Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.6338

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (2):** 47-56 (2018)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Quorum Sensing Inhibition Bioactivities of Philippine Ethnobotanicals against *Pseudomonas aeruginosa*

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Received: 15.03.2018 | Revised: 18.04.2018 | Accepted: 23.04.2018

ABSTRACT

The quorum sensing inhibition (QSI) activities of ethanolic extracts of selected Philippine ethnobotanicals against Pseudomonas aeruginosa BIOTECH 1335 were evaluated in the study. Ethnobotanicals tested were Bidens pilosa L. (Anwad), Cestrum nocturnum L. (Dama de Noche), Sarcandra glabra (Thunb.) Nakai (Hag-ob), Oreocnide trinervis (Wedd.) Miq. (Lal-latan), Derris elliptica Benth. (Opay), Alstonia scholaris (L.) R. Br. (Palay), Ageratina adenophora (Spreng.) R. M. King & H. Rob (Panawel) and Ayapana triplinervis (Vahl) R. M. King & H. Rob (Pantallion).Ethanolic extracts were subjected to assays on the phenotypic expression of virulence factors for P. aeruginosa: pyocyanin production and swarming motility.The ethanolic extracts of O. trinervis, C. nocturnum and A. triplinervis showed presence of QSI in both virulence assays of P. aeruginosa; whereas the ethanolic extracts of S. glabra, D. elliptica, A. scholaris and A. adenophora showed presence of QSI only on the swarming motility but not in pyocyanin production. All ethanolic extracts of the ethnobotanicals showed great potential in the development of anti-quorum sensing drugs as new strategy to combat bacterial infections and inhibition of quorum sensing related virulence processes.

Key words: *Quorum sensing inhibition, Virulence factors, Ethnobotanicals, Pseudomonas aeruginosa.*

INTRODUCTION

Infectious diseases have been among the principal threats to the health of people throughout the history of man. The fight against pathogenic bacteria that cause diseases relies largely on our knowledge of microbe's interactions with the human body. Understanding how certain pathogenic bacteria cause disease may result in better treatment, vaccination, or prevention of infectious diseases[(1,2)]. The control of bacteria without the development of resistant strains could be favorable; and one way of doing that is through quorum sensing inhibition (QSI). Quorum sensing is a cell density-dependent signaling process used by bacteria for coordination of population-wide phenotypes, such as expression of virulence [3].

Cite this article: Limos, G.B.B., Judan Cruz, K.G. and Jacinto, W.R., Quorum Sensing Inhibition Bioactivities of Philippine Ethnobotanicals against *Pseudomonas aeruginosa*, *Int. J. Pure App. Biosci.* **6(2):** 47-56 (2018). doi: http://dx.doi.org/10.18782/2320-7051.6338

Communication among bacteria is achieved via the production, diffusion, detection and responses to chemical signaling molecules known as autoinducers. When a threshold concentration is reached, autoinducers are detected and this leads to quorum sensing and eventually, gene regulation. This process is able to regulate bacterial behaviors such as formation and release of virulence factors, antibiotic production and also biofilm formation[4, 5, 6]. Many opportunistic pathogenic bacteria depend on quorum sensing systems to coordinate their virulence expression; the interference with quorum sensing has been regarded nowadays as the novel way to control bacterial infections.

Pseudomonas aeruginosa is considered one of the most important pathogens in modern hospitals, which often proved to be resistant to antibacterial drugs[7]. *P. aeruginosa* is a gram-negative bacterium considered as one of the most common human pathogens associated with a wide range of hospital-acquired infections, particularly with cystic fibrosis and burnt patients, with a high mortality rate[3, 8, 9]. *P. aeruginosa* uses quorum sensing to collectively produce a suite of virulence factors that contribute to its disease-causing ability[10].

Among the recent means of controlling bacteria without fear of forming resistant strains is through quorum sensing inhibition (QSI). Anti-quorum sensing compounds are known to have the ability to attenuate bacterial pathogenicity. Since the regulation of many bacterial processes is controlled by quorum sensing, the finding of natural compounds acting as quorum sensing inhibitors suggest an attractive tool to control and handle infections caused by human, animal, and plant pathogens[4, 6,11 ,12] .

Many researches have evaluated quorum sensing inhibition potential of many plants. Just like animals and humans, plants are constantly exposed to bacterial infections, thus it is logical to expect that plants have developed sophisticated chemical mechanisms to combat pathogens. Biologically active constituents of natural products, especially plant-derived ones, have led to the discovery of new drugs used for treatment of numerous diseases[4, 13].

Of the plants, the ethnobotanicals are among the most promising in drug discovery. Ethnobotanicals grown in the wild have played important roles in local healing practices such as management and treatment of various ailments, diseases and infections, and is still utilized today[14,15]. However, most of these plants are unidentified, and remain unexplored, and thus, are potential prospects for research, especially in pharmacognosy. In this study, the ethnobotanicals from the Igorots of the Kalahan community, the indigenous people in the province of Nueva Vizcaya in the northeast part of the country were screened for the presence of quorum sensing inhibitors against *P. aeruginosa*. The ethnobotanical resources of the Igorot ancestral domain of Imugan, Sta. Fe, Nueva Vizcaya, whose wild grown plants have not been fully documented have been found to have medicinal properties, hence, results may validate existing traditional medicinal uses of the plants to the community and append newer application as alternative medicine.

The search for quorum sensing inhibitors presents a new perspective in the application of natural compounds as therapeutic agents[16], and the revival of opportunities in using our country's botanical resources as old sources but of novel compounds.

MATERIAL AND METHODS

Collection of Plant Samples

Plants included in the evaluation were pre-determined in an ethnobotanical survey conducted by Undan *et al.*[17] with the permission of the council of elders. Leaves of mature plants were collected by hand picking during the day when the photosynthetic activities of the plants are most active then placed in clean, sealed plastic bags and transported to the laboratory for processing. The place, time, season, and the name of the collector were recorded.

Ethanol Extraction

The leaves of plant samples were rinsed in running tap water to completely eliminate foreign matters on the surface. This was followed by second rinsing using distilled water and then with 70% (v/v) ethanol[18]. Washed plant materials were dried in shade instead of direct sunlight to avoid losing the active constituents[19]. Dried plant materials were ground to fine particles using a blender [18]. Excess ground plant materials were stored in amber bottles or sealed plastic bags in a cool dry place away from sunlight until use for up to six months.

Fifty (50) grams of each ground dried plant material were soaked in 500 ml of 95% ethanol in a stoppered flask for 72 hours. The mixture was then filtered using Whatman no.1 filter paper and the solvent was completely removed using a rotary evaporator [18]. The resulting extracts were stored in tightly stoppered sterile amber bottles [20] at temperatures between 0-5 °C. The containers were labeled with the name of plants, weight of the extract in grams (g), and the date of extraction.

The extracts were sterilized by centrifugation at 10,000 rpm for 30 minutes, followed by syringe filtration (Acrodisc 25mm Syringe Filter) with a 0.45 µm pore size. Sterility of the extracts was monitored by inoculating 100 µl in brain heart infusion agar (BHIA) from time to time. The sterile extracts were kept at 2-8 ℃ prior to use [20].

Evaluation of Quorum Sensing Inhibition in *Pseudomonas aeruginosa* BIOTECH 1335 Through Virulence Factor Assays

A. Pyocyanin Production Assay

A 48-hour culture of *P. aeruginosa* in Luria-Bertani broth diluted to 0.082 OD_{600nm} was aliquoted in 4.5 ml in separate test tubes in triplicates. The aliquotes were added with 0.5 ml plant extracts each and incubated for 24 hours at 37°C. Incubation was followed by centrifugation at 4000 rpm for 15 minutes in refrigerated centrifuge (MR1822, Jouan) to remove bacterial cells and supernatant transferred and added with 3 ml of chloroform to extract the pigment. The mixture was then added with 1 ml of 0.2 M HCl, and the pink layer that formed subjected to absorbance reading at OD_{520nm} using UV-visible spectrophotometer (Beckman Coulter, DU 530, Life Science). Sterile BHIB processed similarly was used as blank, while broth culture of *P. aeruginosa* BIOTECH 1335 added with 0.5 ml sterile distilled water was used as control. Pyocyanin OD measurements among the treatments lower than the control were interpreted as QSI caused by the extracts, and were then subjected to statistical analysis to determine significance.

B. Swarming Motility Assay

Ten (10) ml of pre-solidified swarming agar containing glucose (1% w/v), Bacto peptone (0.05% w/v), bactopeptone (0.05% w/v) and yeast extract (0.02% w/v) was overlaid with 9 ml of the agar supplemented with 1 ml plant extract. The agar was, then, inoculated onto the center with an overnight culture of *P. aeruginosa*. The plate was incubated for 24 hours at 37 °C. Restricted swarming motility of *P. aeruginosa* indicated by limited spreading was interpreted as quorum sensing inhibition activities of the plant extract.

Data Gathering and Statistical Analysis

QSI activities in *P. aeruginosa* BIOTECH 1335 is present when restriction of swarming motility is observed in swarming plates when compared with the control; and pyocyanin production was reduced in the treated culture at OD_{520nm} in comparison with the control. Significance in the measurements was determined using non-parametric Mann-Whitney U Test with 0.05 level of significance using SPSS 13.0 program.

RESULTS AND DISCUSSION

Phenotypic Detection of Quorum Sensing Inhibition of Plant Extracts

Ethanolic ethnobotanical extracts which did not exhibit antibacterial activity against the test bacteria were screened for quorum sensing inhibition (QSI). The production of the virulence factors in the test bacteria while exposed to the ethanolic extracts denoted inability of the plant extracts to inhibit quorum sensing.

Pyocyanin Production Assay

O. trinervis showed the highest significant decrease in pyocyanin production at OD_{520} with 0.017 mg/ml followed by *C. nocturnum* with 0.20 mg/ml and *A. triplinervis* with 0.23 mg/ml. The three ethanolic extracts obtained significantly lower levels of pyocyanin compared to the control (0.053 mg/ml) indicating inhibition of quorum sensing. Other extracts also showed a decrease in the production of pyocyanin though not significant as compared to the control. *A. adenophora* showed an increase in pyocyanin levels (0.057 mg/ml) though not significantly different to the control, indicating enhanced pyocyanin production.

Pyocyanin, a blue-redox reactive toxic exoproduct which causes cellular damage [21, 22], contributes to the persistence of *P. aeruginosa* infections. Pyocyanin can inhibit ciliary beating of airway epithelial cells, enhance superoxide production and increase apoptosis in neutrophils; and further inhibit other components of the immune system [22].

Infection of *P. aruginosa* is hard to eradicate due to development of strong resistance to most conventional antibiotics[23], its biosynthesis is regulated by intercellular communication. Pyocyanin production is a *pqs* (2-heptyl-3-hydroxy-4-quinolone) system-controlled phenotype where the genes *phzABCDEFG* and *phzM* control the expression of the virulence factor [23, 24]. *O. trinervis*, *C. nocturnum* and *A. triplinervis* may have suppressed the expression of these genes and hence, the phenotypic expression of the virulence factor.

Swarming Motility Assay

Swarming is an uncoordinated surface-associated motility of bacterial cells which functions to colonize a niche [25]. All ethanolic extracts showed inhibition of quorum sensing against *P. aeruginosa* swarming motility. Growth of the bacteria in the control showed motility where the bacteria 'swarmed' in all directions on the swarming agar plate. All the plates containing ethanolic extracts, on the other hand, showed suppression of swarming motion of the test bacteria after 24 hours of incubation.

O. trinervis, *C. nocturnum* and *A. triplinervis* showed presence of QSI in both virulence assays for *P. aeruginosa*. All other ethanolic extracts, *B. pilosa*, *S. glabra*, *D. elliptica*, *A. scholaris* and *A. adenophora* showed presence of QSI only on the swarming motility of *P. aeruginosa*.

Swarming is dependent on cell-to-cell communication for bacterial surface translocation. This bacterial motility is pivotal in microbial surface colonization and in the spreading of bacteria across the surface contributing to the formation of biofilms [26]. It is a flagellar motility that is under regulation of QS-related gene expressions[24] with other virulence factors, such as biofilm formation and proteolytic activity [27]. In a study by Köhler *et al.*[27,28], strains of *P. aeruginosa* with *lasI/lsR* mutant genes reduced and delayed swarming while strains having the *rhlI/rhlR* mutant genes completely diminished swarming ability.

The production of rhamnolipids, a lipopeptide biosurfactant, also plays a crucial role in swarming motility and biofilm development, benefiting *P. aeruginosa* in immune evasion [23, 24] and thus, involves QS regulation in its production [23]. The ethanolic plant extracts may have also prevented the expression of the QS regulated gene involved in the production of rhamnosyl-transferases (rhamnolipids): *rhlAB*. The compounds in the extracts may have acted by interrupting the QS system or by deregulating the synthesis of rhamnolipids.

Researches in the past two decades have revealed that *P. aeruginosa* follows four (4) quorum sensing interconnected systems: the *las*, *iqs*, *pqs* and *rhl* systems [23]. These QS systems orchestrate a symphony of virulence factors, and these could be interfered to attenuate the bacterial pathogenicity [29]. As illustrated by Lee and Zhang [23], the different quorum sensing systems in *P. aeruginosa* are concatenated and lead to several definitive virulent phenotypes. Among these are QS systems that translate cytotoxicity through production of pyocyanin, T3SS, Exotoxin A and HCN; and activate flagella and pili leading to swarming. Hence, suppression of these

phenotypic characters may indicate constraint in the quorum sensing mechanisms of the bacterium, either separately or collectively upon exposure to the plant extracts. The mechanism of inhibition, however, is not elucidated but may be 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. These strategies, likewise, can be applied to inhibition of AIPs-mediated quorum sensing in gram-positive bacteria [30].

A summary of the quorum sensing inhibition activities of the ethanolic ethnobotanical extracts of Imugan, Nueva Vizcaya in both *P. aeruginosa* is shown in Table 1.

Table 1: Summary of quorum sensing inhibition (QSI) of plant extracts on *Pseudomonas aeruginosa* BIOTECH 1335 virulence factors

Plant Extracts	*Pseudomonas aeruginosa*	
	Pyocyanin Production	**Swarming Activity**
Bidens pilosa		
Cestrum nocturnum		
Sarcandra glabra		
Oreocnide trinervis		
Derris elliptica		
Alstonia scholaris		
Ageratina adenophora		
Ayapana triplinervis		

Note: GREEN = with QSI; RED = without QSI; _____ = with antibacterial activity

O. trinervis, *C. nocturnum* and *A. triplinervis* inhibited and/or decreased the phenotypic expression of virulence factors in all assays making them the most promising for quorum sensing inhibition. The remaining ethanolic extracts, *B. pilosa*, *S. glabra*, *D. elliptica*, *A. scholaris* and *A. adenophora* showed decrease in virulence factor expression in only one of the two virulence factor assays.

Natural inhibitors against quorum sensing systems are reported and several studies have proven that higher plants produce and secrete secondary metabolites that interfere with the quorum sensing of bacteria, such as those by Adonizio *et al.*[6], Rezaei *et al.* [31], Song *et al.*[12], Chu *et al.*[32] and Tan *et al.*[18], among others. These secondary metabolites have proven anti-quorum sensing effects.

Some of the ethnobotanicals under study have been determined to contain phytochemicals which may have inhibited quorum sensing in *P. aeruginosa* as summarized in Table 2.

It is still difficult to indicate the nature and identity of the active compound/s present in the ethanolic extracts of the ethnobotanicals responsible for its quorum sensing inhibition activities due to the novelty of some of the species. It is possible, nevertheless, that both direct and indirect mechanisms are responsible for the QSI activities of the plants[33].

Table 2: Phytochemicals found in ethnobotanicals proven to have QSI

Ethnobotanicals	Phytochemicals present with proven QSI	References
Bidens pilosa	Flavonoids, terpenoids	(34,35)
Cestrum nocturnum	Alkaloids, flavonol	(36)
Sarcandra glabra	coumarins, flavonoids, rosmarinic acid, sesquiterpenoids	(37)
Oreocnide trinervis	flavonoids	(38)
Derris elliptica	tannins, alkaloids, terpenoids	(39)
Alstonia scholaris	alkaloids, tannins, triterpenoids, flavonoids phenolic acid	(40,41)
Ageratina adenophora	sesquiterpenes, alkaloids, coumarins	(42,43,44)
Ayapana triplinervis	coumarins, tannins, phenols, flavonoids, alkaloids	(45,46)

Quorum sensing inhibition does not kill or inhibit bacterial growth; the inhibition of pathogenesis of bacteria could be accomplished without growth inhibition, thus potentially avoiding selective pressures for drug-resistance [47, 48]. All ethanolic extracts of the ethnobotanicals showed, to some degree, quorum sensing inhibition in both species of bacteria, thus, are potential sources of new drugs in this therapeutic direction to combat bacterial infections. While the mechanisms of action are still unknown, evidence for this anti-pathogenic approach abound. The study infers that multiple phytochemicals in the extracts affected quorum sensing in various ways. Also, the use of ethanol as solvent may have influenced the potency of the extracts as quorum sensing inhibitors since ethanol penetrates the cellular membrane of plants easier to extract the intracellular ingredients[49]. Tiwari *et al.*[50] and Cowan[51] indicated that ethanol is capable of extracting tannins, polyphenols, flavonol, terpenoids, and alkaloids. Moreover, Sultana *et al.*[52] stated that phenolics are often extracted in higher amounts in more polar solvents such as ethanol. These active constituents of plants belong to the group of phytochemicals which are proven to have anti-quorum sensing abilities as identified by Nazzaro *et al*[53]. The extract yields of plant materials are strongly dependent on the nature of the extracting solvent, this is due to the presence of different compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent[52].

CONCLUSION

These ethnobotanicals are traditionally used by the Igorots as medicinal and toxic plants and have not yet been domesticated or cultivated. This presents a trend in which these ethnobotanicals present remarkable pharmacological potential. Aside from having anti-quorum sensing activity, these ethnobotanicals were also found to be anti-inflammatory, analgesic, antioxidant, anti-gout, glucosidase inhibitory (anti-diabetic) as well as antibacterial. Along with these prior researches, this study further confirms the medicinal significance of these ethnobotanicals to the Igorot community of Imugan.

Acknowledgements

The authors would like to acknowledge the permission and assistance of the people of Imugan, Sta. Fe, Nueva Vizcaya, Philippines. This piece of work is dedicated to them.

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