quorum sensing activity.

Lumbang (*Aleurites moluccana* (L.) Wiild.) is a medium-sized tree native to the Philippines and other parts of the Indo-Pacific. It belongs to family Euphorbiaceae. Its plant parts are folklorically used in treating tumors, headaches, fevers and diarrhoea [14]. Phytochemical analyses of this plant yielded sterols, tannins and flavonoids [15,16]. Its anticinoceptive [17] and antipyretic activity [18] were credited to its phytochemicals.

Malakatmon (*Tetracera scandens* (L.) Merr.) is an evergreen from family Dilleniaceae native to the Philippines and South East Asia. Some folkloric uses of its plant parts are used in treating hypertension, rheumatism, urinary disorders, and inflammation in different countries. Phytochemical investigation of the plant yielded flavonoids and terpenoids credited with its variety of bioactivity [19]. A study on the ethanolic extract of its leaves showed its capability to protect the liver from hepatotoxicity [20]. Further studies on its extracts rich in flavonoids yielded anti-hyperglycemic [21] and antidiabetic activity [22].

Due to the continued threat of antibiotic resistance to public health and humanity in general, novel strategies to combat pathogens need to be sought. The rich phytochemical composition of *A. moluccana* and *T. scandens* is said to be responsible for the numerous recorded bioactivities of its plant parts. With this, the team aimed to further investigate the potential uses of these plants including its anti-quorum sensing activities. The determination of the quorum sensing inhibition activities of the two plants endemic to the Philippines may help to further understand the alternatives to antibiotics, as well as to increase their availability, in combating bacterial infections without the risk of resistance.

2. METHODOLOGY

2.1 Sample Preparation and Extraction

2.1.1 Collection of leaves

Fresh and healthy *Tetracera scandens* leaves were collected from Baler, Aurora, and *Aleurites moluccana* leaves from Legazpi, Albay. *Psidium guajava* leaves, which served as the positive control, were collected from Tondo, Manila. The collected leaves were verified by an expert at the Botany Division of the National Museum of the Philippines. Upon verification that the collected plant specimens were correct, the leaves were transported to the laboratory and then washed and dried at room temperature for five days and then pulverized using a blender.

2.1.2 Extraction of *T. scandens* and *A. moluccana* leaves

The pulverized leaves were steeped in analytical grade absolute methanol for 3 days in a container covered with aluminum foil. The extracts were then filtered using Whatman #1 filter paper. The extracts were concentrated using rotary evaporator at 50℃ to obtain the crude extract. The remaining solvent was evaporated using a water bath set at 50℃. The crude methanolic extracts were stored in amber bottles at 0-5℃ prior to use.

2.1.3 Preparation of positive and negative control

The *P. guajava* leaf extract was diluted in methanol to obtain a concentration of 1 g/mL and then swirled for homogenization using a vortex. The Minimum Quorum Sensing Inhibitory Concentration (MQSIC) of the positive control was determined using two-fold dilution [23]. Methanol served as the negative control.

2.1.4 Preparation of leaf extracts for assay

The leaf extracts were diluted in methanol to obtain a concentration of 1 g/mL in preparation for the succeeding microbiological assays and then swirled using a vortex for homogenization [24].

2.2 Preparation of Test Organism

2.2.1 Test organism

Chromobacterium violaceum was obtained from the Industrial Technology Development Institute of the Department of Science and Technology, Philippines.

2.2.2 Preparation of inoculum

A loopful of a 24-hour old culture of *C. violaceum* was added to 10 mL Mueller-Hinton Broth in a screw-capped tube. The culture broth was standardized using 0.5 McFarland standard ($1.5x10^{-8}$ CFU/mL) and sterile saline solution was added if the turbidity of the culture broth was different with the 0.5 McFarland standard [23].

2.2.3 Preparation of assay plates

Approximately 20 mL of Mueller-Hinton Agar (MHA) were poured on Petri plates. A sterile cotton swab was moistened in the inoculum suspension to collect the bacteria. The cotton swab was then streaked for four times to create a bacterial lawn on the entire surface of the solidified Mueller-Hinton Agar plates, and the plate was rotated 60° after each application to ensure that the inoculum was equally distributed on the media [23].

2.3 Qualitative Screening

2.3.1 Agar well diffusion assay

The anti-quorum sensing activity of *T. scandens* and *A. moluccana* crude methanolic leaf extracts as well as the positive (*P. guajava*) and negative controls were screened qualitatively by Agar Well Diffusion Assay. A sterile cork borer was used to bore holes on the agar plate previously swabbed with the test organism to create three wells. The agar plugs in the cork borer were collected and disinfected with 1% aqueous Chlorox solution before disposal. Using a pipettor, twenty microliters of the extract was delivered into the wells. The plates were incubated at 35℃ for 24 hours [23]. The presence of an opaque halo around the wells indicates the inhibition of the quorum-sensing dependent violacein production of *C. violaceum*. A transparent halo indicates growth inhibition instead.

2.4 Determination of Minimum Quorum Sensing Inhibitory Concentration (MQSIC)

Two-fold serial dilution was done to obtain different concentrations of the extract. The lowest concentration of the plant extract which inhibited the violacein production of *C. violaceum* with slight growth inhibition served as the Minimum Quorum Sensing Inhibitory Concentration (MQSIC) [23]. Thirteen wells were used in the determination of MQSIC. Wells #1-10 contain different concentrations of the plant extract and a uniform amount of inoculum. Whereas, Wells #11, 12, and 13 served as the negative, medium, and positive controls, respectively. The wells were incubated for 24 hours at 35℃. The same procedure was conducted to determine the MQSIC of the *P. guajava* as the positive control.

2.5 Determination of Minimum Bactericidal Concentration (MBC)

The wells used in the identification of the MQSIC were used in the determination of Minimum Bactericidal Concentration (MBC). Each wells were homogenized and were streaked separately

on properly-labeled MHA plates. The MHA plates were then incubated at 35℃ for 24 hours, and the presence of colonies were observed [23]. The concentration of the extract which produced at least a single colony will prove the anti-QS activity of the MQSIC. The lowest concentration where no growth was observed was the MBC.

2.6 Quantitative Screening

2.6.1 Paper disc diffusion assay

The anti-quorum sensing activity of the MQSIC of *T. scandens* and *A. moluccana* extracts, the other effective concentrations and the positive control (*P. guajava*) were determined through paper disc diffusion assay. One hundred microliter of the extracts and controls were deposited on sterile paper discs with 6 mm in diameter, and laid in the seeded MHA plates arranged like the agar wells. The discs were gently tapped to ensure that the whole surface of the discs are in contact with the medium. The plates were then incubated for 24 hours at 35℃ [23]. The presence of an opaque halo indicates the anti-quorum sensing activity of the extracts and the presence of a transparent halo is an indication of growth inhibition. The zones of inhibition were measured using a vernier caliper.

2.7 Statistical Analysis

One-way Analysis of Variance (ANOVA) were used to determine the significant difference among the zones of inhibition by the MQSIC and the other effective concentrations of both *T. scandens* and *A. moluccana,* as well as to determine the significant difference between the zones of inhibition by the MQSIC and other effective concentrations with the positive control. Tukey's test served as the post-hoc test if a significant difference among the zones of inhibition.

3. RESULTS AND DISCUSSION

3.1 Qualitative Screening

The absence of a violet pigmentation in the form of opaque halos with visible growth around the wells is an indication of quorum sensing inhibition in *C. violaceum* [25]. Both *T. scandens* and *A. moluccana* crude methanolic leaf extracts inhibited the quorum sensing-dependent violacein production of *C. violaceum* as evident in the observed opaque halo around the well. As expected, the positive control, *P. guajava*, also showed an opaque halo around the well as an indication of its anti-QS activity. The negative control, methanol, did not show anti-QS activity.

A transparent halo, as an indication of growth inhibition, was observed in *T. scandens*, *A. moluccana*, and *P. guajava* around the wells before the opaque halo. This shows that as the extract diffuses away from the wells and as the concentration decreases, anti-quorum sensing activity is increased. The results indicate a concentration-dependent anti-quorum sensing activity of the extracts as observed in previous studies [11] in guava which was used as the positive control.

3.2 Minimum Quorum Sensing Inhibitory Concentration

As observed in the preceding qualitative assay and previous studies concerning the concentration-dependent anti-quorum sensing activity, the Minimum Quorum Sensing Inhibitory Concentration (MQSIC) and Minimum Bactericidal Concentration (MBC) were determined to attest with the observation. MQSIC is defined as the lowest concentration that showed inhibition of quorum sensing, whereas, MBC is the lowest concentration that showed no growth at all [23].

The results of the two-fold dilution assay showed that well #2 (500 mg/mL) as the potential MQSIC for *T. scandens* because of the absence of pigmentation and as white pellets were observed indicating growth. Wells #3 (250 mg/mL) and #4 (125 mg/mL) were also able to yield less pigmentation than the negative control. A two-fold dilution assay was also employed to determine the MQSIC for the other treatment material, *A. moluccana*. Similarly, the results showed that similar to *T. scandens*, the well #2 (500 mg/mL) was the potential MQSIC for *A. moluccana* as pigmentation was absent but growth was still observed. Wells #3 (250 mg/mL) and #4 (125 mg/mL) also resulted to less pigmentation than the negative control.

Same procedure was employed to determine the MQSIC of the positive control, *P. guajava*. It was observed that at 125 mg/mL concentration, the positive control inhibited pigmentation but was still able to yield growth.

3.3 Minimum Bactericidal Concentration

To confirm the results of the MQSIC and prevent biased results, the contents of each wells were

streaked in Mueller-Hinton Agar to determine the MBC. The results for *T. scandens* showed that 500 mg/mL was able to inhibit pigmentation but was able to produce growth. The highest concentration of 1 g/mL, showed no growth which indicates that it is the MBC for *T. scandens*. The MBC for *A. moluccana* was also determined by streaking the contents of each wells on MHA as a confirmatory test. It was determined that 500 mg/mL inhibited the pigmentation but was able to yield growth. Similar to the results of *T. scandens*, the 1 g/mL concentration of *A. moluccana* produced no growth which makes it the MBC. The same procedures were done with the positive control, *P. guajava*, wherein well#4 (125 mg/ml) is the minimum concentration that yielded growth on MHA while manifesting pigment inhibition making it the MQSIC. Furthermore, well #3 (250 mg/ml) was determined to be the MBC as it was the lowest concentration that showed no growth.

The results proved the observations from the previous studies done on guava [11] which was used in this study as the positive control, and on *T. catappa* [13] that as the concentration of the extract decreases, quorum sensing inhibition increases until no more activity was observed. Furthermore, the results showed that both the *T. scandens* and *A. moluccana* have relatively similar activities in terms of the MQSIC since the MQSIC of both extracts is at 500mg/mL. However, the MIC of the positive control, which is 125 mg/mL, is lower than that of the treatments.

3.4 Quantitative Anti-Quorum Sensing Activity

The concentrations of *T. scandens* and *A. moluccana* deemed to show QS inhibitory activity were subjected to disc diffusion assay to quantify their effectiveness in terms of the measurement of their zones of QS inhibition.

The MQSIC of *T. scandens* (500 mg/ml) and other effective concentrations (250 mg/ml and 125 mg/ml) showed QS inhibition in terms of the presence of an opaque halo around the discs. Similar results were observed with the MQSIC of *A. moluccana* (500 mg/ml) and its other effective concentrations (250 mg/ml and 125 mg/ml) as shown in Fig. 1. The positive control, *P. guajava* (125 mg/ml), also exhibited the presence of an opaque halo. The presence of transparent halo indicating growth inhibition in the different treatment concentrations was due to the phytochemical composition of the crude extracts that may include phytochemicals that exhibit both antibacterial and anti-quorum sensing activities.

Fig. 2 shows the means of zones of inhibition of the different concentrations of *T. scandens* and *A. moluccana*. It can be observed that the diameter of the zones of growth inhibition decreases as the concentration decreases. This observation proves that the growth inhibitory and QS inhibitory activities of the plant extracts work on different and independent mechanisms. A smaller zone of growth inhibition does not guarantee that a larger zone of QS inhibition will be yielded. QS inhibition may either be by the degradation of the autoinducers or receptors [26], by inhibition of the formation of LuxI/LuxR complex by competitive [10] or non-competitive inhibition [27].

Furthermore, it can also be observed that the determined MQSIC for both extracts, which was previously identified through the basis of visible absence of pigmentation in wells and as confirmed by streaking them to the agar plates, yielded the largest zones of QS inhibition - suggesting that the MQSIC of both plants are the most effective among all concentrations.

To determine whether the difference between the zones of QS inhibitions yielded by the different concentrations of the extracts, statistical treatment through one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's test was employed.

Through the statistical analysis, it was deemed that the difference concentrations of *T. scandens* yielded a significant difference against the negative control ($P<.001$). However, no significant difference were observed among all concentrations tested ($P>0.05$). The zones of QS inhibition for all concentrations also fall under the partially active category, whereas, the different concentrations were inactive based on their zones of growth inhibition [23]. Similarly, no significant difference was observed among the zones of growth inhibition by the different concentrations ($P>.05$).

The results of the *A. moluccana* showed that a significant difference exists among all concentrations tested ($P=0.031$). *Post hoc* test revealed that the significant difference lies between the 500 mg/mL and 125 mg/mL concentrations. Similarly, the different concentrations of *A. moluccana* yielded significant differences with the negative control

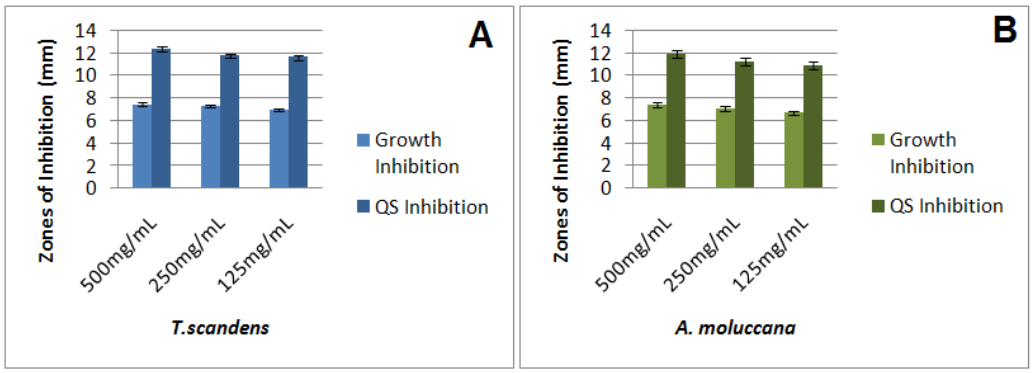

Fig. 2. Quorum Sensing and Growth Inhibitory Activities of *T. scandens* and *A. moluccana* against *C. violaceum.*

Mean zones of growth and quorum sensing inhibition of the different concentrations of T. scandens (A) and A. moluccana (B) leaf extracts against C. violaceum

(*P*<.001). Furthermore, the zones of QS inhibition of all concentrations were also partially active, whereas, their zones of growth inhibition were deemed inactive [23].

These observations on *T. scandens* and *A. moluccana* hint that the concentrations tested for their anti-quorum sensing activity were effective while maintaining their inactive growth inhibitory effects. This may be further studied by isolating the bioactive compounds and check for their activities.

Furthermore, the MQSIC of the two treatment materials were compared against the MQSIC of the positive control. Table 1 shows the zones of QS inhibition yielded by the extracts and the negative control, and their corresponding interpretations. It was revealed that the positive control was more active than both treatments and was able to do so at a lower concentration. Furthermore, statistical analyses confirmed that there is a significant difference between *T. scandens* (*P*=0.001), and *A. moluccana* (*P*=0.001), against the positive control.

The potential quorum sensing inhibitory activities by *T. scandens* and *A. moluccana* and the positive control *P. guajava* were observed in the assays employed. This shows that the plant extracts used contain phytochemicals that were able to inhibit quorum sensing in *C. violaceum*.

The mechanisms as to how the plant extracts were able to exhibit anti-quorum sensing activity depend on the phytochemicals present. In *P. guajava* which was used in this study as the positive control, the flavonoid fraction of the extract were able to inhibit the pigment production of *C. violaceum*. In previous studies, this activity was credited to the flavonoids present in the extract, particularly quercetin and quercetin-3-O-arabinoside [11]. A similar study on isolated flavonoids such as quercetin and kaempferol of *Centella asiatica* yielded an anti-QS activity against *C. violaceum* [12]. A concentration-dependent QS inhibitory activity was also observed in this study whereas the extract is growth inhibitory at higher concentrations and is QS inhibitory at lower concentrations.

A study on the tannins isolated from *Terminalia catappa* [13] showed that tannins were able to inhibit the violacein production of *C. violaceum* in a concentration-dependent manner. A study conducted by [28] in *Szygium cumini* showed that the flavonoids and phenols isolated from the extract exhibited anti-QS activity against *C. violaceum*.

Phytochemical analyses of *T. scandens* yielded flavonoids and terpenoids [19] including quercetin and kaempferol [22]. A quantitative analysis of the phytochemicals in methanolic leaf extract of *T. scandens* showed that there are 6.34±0.02 QE mg/g of flavonoids and 7.26 ± 0.03 GAE of phenolic compounds. Moreover, phytochemical analyses of *A. moluccana* yielded flavonoids [15], sterols and triterpenes [16] which were proven to exhibit anti-QS activity by the previous studies.

Furthermore, it was previously observed that the flavonoids were able to inhibit quorum sensing through the interference on the interaction of the

Table 1. Descriptive values of the minimum quorum sensing inhibitory concentrations of the treatment materials

Treatment	MQSIC (mg/mL)	Zone of QS Inhibition (mm)	Interpretation*
T. scandens	500	12.3	Partially Active
A. moluccana	500	11.9	Partially Active
P. guajava (+ control)	125	14.77	Active
Methanol (- control)	NA	0	Inactive

**Based on the categories of zones of inhibition [23]*

autoinducer AHL with the CviR receptor [16]. This is in accordance with the results of a study that there are plant compounds which are capable of non-competitive inhibition by binding on a site in the substrate other than the AHL binding site which results to the alteration of the structure of the substrate that in turn prevents the formation of autoinducer-receptor complex therefore inhibiting quorum sensing [29].

The continuous search for QS inhibitory compounds is needed to combat bacterial infections without the risk of antibiotic resistance. Resistance arises due to the indiscriminate use of antibiotics that puts pressure on the bacteria. QS inhibition works without pressure thereby avoiding the risk of developing resistance. This is due to the fact that inhibiting quorum sensing does not kill the bacteria but only renders them susceptible to the innate immunity by the host and therefore a low dose of antibiotic is enough to eradicate the pathogen from the body of the host [30].

4. CONCLUSION

The results of this study show the capability of *Tetracera scandens* and *Aleurites moluccana* methanolic leaf extracts to inhibit quorum sensing-dependent pigmentation in *Chromobacterium violaceum*. Similar to previous studies, concentration-dependent activities were also observed in both plants. The two leaf extracts were also partially active in their anti-quorum sensing activities based on their zones of inhibition. Their activity of which are due to their phytochemical compositions. This research laid foundation for characterization of the specific phytochemical constituents of these plants, and to evaluate their efficacy on inhibiting quorum sensing in *C. violaceum*.

ACKNOWLEDGEMENTS

The authors sincerely express their gratitude to the College of Science, Pamantasan ng Lungsod ng Maynila for the support including the use of the facilities; the National Museum of the Philippines for the help in collection and identification of the plant specimens; the Industrial Technology Development Institute, Department of Science and Technology for the bacterial culture; and Professors Bea Mateo, Annie Gallardo, Michelle Ventura, Fe Jacinto, and Eileen Vitug for their valuable inputs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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