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Quorum sensing-inhibition activities of Philippine ethnobotanicals against virulence factors in *Pseudomonas aeruginosa*

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Abstract

The global health issue of antibiotic resistance has brought about the discovery of a promising approach to control bacteria without developing resistant strains through quorum sensing inhibition (QSI) compounds. Among the most promising sources of novel QSI agents are the ethnobotanicals, plants that are utilized by ethnic communities as sources of medicine. Ten ethnobotanicals from the Igorot community of Imugan, Nueva Vizcaya, Philippines were screened to determine QSI activity against *P. aeruginosa* PNCM 1335.The methanolic extracts were tested for the presence of antibacterial activity using disk diffusion assay. In the absence of antibacterial property, the methanolic extracts were tested for QSI activities in reference to the expression of virulence factors swarming motility and pyocyanin production when the extracts were incorporated into the growth media. None of the methanolic extracts showed antimicrobial activity against *P.aeruginosa* PNCM 1335. All extracts manifested effect on swarming motility on the contrary; no methanolic extracts were found to inhibit pyocyanin production in *P. aeruginosa* PNCM 1335. The ethnobotanicals show considerable potential as sources of QS inhibitory compounds for new therapeutic direction in preventing pathogenicity without the threat for development of bacterial resistance.

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Introduction

Eradication of most infectious diseases is based on compounds that kill or inhibit the growth of bacteria. But a major concern in using this approach is the frequent development of bacterial resistance to antibiotics which is now considered a global health issue. Bacteria can adapt by mutation to the selective pressures imposed by antibiotics (Hentzer *et al.*, 2003) and this emergence of antibiotic-resistant bacteria to multiple classes of antibiotics poses a major threat to public health (McCormick, 1998). Over the last few decades, this threat is exacerbated by the failure of drug discovery programs to develop new antibacterial agents (Qazi *et al.*, 2006). As a result, there is renewed interest in antibacterial targets that interrupt the capability of pathogenic bacteria to cause infection by attenuating virulence (Alksne and Projan, 2000). The findings that many bacteria rely on quorum sensing (QS), or cell-to-cell communication, mechanisms to synchronize microbial activities that are essential for infection and survival in the host suggests a promising disease control strategy. QS allows bacteria to monitor their environment and alter behavior on a population-wide scale as a reaction to changes in the number present in a community. Using these signal-response systems, bacteria synchronize diverse physiological processes such as bioluminescence, virulence factor expression, antibiotic production and biofilm development (Chen *et al.*, 2002).

One promising mechanism that has evolved which interferes with bacterial cell- cell communication without developing resistant strains is through quorum sensing inhibition (QSI). QS-inhibitory compounds interfere with bacterial signaling molecules and signal biogenesis or signal detection. If efforts to coordinate were blocked, bacteria would lose their ability to form a systematized assault on the host's immune system or would less be able to mount organized community structures that promote pathogenesis (LaSarre and Federle, 2013). Studies have shown that QSI compounds inhibit the expression of QS-controlled behavior, in particular the production of extracellular virulence factors and inhibit the development of antibiotic-resistant biofilms in *Pseudomonas aeruginosa,* a gram-negative bacterium (Hentzer *et al.*, 2003). Halogenated furanones from the red alga *Delisea pulchra* are the only known anti-QS compounds of non-bacterial origin (Manefield *et al.*, 2002). QSI as reported by Adonizio *et al.* (2006) has also been found in a number of southern Florida seaweeds (Cumberbatch, 2002) and a few terrestrial plants (Teplitski *et al.*, 2000; Gao *et al.*, 2003). Still, few of higher plants have been studied, and a small number has been published with regards to QSI activity in medicinal plants.

Natural products are promising sources of quorum sensing inhibitory compounds that have the potential to inhibit QS regulation in bacteria. Ethnobotanicals represent a rich source of these natural products that can be antimicrobial and antipathogenic agents. The long use of these plants by the ethnic community provides a basis for further exploration of a plant's potential antimicrobial and antipathogenic compounds. Such compounds, in contrast to conventional antimicrobial counterparts, can neither be bactericidal nor bacteriostatic and can reduce the risk of bacterial resistance to antibiotics. Quorum sensing inhibitory compounds are less likely to impose a selective pressure for the bacteria to develop resistance to antibiotics for the reason that these compounds do not kill or inhibit microbial growth but interfere bacterial quorum sensing capability that are responsible for various physiological processes such as virulence factor expression and antibiotic production and this suggests a promising disease control strategy against pathogenic bacteria (McCromick, 1998).

Although ethnobotanicals have rich cultural traditions of usage, scientific investigation of these plants is still largely unexplored. In addition, pharmacological studies of the Philippine flora only gained momentum recently (Vital and Rivera, 2011). There is increasing interest in ethnobotanicals because of their uses and importance. The significance of a country's diverse medicinal plants

not only lies in their therapeutic value but also in their potential as sources of new chemical compounds for drug discovery. Although the Philippines is rich in biodiversity and cultural traditions of the usage of plants, scientific understanding of these ethnobotanicals remains largely unexplored (Vital and Rivera, 2011).Since these plants have not yet been profoundly explored, they could be tapped and studied for the discovery and development of drugs as they are locally used as medicinal plants.

Materials and methods

Collection of plant samples

Collected ethnobotanicals were pre-determined in a survey conducted by Undan *et al.* (2014) with the permission of the community elders. The samples were collected from Mount Imanduyan, Brgy. Imugan, Sta. Fe, Nueva Vizcaya, Philippines. The plant samples were placed in clean, sealed plastic bags and were transported to the laboratory for processing.

The identity of the plants was authenticated by an expert botanist from the Philippine National Museum in Manila, Philippines. Reproductive and vegetative parts of the specimens were collected and pressed, at least in duplicates, as required for obtaining the correct identity. The leaves of *Ageratina adenophora* (Panawel), *Ayapana triplinervis* (Pantallion), *Bidens pilosa*(Anwad), *Alstonia scholaris* (Palay), *Sarcandra glabra* (Hag-ob), *Derris elliptica* (Opay), *Pittosporum pentandrum* (Lahwik), *Cestrum nocturnum* (Dama de noche), *Oreocnide trinervis* (Lal-latan) and Lipang daga (no known scientific name) were tested for quorum sensing inhibition activities.

Methanol Extraction Procedure

The leaves were rinsed by running tap water to completely eliminate foreign matter on the surface. This was followed by second rinsing using distilled water and then with 70% (v/v) ethanol (Tan *et al.*, 2013). Washed leaves were air dried in shade instead of direct sunlight to avoid losing their active constituents (Handa *et al.*, 2008).Dried leaves were grounded to fine particles using a grinder (Tan *et al.*, 2013).Fifty (50) grams of each ground, dried leaves were soaked in 500 ml of 80% methanol in a stoppered flask for 72 hours. The mixture was then filtered using Whatman no.1 filter paper. Methanol was then completely removed using a rotary evaporator (Tan *et al.*, 2013). The resulting extracts were weighed and stored in tightly stoppered sterile amber bottles (Srisawat, 2007) at temperatures between 0-5 ℃.

Centrifugation of the extracts was at 10,000 rpm for 30 minutes followed by sterilization by membrane filtration with pore diameter of 0.45 µm (Srisawat, 2007). The sterility of the extracts was monitored by inoculating 100 µl in brain heart infusion agar (BHIA) from time to time. The sterile extracts were stored at refrigerated temperature at 2-8 ℃ prior to use (Srisawat, 2007).

Disk-Diffusion Assay for Antibacterial Activity of Plant Extracts on Pseudomonas aeruginosa PNCM 1335

Three (3) to five (5) colonies of *P. aeruginosa* grown for 24 hours in BHIA at 37°C were transferred to sterile distilled water, and turbidity adjusted to McFarland 0.5 standard (~1.5 x 10^8 CFU/mL). MHA plates were inoculated using a sterile cotton swab moistened with the standardized culture. Streaking of the entire surface was done three times, accompanied by rotation at every application to cover all areas (modified from Rezaei *et al.*, 2011).

Methanolic extracts were placed on sterile empty petri plates, 20 µL of each extract was pipetted onto 6 mm sterile blank disks (Sterile Blank Disk Hi-Media cat# SD067) and allowed to stand for a few minutes to eliminate excess liquid. Using a sterile forceps, infused discs were then transferred carefully onto previously inoculated 15 mm MHA plates equidistant to each other and were incubated at 37 ℃ for 24 hours.

Sterile distilled water served as negative control. Norfloxacin (5 µg; Hi-Media cat# SD184) served as positive control. Plates were prepared in triplicates.

Antibacterial activity is present when there is a clear or translucent zone of inhibition around the disks (Chenia, 2013). Each plant extract in the study that did not exhibit clearing, hence, ruling out possible antibacterial-mediated decrease in virulence factor production, which is required for accuracy of the subsequent assays continued to the evaluation of QSI against *P. aeruginosa* PNCM 1335.

Evaluation of Quorum Sensing Inhibition in Pseudomonas aeruginosa PNCM1335

Pyocyanin Production Assay: Overnight culture of *P. aeruginosa* grown in BHIB was diluted to 0.06 OD at 600 nm using UV-visible spectrophotometer (Beckman Coulter™, DU®530 Life Science UV/Vis Spectrophotometer). Then, the 4.5 mL of *P. aeruginosa* culture was added with 0.5 ml plant extract and incubated for 24 hours at 37°C. The treated culture was added with 3 ml of chloroform followed by mixing of the chloroform layer with 1 ml of 0.2 M HCl. Absorption of the extracted organic layer was measured using the UV-visible spectrophotometer at 520 nm. Sterile BHIB was used as blanks while 4.5 ml of *P. aeruginosa* culture with 0.5 ml sterile distilled water was used as control.

Swarming Activity Assay: Ten (10) ml of pre-solidified swarming agar containing glucose (1% w/v), bactoagar (0.05% w/v), bactopeptone (0.05% w/v) and yeast extract (0.02% w/v) was overlaid with 9 ml of agar supplemented with 1 ml plant extract. Then, the agar was inoculated in the center with an overnight culture of *P. aeruginosa*.

The plate was incubated for 24 hours at 37°C. Impaired swarming motility of *P. aeruginosa* indicated QSI activities of the plant extract.

Statistical analysis

QSI is present in *P. aeruginosa* if swarming motility is inhibited in comparison with the control, and pyocyanin production is reduced in the treated culture at OD520nm. Significance of the OD measurements in pyocyanin production assay was determined using Mann-Whitney U, the nonparametric counterpart of independent samples t-test, with $P<0.05$ considered as statistically significant.

Results and discussion

Disk-Diffusion Assay for Antibacterial Activity of Ethnobotanical Extracts

Absence of zones of inhibition was observed in ten (10) methanolic extracts against *P. aeruginosa* PNCM 1335, indicating absence of antibacterial activity (Table 1).

Table 1. Antibacterial activities of methanolic extracts against *P. aeruginosa* BIOTECH 1335.Note: (+) with antibacterial acitivity (-) no antibacterial activity.

PLANT EXTRACT	Antibacterial activity
Bidens pilosa	-
Cestrum nocturnum	-
Pittosporum pentandrum	-
Sarcandra glabra	-
Oreocnide trinervis	-
Lipang-daga	-
Derris elliptica	-
Alstonia scholaris	-
Ageratina adenophora	-
Ayapana triplinervis	-
Sterile Distilled Water (- control)	-
Norfloxacin (5µg) (+ control)	+

Pyocyanin Production Assay

OD measurements in pyocyanin production assay were analyzed using Mann-Whitney U, with P< 0.05. Some methanolic extracts showed increased pyocyanin levels in *P. aeruginosa* PNCM 1335(Figure 1). *S. glabra* showed the highest pyocyanin production level at OD_{520}nm of 0.120 mg/ml together with Lipang daga, *P. pendatrum, A. scholaris, A. adenophora* but not significant compared to the control with 0.053 mg/ml, indicating absence of QSI and rather possibly nutritive. Some methanol extracts showed a decrease in the pyocyanin production however not significant in comparison with the control. These were *B. pilosa* with optical density measuring at 0.050mg/ml; *O. trinervis, A. triplinervis* and *D.elliptica*, all three with optical densities measuring at 0.037 mg/ml.

Table 2. QSI activities of ethnobotanical extracts against *P. aeruginosa*PNCM 1335 swarming motility.

PLANT EXTRACT	QSI
Bidens pilosa	+
Cestrum nocturnum	+
Pittosporum pentandrum	+
Sarcandra glabra	+
Oreocnide trinervis	+
Lipang-daga	+
Derris elliptica	+
Alstonia scholaris	+
Ageratina adenophora	+
Ayapana triplinervis	+
Sterile Distilled Water (- control)	-

Swarming Motility Assay

Ten (10) out of 10 (100%) ethnobotanicals exhibited inhibition on swarming motility, a QS- controlled phenotype (Table 2). Swarming is a social phenomenon that involves rapid coordinated movement of bacteria across a semisolid surface (Kohler *et al.*, 2000) that is highly dependent on bacterial cell density, nutrient growth medium, and surface condition moistness (Tremblay *et al.*, 2007).

Inhibition of swarming motility was observed in all of the plates incorporated with methanolic extracts, consequently the presence of QSI. Swarming motility of *P. aeruginosa* is evident in all directions on the swarming agar plate with distilled water (negative control) while the swarming agar incorporated with methanolic extracts, on the other hand, showed inhibition of swarming motility of the bacterium after 16 hours of incubation.

Pyocyanin production and swarming motility in*P. aeruginosa* are complex phenotypes that involves several other traits (Overhage *et al.*, 2007) and the results point out that more specific traits need to be examined using additional indicators to further confirm the QSI effect of ethnobotanicals.

No methanolic extracts in the study significantly reduced the production of pyocyanin in *P. aeruginosa* PNCM 1335.In fact, some extracts even increased pyocyanin production. This increase may be due to the nutritive effects of the plant extracts toward the growth of *P. aeruginosa*, therefore increasing its proliferation and pyocyanin production. Pyocyanin (PCN) is a blue redox-active secondary metabolite. Despite *in vitro* studies demonstrating that PCN interferes with multiple cellular functions, its importance during clinical infection is uncertain (Lau*et al.*, 2004).

The ethnobotanical extracts proved to be effective in inhibiting swarming motility in P. aeruginosa PNCM 1335. Swarming of P. aeruginosa is a proven quorum-sensing mediated phenotype (Köhler *et al.*, 2000 as cited by Priya *et al.*, 2013). Swarmer cells of P. aeruginosa are hyperflagellated and elongated compared to the normal single-flagellum and short vegetative cells (Fraser and Hughes, 1999). P. aeruginosa requires flagella and type IV pili to swarm (Kohler *et al.*, 2000). It also produces biosurfactants such as rhamnolipids and 3-(3-hydroxyalkonoyloxy) and alkanoic acids (HAAs), that are also involved in swarming motility, as they aid in overcoming the surface tension between bacterial cells and their environment (Caiazza *et al.*, 2007; Tremblay *et al.*, 2007).

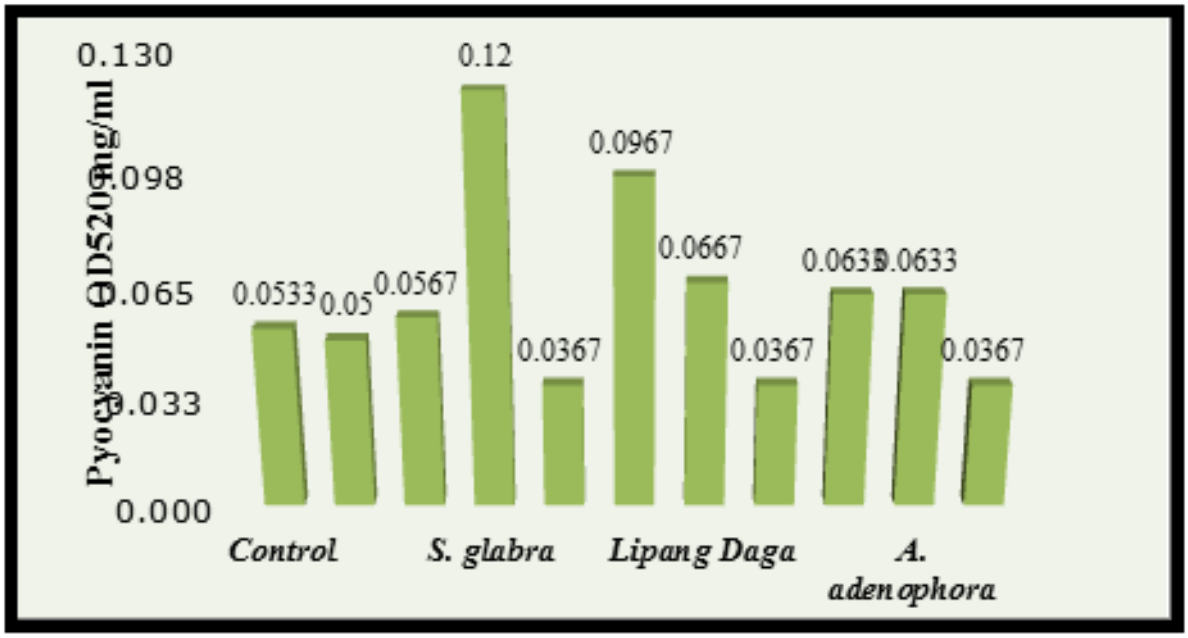

Fig. 1. Mean OD measurement of pyocyanin production in *Pseudomonas aeruginosa* PNCM 1335.

These ethnobotanicals contain substances or phytochemicals that possibly inhibited swarming. The percentage of ethnobotanicals producing substances with QSI properties is higher in the present study than reported in the other ethnobotanical screenings whereby only six (6) out of 50 plants (12%) screened showed QSI (Adonizio *et al.*, 2006). This suggests that the QSI activity of ethnobotanicals on bacteria is more ubiquitous and diverse and that there is distinct potential for further work on ethnobotanical species in this area of research. Although plant extracts substantially inhibited swarming in P. aeruginosa PNCM 1335, it was observed that they have little or no effect on pyocyanin production and some, such as S. glabra, even induced the production of pyocyanin in the bacteria. It appears that the compounds present in the ethnobotanical extracts may have different spectra of effects from beneficial, as in pyocyanin synthesis, to inhibitory, e.g. in swarming. Phytochemicals with proven QSI activities such as gamma aminobutyric acid (GABA), curcumin, furocoumarins, flavanones, flavonoids, flavonols, ursolic acid, rosmarinic acid, salycilic acid, urolithin, chlorogenic acid, aromatic compounds and furanones (Nazzaro *et al.*, 2013), tannins (Taganna *et al.*, 2011) and polyphenols (Huber *et al.*, 2003; Riedel *et al.*, 2006; Sarabhai *et al.*, 2013; Cragg *et al.*, 1997) can be found in the ethnobotanicals under study. In addition, phytochemical analysis revealed that methanol can extract alkaloids, flavonoids and other secondary metabolites like tannins, saponins, steroids, cardiac glycosides (Parekh and Chanda 2007; Anyasor *et al.*, 2010); and flavonoids, phenols, anthraquinones, cardiac glycosides and terpenoids (Anyasor *et al.*, 2010; Parekh and Chanda 2007). The presence of the phytochemicals in the ethnobotanicals could be one of the possible reasons for the QSI against P. aeruginosa.

The ethnobotanical extracts may have also affected the transcription of genes responsible for swarming motility. P. aeruginosa as a major pathogen has evolved numerous QS circuits, which modulate the production of various toxins and regulate parallel QS systems (Martin *et al.*, 2008). The sophisticated hierarchical QS network consisted of sets of connected systems such as las, iqs, pqs and rhl. Among the four, las and rhl circuits activate the expression of QS-responsive genes responsible for some key protein or virulence factors such as pyocyanin, together with elastase, protease, exotoxin A, alkaline protease, rhamnosyl-transferases, lectin and hydrogen cyanide (Lee and Zhang, 2014). These QS-responsive genes constitute nearly 10% of P. aeruginosa genome, and therefore accounts for a majority of the physiological processes and virulence phenotypes (Schuster and Greenberg, 2006).

The mechanisms of inhibition are not precise in any manner such as 1) inhibition of AHL autoinducer synthesis, 2) enzymatic destruction of AHLs molecules by AHL-acylase and AHL-lactonase, and 3) interference with signal receptors or blockage of formation of autoinducer/receptor complex (Lade *et al.*, 2014). Likewise, it was also discovered by Tamber *et al.* (2010); Rutherford and Bassler (2012), and Otto *et al.* (1999) that control of virulence gene expression can be done at the level of transcription and translation. As a consequence, it can be deduced that the methanolic extracts may have affected the mechanism of flagella and pili that aids in swarming activity in P. aeruginosa. At this level, it is also possible that the extracts antagonized the biosurfactant such as rhamnolipids and alkanoic acids of P. aeruginosa interrupting the swarming capability of the bacteria. However, the substance/s that is/are present in the extracts that inhibit swarming activity of P.aeruginosa are not identified. The verification of these possibilities is beyond the coverage of this research.

Not much research has been reported on the ethnobotanicals in the Philippines that show QSI activity. Researches targeting bacterial QS in ethnobotanicals are largely unexplored at present, hence, this offers additional area of research on plants with pharmacological potential leading towards drug development. Many natural substances including those extracted from ethnobotanicals have been evaluated for their ability to interfere with quorum sensing. The ethnobotanicals showed considerable potential as sources of QS inhibitory compounds for new therapeutic direction in preventing pathogenicity without the threat for development of bacterial resistance.

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