PHILIPPINE ETHNOBOTANICALS DOWNREGULATE lasR EXPRESSION LINKED TO QUORUM SENSING-MEDIATED BIOFILM FORMATION IN Pseudomonas aeruginosa

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ABSTRACT

The prevalence of antibiotic resistance has brought about a growing surge to develop novel approaches to control infectious diseases. Targeting Quorum-sensing (QS) - controlled virulence factors in bacteria has indicated a promising strategy for antipathogenic drugs. Extracts of Philippine Ilongot-Egongot ethnobotanicals Stachytrapeta jamaicensis, Adenanthera intermedia, Mikania micrantha, Hypitis suaveolens, Premna odorata, Cymbopogan winterianus, Phyllanthus urinaria, Dillenia phillipinensis, Hydrocotyle vulgaris, Senna alata, Urena lobata, Ceiba pentandra, Ficus sp., Eleusine indica, Diplazium esculentum and Talalahib (no known scientific name) were screened using microtiter plate biofilm formation assay for their QS inhibition activity against biofilm formation in Pseudomonas aeruginosa. RNA extracts of M. micrantha, H. suaveolens flowers, H. vulgaris, A. intermedia, E. indica leaves, D. esculentum and Talalahib have inhibitory effect on P. aeruginosa clinical isolate biofilm formation. Decrease in biofilm formation was shown in extracts of S. jamaicensis, M. micrantha, H. suaveolens, H. vulgaris, U. lobata, C. pentandra, A. intermedia, E. indica, D. esculentum and Talalahib, against P. aeruginosa PNCM 1335. RNA extracts of M. micrantha, Talalahib and A. intermedia exhibited downregulation of lasR in both test bacteria using qRT-PCR analysis through absolute quantification. The prospects of these ethnobotanicals to inhibit bacterial virulence avoiding antibiotic resistance is presented in this paper.

Keywords: quorum sensing, biofilm, Pseudomonas aeruginosa, ethnobotanicals, lasR

INTRODUCTION

The continuous emergence of pathogenic diseases has brought about indiscriminate use of antibiotics. This practice resulted to the development of antibiotic resistance and is now a global threat to public health (Kalia et al., 2007). With the prevalent progression of antibiotic resistance in bacteria, interest for novel approaches to limit infectious diseases is greatly increasing. Pseudomonas aeruginosa is a multi-drug resistant (MDR) human pathogen and known for their biofilm growth making it difficult to eliminate (Driscol et al., 2007). Biofilm formation provides a great advantage to P. aeruginosa where it can degrade host tissues with proteases and toxins, while being spared from antibiotic attack (Adonizio et al., 2008). Its flagellar motility as well as type IV pili-mediated twitching motility has been shown to be essential for surface attachment and colony formation in biofilms. More importantly, however, is the fact that cell-to-cell communication, or quorum-sensing (QS), is critically involved in biofilm formation, particularly in the development of its typical three-dimensional architecture (Antunes et al., 2010). A number of its genes and proteins are under QS regulation, suggesting that regulation of gene expression in populations is material to its successful pathogenesis in plant and animal hosts (Bauer & Mathiesius, 2004).

Hence, an approach referred to as the anti-pathogenic drug principle (Hentzer et al., 2003) restricts this QS-controlled virulence and pathogenesis in bacteria in the hope that invading bacteria will fail to synchronize its activities, and prevent establishment in the host (Rasmussen & Givskov, 2006). Disabling quorum-sensing systems in bacteria has become the new focal point in developing strategies to interrupt bacterial pathogenicity (O’Loughlin et al., 2013).

Current search for new antimicrobials and anti-virulence agents is focused on natural products, specifically, plant-derived compounds. To this day, these compounds have provided the platform for developing new sets of antipathogenic drugs. The Philippines, aside from having an immense plant diversity, hosts a number of diverse ethnic communities that have depended mostly on plants and other natural products to treat diseases (Sia et al., 2002). Among these are the plants utilized by the members of the ethnic community of Ilongot-Egongot of Maria Aurora in the province of Aurora, Philippines. A recent survey of their traditional medicine revealed a varied and huge selection of ethnomedicines for the treatment of many diseases and ailments (Balberona et al., 2018). Their novelty has attracted researchers seeking for agents that may possess quorum sensing inhibiting (QSIs) compounds, and provide additional validation of the traditional medicines (Adonizio et al., 2006). Phytochemicals mimic signals in bacteria and hence, may confuse their quorum sensing regulation (Bauer & Mathiesius, 2004).

The potential of anti-QS compounds has proved to be systemic evaluation for these agents (Adonizio, 2008). While there are worldwide ethnobotanical explorations, Philippine ethnobotanical screenings on pharmacological activities are still quite limited. This paper presents the QSI activity of Philippine Ilongot-Egongot ethnobotanicals against a QS-controlled virulence factor, biofilm formation, in the bioreporter bacterial strain Pseudomonas aeruginosa up to the molecular level through the quantification of the expression of lasR, a QS-linked gene responsible for many virulence factors including biofilm formation.

MATERIALS AND METHODS

Plant Sample Collection and Ethanol Extraction

Through the permission of the tribal chieftains of the Ilongot-Egongot community of Maria Aurora, Aurora, Philippines, plant samples were harvested, then placed in clean, properly labeled sacks, sealed and then transported to the laboratory for processing. The plants tested were: Stachytrapeta jamaicensis (Luzviminda) (leaves), Adenanthera intermedia (Kares) (leaves and seeds), Mikania micrantha (Ola-ola) (leaves), Hypitis suaveolens (Ambangogot) (flowers and leaves), Premna odorata (Asádaong) (leaves) Cymbopogan winterianus (Taday) (leaves), Phyllanthus urinaria (Taltalkidok) (leaves), Dillenia phillipinensis (Katmon) (leaves), Hydrocotyle vulgaris (Gotu kola) (leaves), Senna alata (Bensola) (leaves), Urena lobata (Pukot) (leaves), Ceiba pentandra (Beëlk) (leaves), Ficus sp.(Balete) (leaves), Eleusine indica (Pag) (roots and leaves), Diplazium esculentum (Pako-pako) (leaves) and Talalahib (whole plant) (no known scientific name).
The protocols of Tan et al. (2013) and Srissawat (2007) as followed by Velasco et al., (2020) were used for ethanol extraction with modifications. The plant samples were cleaned using distilled water and 70% (v/v) ethanol then air-dried. Fifty (50) grams of each ground plant sample was soaked in 500 ml of 80% ethanol for 72 hours. This was filtered and the solvent removed using a rotary evaporator. The resulting extracts were stored in sterile bottles at temperatures between 0-5°C. Sterilization of the extracts was done through centrifugation at 10,000 x g for 30 minutes, then membrane filtration (Acrodisc 25mm Syringe Filter) with a pore diameter of 0.45 μm pore size. The sterile extracts were stored at 2-8°C. The sterility of the extracts was monitored by inoculating 100 μl in brain heart infusion agar (BHIA).

**Preparation for Bacterial Culture**

Brain-heart infusion broth (BHIB) and agar (BHIA) were used to revive and maintain cultures of *P. aeruginosa*. Maintenance in special culture media was done as necessary. A reference strain, *P. aeruginosa* PNCM 1335 and a clinical isolate obtained from the University Medical Center, De La Salle Health Sciences Institute, Dasmarñas, Cavite, Philippines were used as test bacteria.

**Antibacterial Assay of Plant Extracts Against *P. aeruginosa***

The protocol of Rezaei et al. (2011) was used with some modifications. Colonies of *P. aeruginosa* from each strain cultured for 16-18 hours in BHIA were transferred to sterile distilled water, the turbidity was adjusted to McFarland 0.5 standard (~ 1.5 x 105 CFU/mL). Mueller Hinton Agar (MHA) plates were inoculated and streaked in three different directions over the surface of the agar to ensure the even distribution. On, sterile petri plates, 20 μl of each extract was pipetted onto 6-mm sterile blank antibiotic discs and was allowed to stand for 4 minutes to eliminate excess liquid. Using sterile forceps, infused discs were then transferred carefully and equidistant to each other onto 15-mm MHA previously inoculated with *P. aeruginosa*. Norfloxacin and sterile distilled water served as positive and negative control; Triplicate plates were prepared for all of the treatments. After 24 to 48 hrs period of incubation, the antibacterial activity of the plant extracts was noted for the appearance of zone of inhibition. Plant extracts should not exhibit zone of inhibition which was required for accuracy of the subsequent assay to rule out antibacterial-mediated decrease in virulence factor production (Fernando and Judan Cruz, 2020; Velasco et al., 2020).

**Microtitre Plate Biofilm Formation Assay**

The effect of plant extracts on the attachment phase of biofilm formation was measured by using a microtitre plate assay. 180 μl of overnight cultures of *P. aeruginosa* were transferred to wells in the plates added with 20 μl of each plant extract. This was incubated at 30°C for 40 hours without shaking. To remove planktonic cells, the plates were rinsed with sterile distilled water and air dried for 45 minutes. For staining, 150 μl of 1% crystal violet solution in water for 45 minutes was used (Fernando and Judan Cruz, 2020; Velasco et al., 2020). For quantification of biofilm, 200 μl of 95% ethanol was added to destain the wells. 100 μl from each well was then transferred to a new microtiter plate and the OD value was measured at 595 nm (Djordjevic et al., 2002). Presence and/or absence of growth inhibition was noted in biofilms and was then quantified using UV-visible Spectrophotometer (Biock Instruments, Inc., USA) (Judan Cruz, 2018, Velasco et al., 2020).

**lasR Gene Expression**

Four plant extracts that showed the lowest values in biofilm formation in the QSI Assay were chosen for the quantification of lasR expression.

**RNA Extraction**

RNA extraction was done using RNeasy Mini kit (Qiagen, GmbH, Germany). For each sample, 25-50 mg acid-washed glass beads (150-600 μm diameter) were weighed in a 2ml safe-lock tubes. Bacteria were collected by centrifugation at 5000 x g for five minutes at 4°C. The supernatant was decanted and aspirated to ensure removal of remaining media. Buffer RLT was added (350 μl for <5 x 108 and 700 μl for 5 x 108 - 1 x 109 number of bacteria). The suspension was transferred into the 2ml safe-lock tube containing the acid-washed beads. Cells were disrupted in the TissueLyser for five minutes at maximum speed. The suspension was centrifuged for 10 seconds at maximum speed. The supernatant was transferred into a new tube and the volume of the sample was determined. An equal volume of 70% ethanol was added and mixed by pipetting. Up to 700 μl lysate was transferred to a spin column placed in a 2ml collection tube and was centrifuged for 15 seconds at ≥8000 x g. Flow-through tube was discarded. 700 μl Buffer RW1 was added to the spin column. With lid closed gently, the spin column was centrifuged for 15 seconds at ≥8000 x g to wash the spin column membrane. The spin column was placed in a new 2ml collection tube with the Lids was closed gently and centrifuged at full speed for one minute. Spin column was then placed in a new 1.5ml collection tube. 30-50 μl RNase-free water was added directly to the spin column membrane and was centrifuged for one minute at ≥8000 x g to elute the RNA. This method followed the RNeasy Mini kit protocol (Qiagen, GmbH, Germany).

**qRT-PCR Analysis through Absolute Quantification**

The expression of lasR in both the test bacteria was determined to evaluate QSI activity through qRT-PCR analysis. The specific primers were: lasR (F) 5′-AAGTGGAATATTGGGAGAGCAG-3′ and lasR (R) 5′-CTATGCGGGCATCAGCCTG-3′ (Sahharwal et al., 2014). An internal standard 16S rRNA was used with the following primers: 16S rRNA (F) 5′-AGAGTGTGATCMTGGCTCWAC-3′ and 16S rRNA (R) 5′-CGYRAMCCTTWACGCT-3′ (Tripathi et al., 2013). The qRT-PCR program is as follows: incubation at 42°C for 5 min for reverse transcription; 1 cycle at 95°C for 2 min; then 45 cycles at 94°C for 20 s, 60°C for 20 s and 72°C for 30 s (Wada et al., 2009). KAPA One Step RT-PCR kit (KAPA Biosystems) was used for amplifications consisting of a mixture of 2.4 HPLC water, 5.0 μl KAPA Universal Mx, 0.2 μl dUTP, 0.5 μl of reconstituted forward and reverse primers, 0.2 μl RT Mix, and 1.0 μl RNA template. Several concentrations of RNA with 16S were used as internal control to quantify RNA transcript levels. Absolute quantification of the amplified transcripts was done through Bio-Rad CFX600 Real-Time System Thermal Cycler, which plots a standard curve from where critical threshold (Ct) values are derived. Through this, the concentration of its PCR signal (Cq) is quantified into this standard curve (Ilumina Inc., 2010).

**Statistical Analysis**

The non-parametric Mann-Whitney U Test with 0.05 level of significance using SPSS 13.0 program was used for the analysis of quantified biofilm as affected by the plant extracts. ct values was determined using qPCR delta ct (ddCt) method. Kruskal-Wallis test (non-
parametric ANOVA) where means between the control and experimental set-up were compared and the significance determined if the F-values was greater than the F-crit at 0.05 level of significance was used. Statistical analysis of the critical threshold value was done through ANOVA (Velasco et al., 2020).

RESULTS

Antibacterial Activity of Plant Extracts Against P. aeruginosa

Three (3) ethanolic extracts (A. intermedia seeds, C. winterianus leaves, H. suaveolens leaves) have antibacterial activity against the clinical isolate and two ethanolic extracts (A. intermedia seeds; C. winterianus leaves) have antibacterial activity against the reference strain P. aeruginosa PNCM 1335. Sixteen (16) ethanolic extracts tested in clinical isolate while seventeen (17) extracts tested in reference strain did not exhibit antibacterial activity making them qualified for the biofilm virulence assay. Results are shown in Table 1.

Table 1 Antibacterial activity of plant extracts against P. aeruginosa clinical isolate and P. aeruginosa PNCM 1335

<table>
<thead>
<tr>
<th>SCIENTIFIC NAME</th>
<th>LOCAL NAME</th>
<th>CLINICAL ISOLATE</th>
<th>REFERENCE STRAIN</th>
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<tbody>
<tr>
<td>Stachydrpepta jamaicensis</td>
<td>Lacyiminda</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Adenanthera intermedia Merr.</td>
<td>Kares stems</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Mikania micrantha Kunth.</td>
<td>Ola-ola</td>
<td>-</td>
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<td>Hypsia suaveolens Poir.</td>
<td>Ambabangot flowers</td>
<td>-</td>
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<td>Premna odorata Blanco</td>
<td>Asédaong</td>
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<tr>
<td>Cymbopogon winterianus Jowit.</td>
<td>Taday</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Phyllanthus urinaria L.</td>
<td>Tallalikod</td>
<td>-</td>
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<td>Dilleana philippinensis Rolfe</td>
<td>Katom</td>
<td>-</td>
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<td>Hydrocotyle vulgaris</td>
<td>Gotu kola</td>
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<td>Senna alata</td>
<td>Bensola</td>
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<td>Urena lobata L.</td>
<td>Pakot</td>
<td>-</td>
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<td>Ceiba pentandra</td>
<td>Béék</td>
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<td>Ficus sp.</td>
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<tr>
<td>A. Intermedia Merr.</td>
<td>Kares seeds</td>
<td>-</td>
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<tr>
<td>Eleusine indica L.</td>
<td>Pag roots</td>
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<td>Eleusine indica L.</td>
<td>Pag leaves</td>
<td>-</td>
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<td>Diplazium esculentum</td>
<td>Pako-pako</td>
<td>-</td>
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<tr>
<td>Hypsia suaveolens Poir.</td>
<td>Ambabangot</td>
<td>+</td>
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<tr>
<td>N/A</td>
<td>Talahib</td>
<td>-</td>
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<tr>
<td></td>
<td>Sterile distilled H2O (+ control)</td>
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<td>-</td>
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<tr>
<td></td>
<td>Norfloxacin (+ control)</td>
<td>+</td>
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Note: (+) = with antibacterial activity; (-) without antibacterial activity

Plant Extracts Inhibit Biofilm Formation

In the clinical isolate, seven (7) ethnobotanical extracts namely M. micrantha leaves (0.11mg/ml); H. suaveolens flowers (0.78mg/ml); H. vulgaris leaves (0.00mg/ml); A. intermedia leaves (1.11mg/ml); E. indica leaves (1.11mg/ml); D. esculentum (0.78mg/ml); and Talahib (0.67mg/ml) exhibited significant decrease in optical density (OD) values in biofilm formation compared to the negative control (without extract) with a value of 2.12mg/ml. In contrast, six (6) plant extracts not only had no effect against biofilm formation and even showed significantly higher OD values in comparison to the control. These plants are P. urinaria leaves (3.56mg/ml); D. philippinensis leaves (2.88mg/ml); S. alata leaves (2.22mg/ml); U. lobata leaves (2.25mg/ml); C. pentandra leaves (2.33mg/ml); and Ficus sp. leaves (2.44mg/ml), respectively. These showed no QSI activity but rather enhanced the formation of biofilm. Results are shown in Figure 1. Twelve (12) extracts showed notable lower values in biofilm formation on the reference strain (PNCM 1335) showing QSI activity. The extracts of M. micrantha (0.00 mg/ml); H. suaveolens leaves (0.11mg/ml); H. suaveolens flowers (0.11mg/ml); H. vulgaris (1.00 mg/ml); A. intermedia (0.00mg/ml); D. esculentum (0.33mg/ml); S. jamaicensis (1.00 mg/ml); U. lobata (1.00 mg/ml); C. pentandra (1.00 mg/ml); E. indica roots (0.78 mg/ml); E. indica leaves (1.00 mg/ml) and Talahib (0.00mg/ml) showed significantly lower OD measurement than the control (2.33mg/ml). The plant extracts, P. urinaria, D. philippinensis and S. alata significantly increased biofilm in the test isolates. Results are shown in Figure 2.
The plant extracts with the lowest recorded OD values in the biofilm formation assay were subjected to gene expression analysis. These were *M. micrantha*, *H. vulgaris*, *A. intermedia* and Talahib. 

*lasR* ct values in the clinical isolate treated with *M. micrantha*, *A. intermedia* and Talahib showed significant decrease in ct values validating the results in QSI biofilm quantification. Results are shown in Figure 3.

Similar to the clinical isolate, *lasR* ct values in the reference strain PNCM 1335, showed significant downregulation as affected by the plant extracts (*M. micrantha* with 1.87 and *A. intermedia* with 1.85) having 10.75 mean ct value. Results are shown in Figure 4.
DISCUSSION

QS-linked genes often control the production of virulence and gene products essential for bacterial host connections (Pirhonen et al., 1993; Parsek & Greenberg, 2000; Pearson et al., 2000). *P. aeruginosa* QS system consists of a transcriptional activator, lasR or rhlR, and an autoinducer synthase, lasI or rhlI, which manages the autoinducer PAI-1, N-(3-oxododecanoyl)-L-homoserine lactone; RhlI on the other hand, controls the synthesis of the autoinducer PAI-2, N-butyryl-L-homoserine lactone (Pesci et al., 1999). Blocking the path to the LasR receptor may have an effect on virulence productions synthesized by other signal receptors due to their overlapping mechanics. As such in the case of *P. aeruginosa*, QS molecules C4-HSL and 3-oxo-C12-HSL, synthesized by LasI and RhlI, respectively, are identified by their corresponding receptors LasR and RhlR, when threshold quorum concentration is attained. The las and rhl systems in combination, with two interconnected acyl-HSL signal-receptor pairs, 3-oxo-dodecanoyl-HSL-LasR and butanoyl-HSL-RhlR, control as much as 353 genes that comprise around 6 percent of the *P. aeruginosa* genome (Tay & Yew, 2013). These virulence system receptors can be blocked from synthesizing signals that mimic their complexes thereby confusing the bacterial QS, as shown when these plant extracts were applied (Li-vorsi et al., 2011).

*lasR* is affected by the plant extracts was significantly downregulated. This antagonistic effect may mean that the compounds in the plant extracts may have blocked the lasR pathway which control several QS virulence factors, particularly biofilm formation. As a consequence, blocking the LasR receptor may mean decrease in virulence factor production, e.g., biofilms. In other QS researches, specifically in *P. aeruginosa* biofilms, strains showing deficient production of the las signal molecule, 3O-C12-HSL, produced significantly reduced biofilms without their typical three-dimensional structure (Davies et al., 1998), confirming that the las QS system is critical in biofilm formation (De Kiviet et al., 2001). LasR coordinates pathogenicity in *P. aeruginosa* (Adonizio et al., 2008) and activates virulence genes lasB, lasA, apr, and toa (Gambello et al., 1993; Pearson et al., 1997). Therefore, if QSI compounds can block las expression, then all of the other QS-linked genes would be regulated as well, especially those involved in biofilm formation (De Kiviet et al., 2001). It can be observed that the plant extracts showed more antivirulence activity in biofilm formation in the reference strain *P. aeruginosa* PNCM 1335 than the clinical isolate. Mutations in lasR have been reported from clinical isolates of *P. aeruginosa* in cystic fibrosis patients (Hoffman et al., 2009) suggesting more possible complications in controlling its virulence system compared to the wildtype *P. aeruginosa* in which additional required autoinducer is normally provided by the las system (Papenfort & Bassler, 2016).

Although no confirmation was done, it is hypothesized that the plant extract *H. vulgaris* downregulates another gene/s involved in biofilm formation, hence, its slightly upregulated expression. *P. aeruginosa*, possess numerous virulence systems aside from las, which in turn, could have been the one affected by the plant extract. These systems are rhl, qsc, and pqs (Gilbert et al., 2009; Chugani et al., 2001; Pesci et al., 1999; Dubern & Diggle, 2008; Tay & Yew, 2013). Possible targeting of these other systems by the plant extract might have occurred.
by blocking their receptors. QS in *P. aeruginosa* consists of an intricately organized hierarchical system, where substantial communications through pathways often lead to an organization of a notable number of genes (Wilder et al., 2011). As such, *H. vulgaris* may have affected other virulence factors such as pyocyanin production, DNAse and swimming motility. This result may also mean that other bacterial pathogens in which QS is controlled by *lasR* may benefit from the potential antagonistic or inhibitory effect of the plant extracts.

The plants that showed QSI activities are known to possess phytochemicals with known QSI activities such as phenols, tannins flavonoids, alkaloids and other secondary metabolites such as saponins (da Gama et al., 2014). It has been found that compounds from plants have already proven promising candidates for inhibiting QSI (Adonizio et al., 2008; Pawar & Arumugan, 2011; Tan et al., 2014; Zhou et al., 2013; Husain et al., 2013).

Understanding QS gene systems crucial for virulence factors will present better insights of the connected genetic and phenotypic aspects, particularly biofilm development, and this information may ultimately pave the discovery of novel approaches for preventing and controlling complex and resistant biofilms. Since the discovery of QSI, plants have been a basis of medicines and presently continues to contribute significantly to pharmaceutical development (Craig et al., 1997 as cited by Adonizio et al., 2006) and ironically, only around 5–15% of the higher plants have been scientifically explored for their bioactive molecules (Pieters & Vlietinck, 2005). Likewise, research on the QSI activities of herbal plants is limited and it is highly probable that with in-depth studies, antimicrobial efficiency mediated by QS control will be thoroughly deliberated (Adonizio et al., 2008). The ethnomedical extracts found to have QSI activity against a human pathogen multi-drug resistant *P. aeruginosa* can be one of the possible means of treating infections and alleviating the emerging antibiotic resistance of many bacterial species of today. In *P. aeruginosa*, a switch to the biofilm development is connected to increased antibacterial resistance and thereby creates a distinctly more severe infection (Adonizio et al., 2008). Hence, it is now becoming more crucial to discover QSI compounds to control pathogenicity.

Strategies and approaches designed to block biofilm formation in clinical and industrial cases is currently the target of researchers on drug development. The confirmation of QSI of these plants is one of these strategies. The reduction of *lasR* expression and its effect on biofilm formation provide some understanding on how these can be used in the future to combat *P. aeruginosa* a multi-drug resistant (MDR) human pathogen, and other bacterial infections.

**CONCLUSION**

The ethnomedical extracts of the Ilongot-Égogot community showed antibacterial as well as QSI activity through inhibition of the biofilm formation in *P. aeruginosa* which was molecularly confirmed through gene expression analysis of *lasR*. This indicates the prospects of these ethnomedicals for therapeutic approach to control bacterial virulence without developing resistance. It is recommended for future researches to test the extracts’ QSI activity against other the *P. aeruginosa* virulence system *rhl* and analyze its coregulation with the other QS systems.

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Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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