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ORIGINAL ARTICLE

Philippine Ethnobotanicals Inhibit Quorum SensingMediated Swarming Motility in *Pseudomonas aeruginosa*

Krystel Grace V. Padilla[1], Wilson R. Jacinto[2] and Khristina G. Judan Cruz[1]*
[1]Department of Biological Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines
[2]Biological Sciences Department, De La Salle University -Dasmarinas, City of Dasmarinas, Cavite, Philippines
***Corresponding author: Khristina G. Judan Cruz : E-mail: karenjudancruz@gmail.com**

ABSTRACT
Exposure to antibiotics intrinsically creates resistance among microorganisms. Quorum sensing (QS) or bacterial cell-to-cell communication is one of the promising approaches that target antibiotic resistant microorganisms. The importance of QS in bacterial pathogenesis has motivated research of inhibition through the use of QS inhibitors (QSI) compounds. Philippine ethnobotanicals were tested for its inhibition of swarming motility in Pseudomonas aeruginosa. Leaves of B. pilosa, C. nocturnum, S. glabra, P. pentandrum, O. trinervis, D. elliptica, A. scholaris, A. adenophora, A. triplinervis and Lipang Daga (no scientific name)from Imugan, Sta. Fe, Nueva Vizcaya were used for extraction using 95% n-hexane. Extracts were first tested for antibacterial activity against P. aeruginosa BIOTECH 1335. Extracts that were negative in the antibacterial testing proceeded to the QSI assay for swarming motility.None of the ten extracts exhibited antibacterial activity against P. aeruginosa. Swarming motility was inhibited by all n-hexane extracts, hence, presence of QSI. All ethnobotanical extracts display great potential for the development of drugs that inhibit QS regulated virulence in P. aeruginosa.

Keywords: *quorum sensing, swarming motility, Pseudomonas aeruginosa, ethnobotanicals*

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INTRODUCTION

When antibiotics were discovered and developed, humankind turned a corner in its ability to fight pathogens. Antibiotics rapidly became the treatment of choice for staving off infections and saving lives. However, exposure to antibiotics intrinsically creates resistance among microorganisms [1]. Their short generation time and the competence with which they develop and share resistance genes imply that no antibiotic remains effectual forever [2]. The worldwide practice of indiscriminate and constant use of antibiotics for the control and prevention of bacterial pathogens has led to the development of bacterial resistance to most available antimicrobials [3]. This multi-drug resistance is now recognized as a global health problem [4]. Therefore, there is a great need for the development of novel therapeutic measures to prevent infection among drug-resistant bacterial pathogens [5].

One of the most important pathogens in modern hospitals and often proved to be resistant to antibacterial drugs is *Pseudomonas aeruginosa. P. aeruginosa* is a Gram-negative bacterium [6], an opportunistic pathogen in humans and common nosocomial pathogen [7] that causes infections with a high mortality rate [8]. The latter is, in part, attributable to the organism's essentially high resistance to many antimicrobials [9] and the development of increasedmultidrug resistance in healthcare settings [10]. Expression of many virulence factors in *P. aeruginosa* is controlled by a quorum-sensing (QS) system [11], an intercellular communication system in which bacteria are able to notice the population density (via signalling molecules and receptors) and control gene expression consequently [12].

QS is a bacterial cell-to-cell communication mediated by extracellular signalling molecules called autoinducers (AIs), which accumulate in the environment in proportion to cell density [13]. Many

physiological processes in the bacteria including luminescence, virulence, motility, sporulation, biofilm formation, development of genetic competence, production of secreted proteolytic enzymes, synthesis of peptide antibiotics and fluorescence are coordinated by QS [14]. The interruption of this communication system can weaken microbial virulence because many important human pathogens depend on QS signalling systems to coordinate expression of virulence genes [15]. Strategies designed to interfere with these signalling systems will likely have broad applicability in the biological control of QS-dependent bacterial infections [16].

The importance of QS during bacterial pathogenesis has motivated research oon the control of this mechanism through the use of QS inhibition (QSI) compounds [17]. These approaches provide the advantage of interfering with this communication system and control the infectious bacteria without halting their growth, thus avoiding the development of bacterial resistance to antibiotics [18]. QSI can be achieved in several ways such as through the enzymatic destruction of QS signal molecules, the development of antibodies to QS signal molecules or via agents which block QS [19].

QS inhibitors offer a way of controlling microbial infections with the advantage of reducing risks of resistance development [20]. The continuing search for new and novel antimicrobials and anti-pathogenic agents has focused on exploiting the fact that plants surviving in an environment with high bacterial density are known to possess protective means against infections [21]. Search for quorum sensing inhibitors offers a new perspective on the application of natural or pure compounds as therapeutic agents which, by inhibiting this mechanism of cell communication, could be used to control bacterial diseases [22].

The current pursuit for new antimicrobials is therefore aimed at discovering non-toxic inhibitors of QS that can be used for treatment of bacterial infections in humans [23]. Natural products especially plants used in traditional medicines are promising sources for deriving molecules that can potentially inhibit quorum sensing [24]. Quorum sensing inhibitory (QSI) compounds have been identified from a wide range of natural resources, particularly medicinal plants, edible herbs, fruit and vegetables [25], as well as spices [26].

Among the potential sources of plants with pharmacological potential are the ethnobotanicals. However, the use of ethnobotany is not well-documented in the Philippines. Most of these plants are not well known, for they are endemic and can only be found and are only utilized in the areas of these ethnic tribes, hence unexplored for their medicinal potential. The Philippines consist of a large number of indigenous ethnic tribes inhabiting the country and most valuable natural resources are concentrated in their areas [27]. Among these tribes are the Igorots of the Kalahan community called Ikalahans, the indigenous people in the province of Nueva Vizcaya, northeast of the Philippines.

This paper presents challenging avenue of research as part of increasing evaluation of the potential of plants extracts as sources of new therapeutic and anti-pathogenic agents. These agents are non-toxic inhibitors of quorum sensing, thus controlling infections without encouraging the appearance of resistant bacterial strains.

MATERIAL AND METHODS

Collection of Samples

The plant samples were collected from Mount Imanduyan, Barangay Imugan, Santa Fe, Nueva Vizcaya.Plants included in the evaluation were pre-determined in an ethnobotanical survey [28] with the permission of the council of elders, and these were the following: *B. pilosa, C. nocturnum, S. glabra, P. pentandrum, O. trinervis, D. elliptica, A. scholaris, A. adenophora, A. triplinervis* and Lipang Daga (no scientific name). Leaves were collected by hand picking and were placed in clean, sealed plastic bags, and were transported to the laboratory for processing. Vegetative and reproductive parts of the specimens were collected in duplicates as required for obtaining correct identities. The authentication of the plants was carried out by an expert botanist from the National Museum of the Philippines in Manila.

Extraction Procedure with n-Hexane

Plant samples were rinsed by running tap water to completely eliminate foreign matters on the surface, followed by second rinsing using distilled water and finally with 70% (v/v) ethanol [29]. Washed plant leaves were dried in shaded area at room temperature instead of direct sunlight to avoid losing their active constituents [30]. Dried plant materials were ground to fine powder using a blender [29]. Excess ground plant materials were stored in amber bottles or sealed plastic bags in a cool dry place away from sunlight until for use up to six months.

The extraction procedures for 95% n-hexane were modified [29] and [31]. Fifty (50) grams of each ground dried plant material weighed in a flask was soaked with 500 ml of 95% n-hexane to completely submerge the material. The mixture was kept in the stoppered flask for 72 hours. The extracts were

filtered using Whatman No. 1 filter paper and the solvent was completely removed through rotary evaporator [29]. The resulting extracts were weighed, and were stored in tightly stoppered sterile amber bottles [31] at refrigerated temperatures between 0-5℃. Each container was labelled with the name of plant, weight of the extract in grams (g), and the date of extraction.

Crude n-hexane ethnobotanical extracts were prepared by dissolving in Dimethyl Sulfoxide (DMSO) to a final concentration of 20% plant extracts and 80% DMSO (100%) [32] since n-hexane extract was not miscible to the broth and agar (dissolved in distilled water).

Sterilization followed by centrifugation of the crude extracts at 10,000 rpm for 30 minutes, then membrane filtration with pore diameter of 0.45 μm [32]. The sterility of the extracts was monitored by inoculating 100 μl in brain heart infusion agar (BHIA) from time to time. The sterile extract was stored at 2-8℃prior to use [32].

Disk-Diffusion Assay for Antibacterial Activity of Plant Extracts in*Pseudomonas aeruginosa* BIOTECH 1335

Three (3) to five (5) colonies of *P. aeruginosa* BIOTECH 1335 grown for 16 to 18 hours in BHIA at 35℃were transferred to sterile distilled water and the turbidity was adjusted to McFarland 0.5 standard (~1.5 x 108 CFU/ml) [33]. Mueller Hinton Agar (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.

Sterile 6 mm paper discs (Sterile Blank Disc Hi-Media SD067) were placed on sterile empty Petri plate where about 20 μL of n-hexane plant extracts were pipetted and were allowed to stand for a few minutes until excess liquid has flowed out. Using a sterile forceps, infused discs were then transferred carefully onto previously inoculated 15-mm MHA plates equidistant to each other.

Sterile distilled water served as negative control. Norfloxacin (5 μg; Hi-Media SD184) served as the positive control. Plates were prepared in triplicates. Antibacterial activity is indicated by the presence of a clear or translucent zone of inhibition around the discs [34]. Plant extracts in the study should not exhibit clearing to rule out antibacterial-mediated decrease in virulence factor production, which was required for accuracy of the subsequent assays. Only plant extracts negative for antibacterial activity were continued to the phenotypic detection of QSI.

Evaluation of Quorum Sensing Inhibition in *P. aeruginosa*(Swarming Motility Assay)

Ten (10) ml of pre-solidified swarming agar was overlaid with 7 ml of swarming agar supplemented with 3 ml plant extracts. Then, the agar was inoculated in the center with an overnight culture of *P. aeruginosa* BIOTECH 1335. The plate was incubated for 24 hours at 37℃. Plates were observed for the presence of inhibition of swarming

RESULTS

Antibacterial Activity of Plant Extracts in *P. aeruginosa* BIOTECH 1335

All ten n-hexane ethnobotanical extracts namely: *B. pilosa, C. nocturnum, S. glabra, P. pentandrum, O. trinervis, D. elliptica, A. scholaris, A. adenophora, A. triplinervis*and Lipang Daga (no scientific name)did not exhibit antibacterial activity against *P. aeruginosa* making them qualified for the succeeding virulence assays.

Ethnobotanicals Inhibit Swarming Motility in *P. aeruginosa* BIOTECH 1335

All plant extracts in the study, *B. pilosa, C. nocturnum, S. glabra, P. pentandrum, O. trinervis, D. elliptica, A. scholaris, A. adenophora, A. triplinervis* and Lipang Daga (no scientific name), effectively inhibited swarming motility.

DISCUSSION

Swarming is a one of the QS-regulated phenotypes in *P. aeruginosa*. Swarming consists of a flagella-driven movement of differentiated swarmer cells (hyperflagellated, elongated, multinucleated) which enables bacteria to span over a semisolid surface as biofilms [35].QS regulation of swarming motility allows the optimal distribution of bacterial cells when a population was getting too large to dwell in a single niche or when the nutrients in the environment no longer suits their needs [36].

The results could be accounted to the potential QSI compounds of the ten n-hexane ethnobotanical extracts that might have targeted the QS-systems either by disrupting the N-acyl homoserine lactones (AHL) synthase, the signal molecule itself, or AHL target receptors [37].Moreover, the extracts might have affected the flagella or the pili of *P. aeruginosa* which are essential for swarming motility [38].

These QSI activities of the extracts can be attributed to phytochemicals extracted by n-hexane. Several studies have reported phytochemicals with proven QS activity. These are flavanones, flavonoids, flavonols [39], curcumin [40] furocoumarins,[41], rosmarinic acid, salycilic acid, urolithin, chlorogenic acid, aromatic compounds and furanones, tannic acid (tannins) [42] as well as phenols such as vannilin, furanones, and ellagitannins [43, 44]. Flavonoids in *Citrus* (mainly naringin) were found to inhibit motility [39].Furocoumarins were shown to hinder the formation of biofilm in *P. aeruginosa*[41]. Flavanones (naringenin and taxifolin) reduced the expression of several QS-controlled genes (*lasI, lasR, rhlI, rhlR, lasA, lasB, phzA1 and rhlA*) in *P. aeruginosa* PAO1 [45].

Literatures support that some of these phytochemicals with QSI activities are found in the ethnobotanicals under study. This possibly contributed to the capability of these ethnobotanical plants for QSI activity in either *P. aeruginosa.* For example, *B. pilosa* contains flavonoids, alkaloids, tannins, anthraquinones, and saponins [46]; *C. nocturnum* have alkaloids, flavonoids, tannins, glycoside, phenols, sterols, and saponins [47]; *S. glabra* include flavonoids, phenols, rosmarinic acid, isofraxidin, fumaric acid, terpenoids, and saponins [48, 49]; *P. pentandrum* is accounted to have flavonoids, amygdalin, sesquiterpene glycosides, triterpenoids, and essential oils [50]; *D. elliptica* also contains flavonoids, steroids, terpenoids, phenols, saponins, fixed oil and fat, and quinone [51]; *A. scholaris* contains alkaloids, tannins, glycosides, triterpenoids, flavonoids, coumarin, saponins, steroids, and phenolic acid [52]; *A. adenophora* has alkaloids, flavonoids, steroids, tannins, saponins, glycosides, phenols, and terpenoids [53] and *A. triplinervis* includes alkaloids, steroids, terpenes, phenols, tannin, and flavonoids [54]. The phytochemical content of *O*. *trinervis* is not clearly established yet but one species under this genus *O*. *integrifolia* is accounted to be rich in flavonoids [55].

Other n-hexane plant extracts have exhibited suppression of swarming motility. N-hexane extract of *Dalbergia trichocarpa* bark (DTB) inhibited the QS system of swarming motility in *P. aeruginosa*[56]. Clove essential oil has been proven to interfere also with *P. aeruginosa* QS, reducing social phenotypes as swarming motility [57]. *Melicope lunu-ankenda, Piper betle, Piper nigrum*, and *Gnetum gnemon* hexane extracts have shown significant QSI in swarming activity of *P. aeruginosa*[29, 58]. Several plants also showed QSI activity of various fruits and herbs in swarming motility of *P. aeruginosa*[24]. Traditional Chinese medicinal plants *Areca catechu*, Panax ginseng and *Prunus armeniaca* were also found to contain compounds with anti-swarming activity against *P. aeruginosa*[44, 59].

The results imply that these n-hexane ethnobotanical extracts are potential sources of new drugs in this therapeutic direction to stop or weaken bacterial infections. While the specific mechanisms on how these ten extracts inhibited these virulence factors are still unknown, confirmation for this anti-pathogenic approach needs to be studied. It is still premature to indicate the nature and identity of the active chemical substances present in the n-hexane ethnobotanical extracts responsible for QSI activities. The isolation of these active constituents of the plants was not done in the study. It is probable, on the other hand, that both direct and indirect mechanisms are responsible for the QSI activities of the plants [60]. But proven phytochemical contents of these extracts potentially isolated by n-hexane may have possibly affected the QS systems of these bacteria in various ways.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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