IJBPAS, April, 2018, 7(4): 527-537

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

PHILIPPINE ETHNOBOTANICALS INHIBIT QUORUM SENSING-CONTROLLED BIOFILM FORMATION IN *Pseudomonas aeruginosa*

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Received 30th Oct. 2017; Revised 25th Nov. 2017; Accepted 26th December 2017; Available online 1st April 2018

ABSTRACT

The Quorum Sensing Inhibition activities of Philippine ethnobotanicals were determined through the Biofilm Formation Assay against *Pseudomonas aeruginosa* BIOTECH 1335. Leaves of ethnobotanicals were collected from Barangay Imugan, Sta. Fe, Nueva Vizcaya. These were Hag-ob (*Sarcandra glabra* (Thunb.) Nakai), Palay (*Alstonia scholaris* (L.) R. Br.), Panawel (*Ageratina adenophora* (Spreng.) R. M. King & H. Rob), Pantallion (*Ayapana triplinervis* (Vahl) R. M. King & H. Rob), Dama de Noche (*Cestrum nocturnum* L.), Lal-latan (*Oreocnide trinervis* (Wedd.) Miq.), Lipang Daga (local name; no known scientific name), and Opay (*Derris elliptica* Benth.) Statistical analyses were conducted to evaluate the presence of QSI activites in the plant extracts.

Methanolic extracts of *C. nocturnum*; Lipang Daga; and *P. pentandrum* show a significant inhibition against *P. aeruginosa* biofilm formation. These methanolic extracts of ethnobotanicals showed a considerable potential as sources of QSI compounds for a new therapeutic direction in preventing pathogenicity without developing resistance in bacterial strains.

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

INTRODUCTION

In search for answers to control diseases, people looked for drugs in nature [1]. In the advancement of technology and development of mankind, the healing properties of certain medicinal plants were identified, noted, and conveyed to the succeeding generations. Among the benefits of using these medicinal plants is also the development of drugs against pathogenic diseases [2].

Annihilation of most diseases is based on how it kills or inhibits the growth of pathogens. Therefore, the development of antibiotics in 1940 provided physicians an effective tool against bacterial infection [3]. The use of antibiotics is the traditional way of treating infectious diseases that aims to kill or inhibit the growth of bacterial colony. However, the development of resistance to antimicrobial compounds is a major concern with this approach [4]. Antibiotics charge a selective pressure through which the bacteria can adapt by mutation. The rise of resistance against some classes of antibiotics by the bacteria gives a major threat on public health [6]. This becomes more complicated due to the failure of the development of new antibacterial agents [7]. Therefore, we are entering an era against antibiotics with a lower capability to fight microbes [8].

A new interest in the field of antibacterial mechanism that aims to interrupt the capability of pathogenic bacteria that causes infection by virulence [9] is gaining interest. This field that deals with the disruption of bacterial communication is called quorum sensing inhibition (QSI).

Quorum sensing (QS) is a cell density-dependent signalling process used by bacteria for coordination of population-wide phenotypes, such as expression of virulence [10]. It is a bacterial communication in which produce, release, and detect signal molecules known as the autoinducers (AIs) [11]. This allows the bacteria to sense and trigger behaviors with their environment and even their changes in number of population with their community. Bacteria detect the accumulation of a minimal threshold stimulatory concentration of these autoinducers and alter gene expression and in response alter bacterial behavior [12]. Using the signal system, bacteria can synchronize physiological processes such as virulence factor expression, antibiotic production and biofilm development [11].

Pseudomonas aeruginosa is 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 [10]; [13]; [14]. *P. aeruginosa* uses QS to collectively produce a suite of virulence factors that contribute to its disease-causing ability [15]. *P. aeruginosa* is possibly the best understood in terms of the virulence factors regulated among all bacteria that utilize QS as part of their pathogenic life style [16].

P. aeruginosa is commonly used for studies on QSI because (1) the QS network of *P. aeruginosa* is one of the best characterized quorum-sensing systems to date; (2) the QS regulates the expression of numerous virulence-related products and has been shown to be important for *P. aeruginosa* pathogenesis in various model infection systems; and (3) this pathogen gives a large burden on the medical community due to its broad resistance to antibiotics and lack of effective treatment options [17].

The most effective mechanism that disrupts cell to cell communication of bacteria without development of resistant strain is through QSI. QS inhibitors interfere the signaling molecules and signal detection of bacteria. As a result, bacteria lose their systematized form of attack in the host's immune system or they can be less powerful to form a community structure that exhibit pathogenesis [17]. QS inhibitors in medicinal plants is now being studied and continues to gain interest in the field of drug discovery.

Among all the plants, the ethnobotanicals has the most potential to be screened for QSI. Ethnobotanicals are groups of species of plants that are traditionally used by our elders and indigenous groups in preventing and curing diseases. They are potential rich sources of compounds against pathogens and microbes. The ethnobotanicals of Brgy. Imugan, Sta. Fe. Nueva Vizcaya shows a promising pharmacological potential. These plants were found to have anti-gout, antidiabetic, anti-inflammatory, analgesic, anti-oxidant as well as anti-bacterial (personal communication Judan Cruz, 2015) properties. Recently, these ethnobotanicals were proven to prevent the expression of virulence factors in different assays, hence with QSI activities [18]; [19]; [20]).

The failure of development of new drugs against pathogenic diseases results to a continuity of bacterial pathogenicity and even the increase of the resistance of bacteria against antibiotics. This situation gives a huge threat to human health. In order to prevent the increase of bacterial resistance against antibiotics, studies show the potential of QSI. QSI is one of the promising mechanisms that inhibit the

bacterial communication. Unlike the antibiotics, it does not kill bacteria, but in contrast, it destroys their communication that they use in attacking the host's immune system. In response to producing new ways of preventing the development of disease against drug resistant strains of bacteria, this study contributes a developmental approach on the QSI potential of different ethnobotanicals.

MATERIALS AND METHODS

Collection of Plant Samples

Ten (10) ethnobotanical plants previously surveyed [21] with the permission of elders and barangay officials were used in the testing of QSI and these were: *Biden spilosa* L.. *Cestrum nocturnum* L, *Sarcandra glabra* (Thunb.) Naka, *Oreocnide trinervis* (Wedd.) Miq, *Ageratina adenophora* (Spreng) R. M. King H. Rob), *Derris elliptica* Bent. *Alstonia scholaris* (L.) R. Br, *Ageratina adenophora* (Spreng.) R. M. King H. Ro, *Ayaphana triplinervis* (Vahl) R.M. King H. Ro and Lipang daga (local name). Plants were previously tested for antimicrobial activity [19].

Extraction Procedure

The leaves were rinsed in running tap water to completely eliminate unnecessary matters on the surface. This was followed by second rinsing using distilled water and finally with 70% (v/v) ethanol for disinfection [22].

Fifty (50) grams of each ground dried leaves weighed in a flask were treated with 500 ml of 80% methanol to completely submerge the material. The mixture was kept in the stoppered flask for 72 hours to soak. The extracts were filtered using Whatman No. 1 filter paper and the solvent was completely removed through rotary evaporation [22]. The resulting extracts were stored in tightly stoppered sterile amber bottles [23] at temperatures between 0-5°C. Sterilization followed by centrifugation of the extracts at 10,000 x g for 30 minutes, then membrane filtration with pore diameter of 0.45 µm [23] was done. 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 2-8°C prior to use [23].

Quantification of QSI using the Polyvinyl Chloride Biofilm Formation Assay

Stock cultures of *Pseudomonas aeruginosa* BIOTECH 1335 were revived in brain-heart infusion broth (BHIB) and maintained in brain-heart fusion agar (BHIA) plates. Assay for *P. aeruginosa* (biofilm formation) [24] were modified to incorporate the plant extracts and sterile

distilled water as the control in the media. All tests were done in triplicates.

Overnight cultures of *P. aeruginosa* BIOTECH 1335 were resuspended in fresh LB medium in the presence or absence of plant extracts. After a 10-h incubation at 30°C, the plates were rinsed to remove planktonic cells, and the surface-attached cells. Quantification was done by solubilizing the dye in ethanol and measuring the absorbance at OD_{595} nm.

RESULTS AND DISCUSSION

Three (3) of the methanolic extracts showed a significant decrease in biofilm formation in comparison with the negative control showing the presence of QSI activity as shown on Figure 1. The extracts of *C. nocturnum* with 0.1255*;* Lipang daga (local name) with 0.1552; and *P. pentandrum* with 0.0987 had significantly lower OD measurements compared with the control (0.3427 mg/ml) indicating QSI. However, *S. glabra* had a significantly higher OD measurement compared to the control indicative of enhancement of biofilm formation. The other extracts showed no significant difference and even higher OD values in comparison with the control, indicating the absence of QSI.

Biofilm formation is a virulence factor that contributes to a high emergence of *P. aeruginosa* as a major opportunistic pathogens on human health (Adonizio, 2008)[24]. Biofilms are communities of microbes attached to surfaces, which can be found in many medical, industrial and natural settings. *Pseudomonas aeruginosa* has become the primary model organism for bacterial biofilms, and several researches have been done in order to identify the concurrency in biofilm formation and to formulate a general biofilm model [25].

P. aeruginosa has been proven to be a highly resistant strain. It infects a host, by expressing an abundant numbers of virulence factors that allow it to attack the host immune system while evading treatment. An essential defensive feature of this organism is its outer phospholipids membrane which has limited permeability for most molecules. It is also capable of forming a biofilm under physiological conditions that contributes to its persistence despite long-term treatment with antibiotics. The main characteristic property of all biofilms is their highly resistance to eradication by physical and biochemical treatments, including antibiotics. Although this resistance has been recognized for many years, its biological basis has still not been thoroughly explained [26].

Many characteristics of biofilms, such as the exopolysaccharide matrix and specialized virulence gene expression,

contribute to the high emergence of resistant strain against normal antibiotic doses. *Pseudomonas aeruginosa* usually produce one or more number of polysaccharides that provide hydrated scaffolding for the stability purposes and function for the reinforcement of the structure of the biofilm, mediate cell-cell and cell-surface interactions, and provide the bacteria a protection from biocides and antimicrobial agents [27].

Several mechanisms are responsible for the QSI activity of the plant extracts [28]. QSI may be due to the phytochemicals present in the extracts. The ethnobotanicals tested contain phytochemicals with proven QSI activities. These include gamma aminobutyric acid (GABA), curcumin, furocoumarins, falvanoes, flavonoids, flavonols, ursolic acid, rosmarinic acid, salycilic acid, urolithin, chlorogenic acid, aromatic compounds and furanones [29], tannins [30] and polyphenols [31]. These phytochemicals are contained by the ethnobotanicals tested.

The extraction solvent also accounts for the types of phytochemicals in the extracts tested. For example, methanol can extract some phytochemicals such alkaloids, flavonoids and other secondary metabolites like tannins, saponins, steroids, cardiac glycosides [32] [33] which are known for their QSI properties.

While three of the methanolic extracts of the ethnobotanicals namely: *S. Glabra,* Lipang Daga, *P. pentandrum* inhibited the pathway that is accountable for the development of the some of biofilm in *P. aeruginosa,* all of these methanolic extracts showed QSI in another virulence factor, the swarming motility [19].

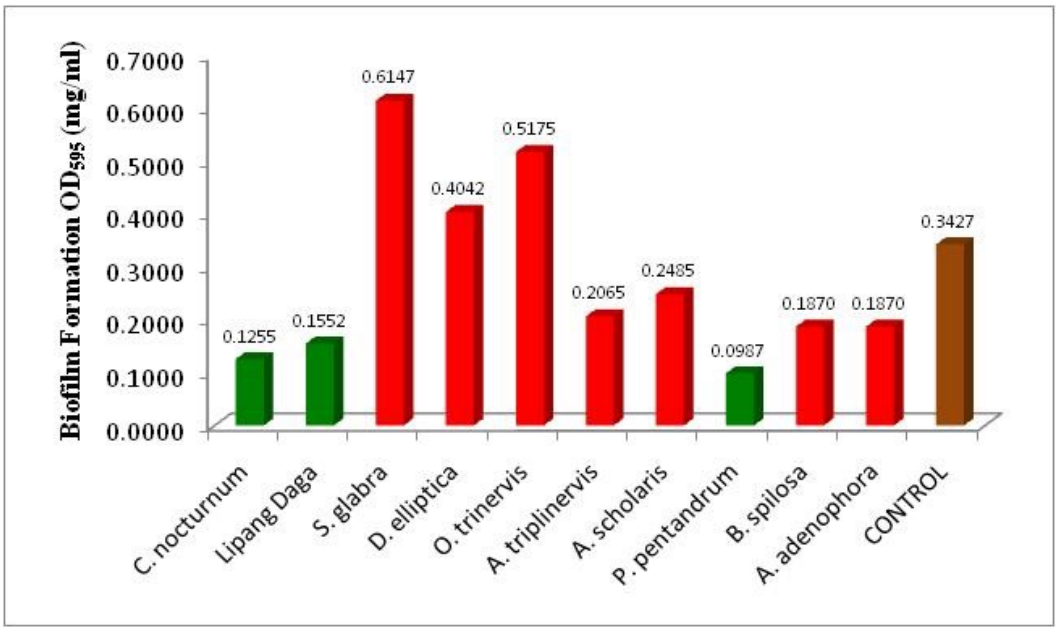

Figure 1: OD measurement of Biofilm Formation Assay in *Pseudomonas aeruginosa* BIOTECH 1335 in comparison with the *Pseudomonas aeruginosa* control. (Green: positive for QSI; Red: without QSI)

CONCLUSION

The potential of the ethnobotanicals as new source of drugs in this therapeutic direction to combat bacterial infections has been shown. This also points out that the QSI activities of these ethnobotanicals on bacteria is diverse. More researches that tap the pharmacological potential should be done, as this may pave for development of drugs targeting bacteria and create an enormous impact in the society especially in pharmacological and biological field.

ACKNOWLEDGEMENT

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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