



Quantifying Production of Quorum Sensing Regulated Pigments in *Pseudomonas aeruginosa* BIOTECH 1335

KRYSTEL GRACE VERGARA PADILLA^{1*} and KING DAVE GLORIA MARTIN¹

¹Chemistry and Environmental Science Department, College of Arts and Sciences, Nueva Ecija University of Science and Technology, Cabanatuan City, Nueva Ecija, 3100, Philippines.

*Corresponding author E-mail: krystelgrace_vergara@yahoo.com

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ABSTRACT

One more method that can be used in the fight against communicable diseases is greatly important. Numerous pathogenic bacteria use intercellular signaling known as quorum sensing (QS) in defining virulence gene expression as well as gene regulatory mechanisms. Among the most promising sources of QSI agents are the ethnobotanicals. Extraction used 95% n-hexane in ethnobotanical leaves of *A. triplinervis*, *B. pilosa*, *C. nocturnum*, *S. glabra*, *P. pentandrum*, *O. trinervis*, *D. elliptica*, *A. scholaris*, *A. adenophora*, and Lipang daga (no scientific name). Extracts that were negative in the antibacterial testing proceeded to the QSI assay for pyocyanin production. The ten ethnobotanical extracts did not exhibit antibacterial activity against *P. aeruginosa* and were found to increase the pyocyanin production therefore the absence of QSI. However, all plant extracts can be used to increase the production of pyocyanin to accumulate more metabolites that are proven to have important biological and biotechnological applications.

Keywords: Quorum sensing inhibition, Pyocyanin, Virulence factor, Ethnobotanicals.

INTRODUCTION

Organisms have established numerous complicated ways to work together and adjust to the setting they occupy through natural evolution. For that reason, they must modify their phenotypes to respond in any diverse situations of external and/or internal pressure. Based on Van't Padje *et al.* (2016), for an organism to thrive in the new environment they must alter their metabolism as well as other activities¹. Since the finding of antibiotics to combat infections, their utilization has been expansive. Microorganisms become accustomed to

the misapplication of antimicrobial drugs resulted in their antibiotic-resistance. According to Van Hecke *et al.* (2017), within a short period of time antibiotics will not function properly and will become unmanageable in terms of communicable disease treatment and prevention, and also conventional contagions will be revived as of the main reason of mortality². In addition, the World Health Organization's (2017) information's are referring to the limitations of treatment selection to fight bacteria with resistance in antibiotics, and global synchronized action is vital to overwhelm this condition³.



According to the report of Starkey *et al.* (2014), the finding that numerous pathogenic bacteria employ cell to cell communication or termed as quorum sensing to control pathogenic effect and production of virulence factor assembly makes this process a striking focus for the strategy and progress of innovative curative agents⁴. Any Gram type of the bacterium uses QS to organize collective actions as the purpose to assemble a large number of cells. The works of Lee and Zhang (2015) showed that the system of quorum sensing has been displayed to govern the creation of a collection of virulence factors outside the cells and the development of biofilm in numerous pathogenic bacteria from *Pseudomonas aeruginosa* is also included⁵.

One of the opportunistic pathogens found in human is *P. aeruginosa* that can specifically contaminate patients with acquired immune deficiency syndrome (AIDS), cancer, burn wounds, cystic fibrosis, cytotoxic drugs exposure, those with infections in the eye, blood, skin, and genitourinary tract infections as well as patients that undergo surgery and their immune system is compromised⁶. Moreover, Girard and Bloemberg (2008) presented that this bacterium produces countless products that are released by the cells externally from where pyocyanin is included, and some are elastase, hydrogen cyanide, LasA protease, exotoxin A, alkaline protease, and rhamnolipids⁷. In order for *P. aeruginosa* to create and sustain the infection, it requires all these virulence factors that are released by the bacterium externally and are controlled by the QS system. Jakobsen (2011) recommended that deactivating the intercellular signaling mechanisms of a pathogen can end an important reduction in the production of these virulence proteins which regulate the disease mechanism of a bacterium⁸. Therefore, the interference of the quorum sensing scheme provides a novel way to combat bacteria that are resistant to multiple antibiotics.

Indigenous peoples in the Philippines usually live in geographically remote areas and commercially valued natural resources are found concentrated in their areas, yet unexplored for their medicinal potential⁹. Among these tribes is the Igorots of the Kalahan community called Ikalahans located in the Nueva vizcaya, Philippines¹⁰. Ethnobotanicals found in this area are used by the tribe for medical purposes, to control pests like rodents, and to paralyze fish for easy catching¹¹.

There are no known studies about QSI potential of the ethnobotanicals in the Igorot community of Imugan, Nueva vizcaya against pyocyanin production using n-hexane extracts which were the main target of the study, with great significance on the prevention and decrease in bacterial virulence.

MATERIALS AND METHODS

Sample Collection

Plants included in the tests were pre-determined in an ethnobotanical survey¹² through the permission of the council of elders of the Igorot community of Barangay Imugan, Santa Fe, Nueva vizcaya. Ten plant samples were identified for testing and were collected from Mount Imanduyan located within the barangay. These were *Bidens pilosa*, *Pittosporum pentandrum*, *Cestrum nocturnum*, *Oreocnide trinervis*, *Derris elliptica*, *Sarcandra glabra*, *Alstonia scholaris*, *Ayapana triplinervis*, Lipang daga (no known scientific name), and *Ageratina adenophora*. Leaves were collected by handpicking 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 cleaned with running tap water next rinse using distilled water and finally with 70% ethanol. Air-dried leaves were crushed to a fine powder using a blender⁹. Surplus pulverized leaves were kept in amber bottles or wrapped in clean plastic bags and stored in a cool dry place away from sunlight while waiting for use up to six months⁹. Dried ground leaves with a weight of fifty (50) g were soaked with a 500 mL of 95% n-hexane and kept in the Erlenmeyer or stoppered flask for 72 hours⁹. Whatman No. 1 filter paper was used for filtration and was subject through a rotary evaporator⁹. The filtrated extracts that were subject in rotary evaporator were kept in securely stoppered sterile amber bottles at refrigerated temperatures of 0-5°C¹³. Dimethyl Sulfoxide (DMSO) was used to dissolve crude n-hexane ethnobotanical extracts a final concentration of 20% plant extracts and 80% DMSO (100%)⁹.

Sterilization followed by centrifugation of the crude extracts at 10,000 rpm for 30 min, then membrane filtration with a pore diameter of 0.45 μm ¹⁴. The extracts sterility was checked by inoculating 100 μl in brain heart infusion agar (BHIA) from time to time. The sterile extract was stored at 2-8°C prior to use¹⁴.

Evaluation of Antibacterial Activity of Plant Extracts on *Pseudomonas aeruginosa* BIOTECH 1335 using Disk-Diffusion Assay

Pseudomonas aeruginosa BIOTECH 1335 with a colony of three (3) to five (5) were full-grown for 16 to 18 h in brain heart infusion agar (BHIA) at 35°C and were shifted to sterile distilled water and McFarland 0.5 standard ($\sim 1.5 \times 10^8$ CFU/mL) was used to adjust the turbidity⁹. Inoculation of Mueller Hinton Agar (MHA) plates was done by using a sterilized cotton swab moistened with the standardized culture. Sterile 6 mm paper discs (Sterile Blank Disc Hi-Media SD067) placed on sterile empty Petri plate with 20 μl of n-hexane plant extracts were pipetted and allowed to stand for a few minutes until excess liquid has flowed out. With the use of disinfected forceps, plant extracts in the discs were transferred onto 15-mm MHA plates equidistant to each other that were previously inoculated. Plates were prepared in triplicates. Norfloxacin (5 μg ; Hi-Media SD184) served as the positive control while sterile distilled water served as a negative control⁹. The presence of a clear or translucent zone of inhibition around the discs shows antibacterial activity¹⁵. To eliminate an antibacterial effect that can decline in the production of virulence factor, which was vital for the precision of the succeeding assay, only ethnobotanical extracts lacking antibacterial activity were qualified for the pyocyanin production assay.

Evaluation of Quorum Sensing Inhibition in *Pseudomonas aeruginosa* BIOTECH 1335 Pyocyanin Production Assay

Overnight culture of *Pseudomonas aeruginosa* BIOTECH 1335 grown in brain heart infusion broth (BHIB) was diluted to 0.06 OD at 600 nm using a UV-Visible spectrophotometer (Beckman CoulterTM, DU@530 Life Science UV/Vis Spectrophotometer)¹⁶. Then, the 4.5 mL of

P. aeruginosa culture was supplemented with 0.5 mL leave extract followed by incubation at 37°C for 24 hours¹⁶. The treated culture was added with chloroform with the amount of 3 mL then mixing of the chloroform layer with 0.2 M HCl with a quantity of 1 mL. The extracted organic layer absorption was quantified with the used UV-Vis spectrophotometer at 520 nm. For the blanks sample, sterile BHIB was used whereas *P. aeruginosa* culture of 4.5 mL with additional sterile distilled water of 0.5 mL was used as the control.

Statistical analysis

Pyocyanin production is reduced in the treated culture at OD₅₂₀nm. The significance of the OD measurements in pyocyanin production assay was determined using Mann-Whitney U, with a P<0.05 level of significance. Plant extracts with significantly lower OD measurements in comparison with the control meant the presence of QSI.

RESULTS

Antibacterial Activity of Plant Extracts in *Pseudomonas aeruginosa* BIOTECH 1335

Antibacterial testing was performed in the n-hexane extracts of the ten (10) ethnobotanicals to select which extracts meet the requirements for the evaluation of QSI. Fig. 1 shows the antibacterial activities of the ten n-hexane ethnobotanical extracts against *P. aeruginosa* BIOTECH 1335. All the ten n-hexane ethnobotanical extracts namely: *A. triplinervis*, *B. pilosa*, *S. glabra*, *P. pentandrum*, *O. trinervis*, *C. nocturnum*, *D. elliptica*, Lipang daga (Unidentified), *A. scholaris*, and *A. adenophora* did not display antibacterial effect counter to the bacterium used in the study making them qualified for the succeeding virulence assays.

The results could be attributed to the polarity of the solvent used the n-hexane, a non-polar solvent extracted different set of phytochemicals but in a lesser amount that cannot suppress or inhibit the growth of the two test bacteria. It could also be accounted that the greater the phytochemical load, the greater the antibacterial activity of a plant extracts. Although there was no antibacterial effect, shifting the focus from the antibacterial to QSI properties of these ethnobotanical plants might lead to the discovery of new QS inhibitor compounds.

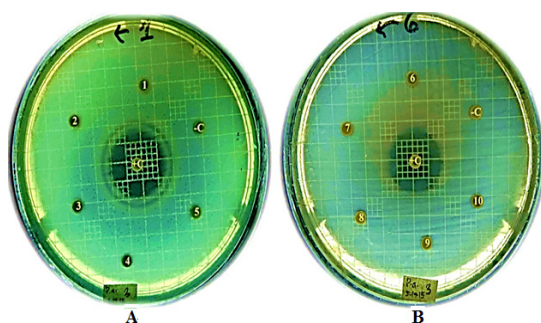


Fig. 1. Representative plates of antibacterial activities of n-hexane extracts against *P. aeruginosa* BIOTECH 1335 (A) 1- *B. pilosa*; 2- *C. nocturnum*; 3- *S. glabra*; 4- *P. pentandrum*; 5- *O. trinervis*; (B) 6- Lipang Daga (Unidentified); 7- *D. elliptica*; 8- *A. scholaris*; 9- *A. adenophora*; 10- *A. triplinervis*; -C - negative control; +C - positive control)

Pyocyanin Production Assay

The mean OD_{520nm} measurements of pyocyanin pigment production in the presence of ethnobotanical n-hexane extracts (mg/mL) which was diluted to 0.13 OD_{600nm} is presented in Fig. 2. All extracts did not result in any reduction in pyocyanin production in the test bacteria. Instead, extracts such as *A. triplinervis*, *B. pilosa*, *S. glabra*, *P. pentandrum*, *O. trinervis*, *C. nocturnum*, *D. elliptica*, Lipang daga (Unidentified), and *A. scholaris*, resulted in higher pyocyanin output to significant levels compared to that of *A. adenophora* also with increased pigment production at 0.060 mg/mL, however, not significantly compared to the control which was at 0.027 mg/mL. As such, none of the ten n-hexane ethnobotanical extracts can be considered QSI to pyocyanin production. The increase may possibly be due to the nutritive effects of the extracts, promoting the expression of the virulence factor in *P. aeruginosa* although this has not been verified in this study.

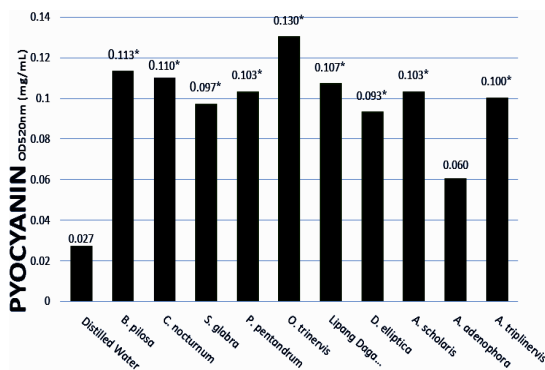


Fig. 2. Mean optical density (OD) measurement of pyocyanin production in *Pseudomonas aeruginosa* BIOTECH 1335 with distilled water and n-hexane ethnobotanical extracts

DISCUSSION

Polar solvent has shown to recover more bioactive components from plants than nonpolar solvents hence, a greater phytochemical load^{17,18}. Many extracted phytochemicals from plants might not be highly effective as antibacterial agents¹⁵. As an alternative, they hold properties against viruses or pathogenic bacteria, which are neither kill nor inhibit the growth of the bacteria and thus do not give the source or cause for the progress of bacteria which are resistant to various antibiotics¹⁵. The reduction of gene expression for pathogenic bacteria and the release of virulence factors was mediated by these compounds by inhibiting communication between cells or intercellular signaling and some connected properties¹⁹.

Some studies have noted the absence of antibacterial effects in some bacteria of n-hexane extracts such as by Khatun *et al.*, (2013) who reported that n-hexane and chloroform extracts displayed moderate inhibitory effect against most of the bacteria (including *S. aureus* and *P. aeruginosa*) in comparison to methanol or ethanol extracts²⁰. Ethyl acetate and n-hexane extracts showed no antibacterial activities against *P. aeruginosa* and *S. typhi*, respectively (Javid *et al.*, 2015)²¹, whereas Singh *et al.*,(2011), confirmed that ethanol, methanol, and water extracts of a plant, *Tecoma stans* can be effective against, *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. typhi*²². Biswas *et al.*,(2013), likewise, indicated that only methanol and ethanol solvent extracts prepared from the leaves of *Psidium guajava*, showed inhibitory activities against bacteria (*S. aureus* and *B. cereus*), and no inhibitory results in n-hexane²³.

Other studies, nevertheless, reported that some plant extracts have no QSI in pyocyanin production in the same bacterium such as the study of Tan *et al.*,(2013), which reported that n-hexane and methanol extracts of *Gnetum gnemon*, and n-hexane extracts of *Piper nigrum* showed no significant inhibition against pyocyanin production²⁴. Ahmad *et al.* (2015), on the other hand, had similar results as this study had seven essential oil compounds increased pyocyanin production in *P. aeruginosa*²⁵. *Panax pseudoginseng* encouraged the making of pyocyanin in cultures that are older. The authors posited that there is an additional compound

existing in the extract of ginseng, one compound is against swarming; the other compounds may disturb a dissimilar signaling trail in *P.aeruginosa* PAO1, inducing the synthesis of pyocyanin²⁶. These researches, on the other hand, specified that the volatiles of the plants are possible to either obstruct or encourage the cell to cell communication of the bacteria.

Although there were no QSI activities, all ethnobotanicals used in the study were able to enhance the production of pyocyanin. In other aspects of microbiology, this metabolite produces by *P. aeruginosa* have proven to use as dye decoloration²⁷, food colorants²⁸, antibacterial²⁸, antifungal²⁹, nematocidal²⁹ and pesticide degradation³⁰, antioxidant, anticancer, plant growth promotion and is a promising biocontrol candidate³¹, can be utilized for solving many agricultural^{27,28,30} and environmental issues³².

Extracts of *A. triplinervis*, *B. pilosa*, *C. nocturnum*, *P. pentandrum*, *Lipang daga*, *O. trinervis*,

A. scholaris, *D. elliptica*, *A. adenophora*, and *S. glabra*, do not exhibit antibacterial activities against *P. aeruginosa*. All ethnobotanical extracts show an increase in pyocyanin production of *P. aeruginosa* therefore the absence of QSI. Moreover, all plant extracts can be used to increase the production of pyocyanin therefore increasing the production of their metabolites that are proven to have important biological and biotechnological applications.

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Conflict of Interests

The authors state that there is no conflict of interest.

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