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Quorum sensing inhibition activities of Philippine ethnobotanicals against virulence factors in *Staphylococcus aureus*

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Abstract Quorum sensing (QS) and quorum sensing-inhibition (QSI) compounds hold a promising approach in combatting pathogenic diseases without the development of resistant strains. The global concern on rising antibiotic resistance paved the way for tapping natural products for their QSI activities. Natural products are ideal sources of QSI compounds that have the potential to inhibit QS regulation in bacteria. Ten ethnobotanicals namely *Ageratina adenophora* (Spreng.) R.M.King & H.Rob. (Panawel), *Alstonia scholaris* (L.) R. Br. (Palay), *Ayapana triplinervis* (Vahl) R.M.King & H.Rob. (Pantallion), *Bidens pilosa* L. (Anwad), *Cestrum nocturnum* L. (Dama de noche), *Derris elliptica* (Wall.) Benth. (Opay), *Oreocnide trinervis* (Wedd.) Miq. (Lal-latan), *Pittosporum pentandrum* (Blanco) Merr. (Lahwik), *Sarcandra glabra* (Thunb.) Nakai (Hag-ob), and Lipang daga (without known scientific name) were evaluated for the occurrence of QSI activity against *Staphylococcus aureus* PNCM 1582. Disk diffusion assay was tested the antibacterial activity of the methanolic extracts. In the absence of antibacterial activity, the methanolic extracts that were incorporated into the growth media were evaluated for QSI against the expression of virulence factors; DNase and α-hemolysin, of *S. aureus* PNCM 1582. Three methanolic extracts, *B. pilosa*, *C. nocturnum*, and *P. pentandrum*, showed antimicrobial activity against *S. aureus* PNCM 1582. Extracts of *O. trinervis* and *D. elliptica* showed QSI against DNase production. No methanolic extracts inhibited the α-hemolysin production in *S. aureus* PNCM 1582. The result represented possibilities for future studies on the biological activities of these plants. These plants are probable sources of natural products against QS to develop drugs against bacterial pathogens without developing resistance.

Keywords: Quorum sensing inhibition, Virulence factors, Ethnobotanicals, *Staphylococcus aureus*

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Introduction

The discovery of quorum sensing (QS) in bacteria and the use of quorum sensing-inhibition (QSI) compounds holds a promising approach in combatting pathogenic diseases without the development of resistant strains. On a population-wide scale, bacteria can monitor their environment and alter behavior through QS as a reaction to changes in the number present in a community. Using QS system, bacteria synchronize diverse physiological processes such as bioluminescence, biofilm development, antibiotic production, and virulence factor expression (Chen *et al*., 2002). Interrupting this monitoring system disrupts the synchrony of microbial activities essential for bacterial infection, thereby decreasing virulence expression without developing resistance. Quorum sensing inhibitory compounds rarely impose selective pressure for the bacteria to develop resistance to antibiotics. These compounds do not kill or prevent microbial growth, but interfere with bacterial quorum sensing capability, suggesting a promising disease control strategy against pathogenic bacteria.

Staphylococcus aureus is a major cause of infections attributed to its multi-drug resistance (Lee *et al.*, 2012). The global concerns on rising antibiotic resistance paved the way for tapping natural products for their QSI activities. Natural products are ideal sources of quorum sensing inhibitory compounds that can prevent bacterial QS regulation. The significance of the country's diverse medicinal plants is based in their therapeutic value and their potential as sources of new chemical compounds for drug discovery. The Philippines is endowed with rich source of natural resources and cultural traditions of the use of plants, but the scientific understanding of plants remains mostly unexplored (Vital and Rivera, 2011). Among these are the ethnobotanicals utilized by ethnic communities in the Philippines for medicinal purposes. These plants are usually found in the wild, and their medicinal claims are typically without scientific evidence. Ethnobotanicals represent a rich source of these natural products that can be antimicrobial and antipathogenic agents. The use of these plants by the ethnic community provides a basis for further exploration of a plant's potential antimicrobial and antipathogenic compounds.

In contrast to conventional antimicrobial counterparts, QSI compounds are not bactericidal nor bacteriostatic and can decrease the risk of bacterial resistance to antibiotics. A growing number of studies have been published, and a handful of higher plants have been studied on their quorum sensing inhibition activity. Despite this, there has been limited research on the QSI of medicinal plants. In this study, we evaluated the ten ethnobotanicals namely *Ageratina adenophora* (Spreng.) R. M. King & H. Rob. (Panawel),

Alstonia scholaris (L.) R. Br. (Palay), *Ayapana triplinervis* (Vahl) R. M. King & H. Rob. (Pantallion), *Bidens pilosa* L. (Anwad), *Cestrum nocturnum* L. (Dama de noche), *Derris elliptica* (Wall.) Benth. (Opay), *Oreocnide trinervis* (Wedd.) Miq. (Lal-latan), *Pittosporum pentandrum* (Blanco) Merr. (Lahwik), *Sarcandra glabra* (Thunb.) Nakai (Hag-ob), and Lipang daga (without known scientific name) for antibacterial activity and for the occurrence of QSI activity against *Staphylococcus aureus* PNCM 1582. This study may prompt numerous researches in a quest to find strategies to attenuate bacterial virulence.

Materials and methods

Collection of plant samples

Collected ethnobotanicals were pre-determined in a survey conducted by Undan *et al.* (2014). Plant samples were collected from Mount Imanduyan, Brgy. Imugan, Sta. Fe, Nueva Vizcaya with the permission of the community elders. The leaves of the collected ethnobotanicals were evaluated for QSI activities. The scientific names and local names are as follows *Ageratina adenophora* (Spreng.) R.M.King & H.Rob. (Panawel), *Alstonia scholaris* (L.) R. Br. (Palay), *Ayapana triplinervis* (Vahl) R.M.King & H.Rob. (Pantallion), *Bidens pilosa* L. (Anwad), *Cestrum nocturnum* L. (Dama de noche), *Derris elliptica* (Wall.) Benth. (Opay), *Oreocnide trinervis* (Wedd.) Miq. (Lal-latan), *Pittosporum pentandrum* (Blanco) Merr. (Lahwik), *Sarcandra glabra* (Thunb.) Nakai (Hag-ob), and Lipang daga (without known scientific name).

Methanol extraction procedure

The leaves were rinsed in running tap water, followed by second rinsing using distilled water and then with 70% (v/v) ethanol. The leaves were air-dried and ground into fine particles using a grinder. Fifty (50) grams of each ground leaves were soaked in 500 ml of 80% methanol in a stoppered flask for 72 hours. The mixture was filtered using Whatman no.1 filter paper and subjected to a rotary evaporator to eliminate the solvent (Tan *et al*., 2013). The resulting extracts were weighed and stored in tightly stoppered sterile amber bottles (Srisawat, 2007) at temperatures between 0-5 C.

The extracts were centrifuged at 10,000 rpm for 30 minutes, followed by sterilization using membrane filtration with a pore diameter of 0.45 µm. The sterility of the extracts was monitored by inoculating 100 µl in brain heart

infusion agar (BHIA). The sterile extracts were stored at a refrigerated temperature at 2-8 C before use (Srisawat, 2007).

Disk diffusion assay for antibacterial activity of plant extracts on Staphylococcus aureus PNCM 1582

Three to five colonies of *S. aureus* grown for 24 hours in BHIA at 37 ℃ were transferred into sterile distilled water and the turbidity was adjusted equal to McFarland 0.5 standard (~1.5 x 108 CFU/mL) (Ortez, 2006). 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 (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, the infused discs were then transferred carefully onto the previously inoculated 15 mm MHA plates equidistant to each other and incubated at 37 ℃ for 24 hours. Sterile distilled water served as the negative control. Erythromycin (15 µg; Hi-Media cat# SD013) served as the 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 a possible antibacterial-mediated decrease in virulence factor production, which is required for accuracy of the subsequent assays, continued to the evaluation of QSI against *S. aureus* PNCM 1582.

Evaluation of quorum sensing inhibition in Staphylococcus aureus PNCM 1582

Deoxyribonuclease (DNase) assay

Late log phase BHIA cultures of *S. aureus* PNCM 1582 were heavily streaked on a plate of modified DNase test agar prepared by the addition of 1 ml of plant extracts to liquefied 9 ml of DNase agar poured over pre-solidified base DNase agar (10 ml). Three one-inch streaks of each *S. aureus* were made as replicates. Plates were incubated for 24 hours at 37 ℃. Then, drops of 1 N HCl were added to highlight clear zones around the bacterial colonies. Excess acid was removed. Liquefied DNase agar with 1 ml sterile distilled water on top agar was used as control. The absence of clear zones indicated inhibition of DNase production.

Alpha (α) - hemolysin assay

Prior to the testing, subcultures of *S. aureus* were grown in BHIA. Nine ml of blood agar plates (BAP), supplemented with one ml of plant extracts was poured over ten ml of pre-solidified BAP. Overnight culture of *S. aureus* PNCM 1582 was streaked onto the agar, followed by incubation at 37 ℃ for 24 hours. Plates were removed not later than 24 hours to prevent blood degeneration caused by over-incubation. The absence of hemolysis in BAP plate indicated the presence of QSI activity of plant extracts.

Data analysis

For the antibacterial activity of plant extracts, presence was denoted by the appearance of a clear zone of inhibition around the disks. Plant extracts should not exhibit bacterial growth inhibition to rule out an antimicrobial-mediated decrease in virulence factor production in the later tests, which is required for the accuracy of the subsequent assays.

For the evaluation of QSI in α-hemolysin, the absence of hemolysis in BAP was meant suppression of the α-hemolytic toxin production, hence, the presence of QSI mechanism in the extracts.

For the DNase test, the absence of the clearing zones near the streaks on the DNase agar plate after the addition of 1 N HCl was meant inhibition of DNase production, hence, the presence of QSI.

Results

Disk-diffusion assay for antibacterial activity of ethnobotanical extracts

Zones of inhibition were observed for methanolic extract tests of *B. pilosa, C. nocturnum*, and *P. pentandrum* against *S. aureus* PNCM 1582 while those of *A. adenophora, A. triplinervis, A. scholaris, S. glabra, D. elliptica, O. trinervis*, and Lipang daga were not inhibitited (Table 1). Nevertheless, the methanolic extracts with antibacterial activity produced smaller inhibition zones than the standard commercial antibiotics Erythromycin (15 μg). Methanolic extracts were not able to produce a zone of inhibition or negative for antibacterial activity that qualified for the subsequent virulence assays.

Table 1. Antibacterial activities of methanolic extracts against *S. aureus* PNCM 1582

Plant Extract	Antibacterial activity
Bidens pilosa	+
Cestrum nocturnum	+
Pittosporum pentandrum	+
Alstonia scholaris	-
Ageratina adenophora	-
Ayapana triplinervis	-
Derris elliptica	-
Oreocnide trinervis	-
Sarcandra glabra	-
Lipang-daga	-
Sterile Distilled Water (- control)	-
Erythromycin (15μg) (+ control)	+

+ presence of antibacterial activity
- absence of antibacterial activity

Evaluation of quorum sensing inhibition in S. aureus PNCM 1582

DNase assay

Two of the seven methanolic extracts that qualified for *S. aureus* virulence assay, namely *O. Trinervis* and *D. elliptica*, inhibited DNase production, and expressed positive for the presence of QSI (Table 2). The remaining five methanolic extracts were negative effects for QSI.

α-Hemolysin assay

No methanolic extracts inhibited the production of α–hemolysin in *S. aureus* (Table 2). Clearing along the streaks of growth was not observed in all cultures as compared to the control that absent QSI.

Discussion

Antibacterial activity of methanolic extract of *B. pilosa* against *S. aureus* was confirmed by the studies of Rabe and Staden (1997); Rojas *et al*. (2006); Deba *et al*. (2008). The methanolic extract of *C. nocturnum*, an ethnotoxic plant, showed antibacterial activity against *S. aureus* PNCM 1582,

confirming the results of Al-Reza *et al*. (2009); Khan *et al*. (2011). Huang *et al*. (2006) and Chatterjee *et al*. (2007) also reported the bactericidal activity of the leaves of *C. nocturnum*. The methanolic extract of *P. pentandrum* showed antibacterial effects against *S. aureus* PNCM 1582. Khare (2008) also noted the antibacterial, antifungal, and anti-inflammatory properties of the extracts from *P. pentandrum.*

The extracts of *O. trinervis* and *D. elliptica* inhibited the production of DNase, indicating QSI activity. The DNase assay is widely used to distinguish *S. aureus* from other staphylococcal species, together with gold pigmentation of colonies, positive coagulase results, and mannitol fermentation (Wilkinson, 1997). *Staphylococcus aureus* produces DNAse enzyme that breaks down DNA.

Table 2. QSI activities of ethnobotanical extracts against *S. aureus* PNCM 1582 virulence factors

Plant Extract	**DNase Assay**	**α-Hemolysin Assay**
Bidens pilosa	-	-
Cestrum nocturnum	-	-
Pittosporum pentandrum	-	-
Alstonia scholaris	-	-
Ageratina adenophora	-	-
Ayapana triplinervis	-	-
Derris elliptica	+	-
Oreocnide trinervis	+	-
Sarcandra glabra	-	-
Lipang-daga (no known scientific name)	-	-
Sterile Distilled Water (- control)	-	-

+ presence of QSI
- absence of QSI

DNAse enzyme is among the myriads of enzymes such as lipase, which digests lipids; staphylokinase, an enzyme that dissolves fibrin and aids in spread; and beta-lactamase for drug resistance (Uwaezuoke and Aririatu, 2004). The expression of DNase allows *S. aureus* to escape extracellular traps of neutrophils (Zarringhalam *et al.*, 2013) while helping the bacterium in its metastatic infections and tissue destruction (Gordon and Lowy, 2008). DNase is also associated with pus-forming infections caused by *S. aureus*.

The methanolic extracts may contain substances or phytochemicals that impede the biphasic strategy of *S. aureus* to initiate the production of DNase and cause disease. At low cell density, the bacteria express protein factors that promote attachment and colonization. In contrast, at high cell density, the bacteria repress these traits and initiate the secretion of toxins and proteases presumably required for dissemination (reviewed in Lyon and Novick, 2004). The Agr quorum-sensing system regulates this switch in gene expression programs. The bacterium's Agr locus regulates the expression of diverse cell surface proteins and exoprotein in concert with cell population density (Novick, 2003; Bronner *et al*., 2004 as cited by Qazi *et al*., 2006).

No ethnobotanical extracts showed activity against the production of α–hemolysin. It is assumed that the extracts could not target the mechanisms for the production of this virulence factor. One of the factors for the virulence of *S. aureus* is its capacity to act on the host cell membrane with membrane-damaging toxins and peptides, one of which is α-hemolysin (Vandenesch *et al.*, 2012), causing cell death due to lysis (Madigan *et al*., 2003). The exotoxin of *S. aureus* is a secreted protein endowed with cytotoxic, dermonecrotic, hemolytic, and lethal properties. The synchronized production of many exoproteins in *S. aureus* appears mainly during the post-exponential or stationary phase (Abbas-Ali and Coleman, 1977; Coleman and Abbas-Ali, 1977) and can be attributed to *S. aureus* QS-controlled processes.

The antibacterial and QSI activities may be attributed to the phytochemicals with proven QSI activities present in the ethnobotanicals under study. The phytochemical content of the genus *Bidens* was listed by Chiang *et al.* (2004). Other studies reported the isolation of chalcones (Redl *et al*., 1994), diterpene (Zulueta *et al*., 1995), flavonoids (Wang *et al*., 1997; Sarker *et al*., 2000), flavone glycosides (Brandão *et al*., 1998), phenyl propanoid glucosides (Sashida *et al*., 1991), and polyacetylenes (Redl *et al*., 1994; Brandão *et al*., 1997; Chang *et al*., 2000; Ubillas *et al.*, 2000). *C. nocturnum* contains alkaloids, saponins, and terpenoids. *P. pentandrum* contains alkaloids, tannins, terpenoids, and saponins. *D. elliptica* contains tannins and alkaloids. Alkaloids contain toxic contents such as rotenone which has been used as a natural insecticide. The tannin compounds have a wide distribution in plant species for their growth regulation and protection against predators and pests (Thorington *et al.*, 2006). No phytochemical analysis was done in *O. trinervis*.

The results of the study presented the antibacterial activities of three plants against *S. aureus*: *B. pilosa*, *C. nocturnum*, and *P. pentandrum* while, the two plants, *O. trinervis* and *D. elliptica*, showed QSI against DNase production.

While only a few plants displayed antipathogenic activities, this may still indicate the possibilities for further studies on these plants' biological activities. These plants are probable sources of natural products against QS to develop drugs against bacterial pathogens without resistance development.

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