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Philippine ethnobotanicals inhibit virulence factors in *Staphylococcus aureus*

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Abstract

A new perspective in the application of natural compounds as therapeutic agents are presented by quorum sensing inhibitors to combat pathogenic diseases without developing antimicrobial resistance. The study evaluated the quorum sensing inhibition (QSI) activities of ethanolic extracts of selected Philippine ethnobotanicals against *Staphylococcus aureus* PNCM 1582. Ethnobotanicals tested were *Bidens pilosa, Cestrum nocturnum, Sarcandra glabra, Oreocnide trinervis, Pittosporum pentandrum,* Lipang-daga (no known scientific name)*, Derris elliptica, Alstonia scholaris, Ageratina adenophora and Ayapana triplinervis* Extracts were subjected to assays on the phenotypic expression of virulence factors of *S. aureus*: DNase and α-hemolysin. *C. nocturnum*, *O. trinervis* and *A. triplinervis* showed inhibition of the phenotypic expression of DNase in *S. aureus* indicating the presence of QSI.*C. nocturnum, S. glabra, O. trinervis, Lipang-daga, D. elliptica, A. scholaris, A. adenophora* and *A. triplinervis* ethanolic plant extracts caused QSI resulting in the absence of α-hemolysin production in the plate culture. Findings of both virulence assays confirm the QSI activities of the ethnobotanicals against the virulence factors in *S. aureus*. Thus, these ethnobotanicals display potential for the development of anti-quorum sensing drugs to combat bacterial infections without developing resistance.

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Introduction

The inappropriate and repeated use of antibiotics without following the proper dosages and administration triggered the development of drug-resistant bacteria, among which are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and multi-drug-resistant *Mycobacterium tuberculosis* (MDR-TB) (Senior, 2014). In the light of the recent emergence of bacteria which are resistant to multiple antimicrobial drugs, the challenges in the treatment of infections and the need to discover new ways to combat such microorganisms becomes pertinent (Gbadamosi, 2012).

Staphylococcus aureus is considered one of the most important pathogens in modern hospitals, which often proved to be resistant to antibacterial drugs (Rice, 2008). *S. aureus*, a versatile pathogen capable of causing wide range of diseases, is among the normal human skin microflora (Gordon and Lowy, 2008; Asif and Acharya, 2012). It is a gram-positive bacterium capable of causing infections that may lead to pneumonia, bacteremia and sepsis (Massey *et al.,* 2006). The ability of *S. aureus* to cause disease depends on the expression of an array of adhesion molecules, toxins and compounds that affect the human system and quorum sensing that regulates the expression of genes encoding these virulence factors (Asif and Acharya, 2012). *S. aureus* quorum sensing system is encoded by the accessory gene regulator (*agr*) locus; the *agr* system contributes to virulence in model biofilm-associated infections (Yarwood *et al.*, 2004; Novick and Geisinger, 2008).

Quorum sensing inhibition (QSI) is among the recent means of controlling bacteria without forming resistant strains. Anti-quorum sensing compounds are known to have the ability to attenuate bacterial pathogenicity. Since quorum sensing controls the regulation of many bacterial processes, an attractive tool to control and handle infections caused by human, animal, and plant pathogens is suggested by finding of natural compounds acting as quorum sensing inhibitors (Adonizio *et al.*, 2006 ; Hong *et al.*, 2012; Song *et al.*, 2012; Koh *et al.*, 2013).

Ethnobotanicals are among the most promising plants in drug discovery. Ethnobotanicals grown in the wild have played important roles in local healing practices that include management and treatment of various ailments, diseases and infections. These plants are still utilized today (Abe and Ohtani, 2012; Gbadamosi, 2012). Most of these plants, however, are unidentified, and remain unexplored, and thus, are potential prospects for research, especially in pharmacognosy. The ethnobotanicals from the Igorots of the Kalahan community, the indigenous people in the province of Nueva Vizcaya in the northeast part of the Philippines were screened in this study for the presence of quorum sensing inhibitors against *S. aureus*. The results may validate existing traditional medicinal uses of the plants by the Igorot community and append newer application as alternative medicine.

A new perspective in the application of natural compounds as therapeutic agents can be presented by the search for quorum sensing inhibitors (Domingo and López, 2003), and the revival of opportunities in using our country's botanical resources as old sources but of novel compounds. The findings in this research work may help establish a new approach in combating infectious diseases caused by microbes, especially bacteria. This new therapeutic direction relies on the inhibition of quorum sensing mechanisms among pathogens, which are proven key activities in mounting infections in the host.

Materials and methods

Collection of plant samples

Ethnobotanical plant samples of *Bidens pilosa, Cestrum nocturnum*, *Sarcandra glabra*, *Oreocnide trinervis*, *Pittosporum pentandrum*, Lipang-daga (no known scientific name), *Derris elliptica*, *Alstonia scholaris*, *Ageratina adenophora* and *Ayapana triplinervis* were collected along a trail of Mt. Imanduyan, Brgy. Imugan, Sta. Fe, Nueva Vizcaya. Plants included in the evaluation were pre-

determined in an ethnobotanical survey conducted by Undan *et al.* (2014) with the permission of the council of elders. Leaves of mature plants were collected by hand picking then placed in clean, sealed plastic bags and transported to the laboratory for processing.

Ethanol extraction

The leaves of plant samples were rinsed in running tap water. This was followed by second rinsing using distilled water and then with 70% (v/v) ethanol (Tan *et al.*, 2013). Washed plant materials were dried were ground to fine particles using a blender. Fifty (50) grams of 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 (Tan *et al.*, 2013). The resulting extracts were stored in tightly stoppered sterile amber bottles (Srisawat, 2007) at temperatures between 0-5 °C. 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 (Srisawat, 2007). 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°C prior to use (Srisawat 2007).

Disk-Diffusion Assay for Antibacterial Activity of Plant Extracts Against S. aureus PNMC 1582

The protocol of Rezaei *et al.*, (2011) was used with some modifications. Three (3) to five (5) colonies of *S. aureus* PNMC 1582 grown for 16-18 hours in BHIA at 35 °C were transferred to sterile distilled water, the turbidity adjusted to McFarland 0.5 standard (~ 1.5 x 108 CFU/mL) (Ortez, 2005). 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.

Ethanolic plant extracts were placed on sterile empty petri plates, 20 µL of each extract was pipetted onto 6-mm sterile blank antibiotic discs (Hi-Media cat# SD067) and allowed to stand for a few minutes to eliminate excess liquid. 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; Erythromycin (15 µg, Hi-Media cat# SD013) as positive control. Plates were prepared in triplicates. Antimicrobial activities of the extracts were detected through the presence of clear or translucent zone of inhibition around the disks. Each plant extracts in the study should not exhibit clearing to rule out antibacterial-mediated decrease in virulence factor production, which is required for accuracy of the subsequent assays. Only plant extracts without antimicrobial activities were used in the detection of quorum sensing inhibition through modified virulence assays.

Evaluation of Quorum Sensing Inhibition in Staphylococcus aureus PNCM 1582 Through Virulence Factor Assays

DNase Assay

(Modified from Brown, 2011; Tan *et al.*, 2013; and Kateete *et al.*, 2010): Late log phase BHIA cultures of *S. aureus* PNCM 1582 were heavily streaked on modified DNase test agar plate 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 *S. aureus* comprised the replicates. Plates were incubated for 18-24 hours at 35 °C. Drops of 1N HCl were added to highlight clear zones around the bacterial colonies. Excess acid was removed. Liquefied DNase agar with 1 ml sterile distilled water in the top agar was used as control. Absence of clear zones indicated inhibition of DNase production.

α-Hemolysin assay

Liquefied Blood Agar (BA, 9 ml) supplemented with plant extracts (1 ml) poured over pre-solidified Blood agar (10 ml) was prepared. Overnight culture of *S. aureus* PNCM 1582 was streaked onto the agar, followed by incubation at 37 °C for 18 to 24 hours.

Plates were removed not later than 24 hours to prevent blood degeneration caused by over-incubation. Absence of hemolysis in BAP indicated the presence of quorum sensing inhibition activity of plant extracts.

Data gathering and statistical analysis

The absence of beta hemolysis in Blood Agar plate meant suppression of the α-hemolytic toxin production, hence, presence of anti-quorum sensing mechanism in the extracts. In DNase test, absence of the clearing zones around the streaks on the DNase agar plate or after addition of 1N HCl was recorded as inhibition of DNase production, hence, presence of QSI.

Results and discussion

Antibacterial testing was performed to select which extracts qualified for the screening of quorum sensing inhibition (QSI). Table 1 shows the antibacterial activity of the ethnobotanicals tested against *S. aureus* PNCM 1582.The extracts tested in the QSI screening should not exhibit inhibition of bacterial growth to rule out antimicrobial-mediated decrease in virulence factor production in the later tests, which is required for accuracy of the subsequent assays. *B. pilosa* and *P. pentandrum* showed clear zones of inhibition around the discs indicating the antibacterial activity of the two plants.

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

Plant Extracts	*S. aureus*
Pittosporum pentandrum	+
Bidens pilosa	+
Cestrum nocturnum	–
Sarcandra glabra	–
Oreocnide trinervis	–
Lipang-daga	–
Derris elliptica	–
Alstonia scholaris	–
Ageratina adenophora	–
Ayapana triplinervis	–
Sterile Distilled Water (-control)	–
Erythromycin (15µg) (+ control)	+

Note: (+) = with antibacterial activity; (–) = without antibacterial activity.

Plant extracts show QSI against DNase and α-hemolysin

Table 2 shows the QSI of plant extracts against *Staphylococcus aureus* PNCM 1582 virulence factors. The ethanolic extracts of *C. nocturnum*, *O. trinervis* and *A. triplinervis* showed inhibition of the phenotypic expression of DNase in *S. aureus* indicating the presence of QSI (Table 2). *C. nocturnum, S. glabra, O. trinervis, Lipang-daga, D. elliptica, A. scholaris, A. adenophora* and *A. triplinervis* ethanolic plant extracts caused quorum sensing inhibition resulting in the absence of α-hemolysin production in the plate culture(Table 2).Four (4) ethanolic extracts: *S glabra*, *D. elliptica*, *A. scholaris* and *A.adenophora*, showed inhibition of quorum sensing by suppressing the phenotypic expression of α-hemolysin only and not DNase, thus, affecting only the second class of virulence factors. Moreover, three (3) ethanolic extracts, *C. nocturnum*, *O. trinervis* and *A. triplinervis*, showed suppression of phenotypic expression on both virulence factors showing QSI targeting the two classes of virulence factors.

As all ethanolic extracts in this study showed QSI on *S. aureus* α-hemolysin virulence factor phenotypic expression, it could be suggested that the ethnobotanicals may have affected the pathogenicity of *S. aureus* by suppressing the phenotypic expression of RNAIII *hla* and/or *hld* at the transcriptional or translational level. Tamber *et al.* (2010) demonstrated that *S. aureus* pathogenesis has virulence progressing in two (2) discrete stages: (1) the production of adhesins and surface proteins during the exponential phase of growth; and (2) the increased toxin production which leads to the increased tissue damage and bacterial spread.

Table 2. Summary of quorum sensing inhibition (QSI) of plant extracts on *S. aureus*PNCM 1582 virulence factors.

Plant Extracts	DNase	α- Hemolysin
Pittosporum pentandrum	NA	NA
Bidens pilosa	NA	NA
Cestrum nocturnum	+	+
Sarcandra glabra	–	+
Oreocnide trinervis	+	+
Lipang-daga	–	+
Derris elliptica	–	+
Alstonia scholaris	–	+
Ageratina adenophora	–	+
Ayapana triplinervis	+	+

Note: (+) = with QSI activity; (–) = without QSI activity; NA= with antibacterial activity.

These virulence factors that greatly impact staphylococcal infection are controlled by the accessory gene regulator (*agr*) system (Novick *et al.,* 1993; Lebeau *et al.*, 1994; Otto *et al.*, 1999; Liu, 2009; Thoendel *et al.*, 2011). The *agr* system is comprised of several genes, *agr DBCA*, and RNAIII the gene for a regulatory RNA molecule (Otto *et al.*, 1999). RNAIII is expressed maximally during post-exponential phase and post-transcriptionally activates virulence factor production as it harbors genes encoding for toxins such as α-hemolysin (*hla*), δ-hemolysin (*hld*) and V8 protease (*sspA*). RNAIII controls virulence gene expression at the transcriptional and translational levels (Otto *et al.*, 1999; Tamber *et al.*, 2010; Rutherford and Bassler, 2012).Many virulence factors of *S. aureus*, including α-toxin, β-toxin, δ-toxin and DNase, are controlled by the accessory gene regulator (*agr*) system (Otto *et al.*, 1999; Antunes *et al.*, 2010; Yarwood and Schlievert, 2003). There are two (2) classes of virulence factors regulated by *agr*: (1) virulence factors involved in attachment to the host and immune evasion, and (2) virulence factor genes involved in the production of exoproteins associated with invasion and toxin production (Bowden *et al.*, 2005). Production of DNase belong to the first class as it allows *S. aureus*to avoid destruction by PMNs (neutrophils) to survive, thereby causing human infections (Voyich *et al.*, 2005; Zarringhalam *et al.*, 2013) while production of exotoxins belong to the second class of virulence factors.

Three (3) extracts, *C. nocturnum, O. trinervis* and *A. triplinervis* inhibited the production of DNase in *S. aureus* which show their antivirulence activity.*S. aureus* normally produces deoxyribonuclease (DNase) (Foster, 1996; Uwaezuoke and Aririatu, 2004; Gordon and Lowy, 2008), an enzyme that breaks down DNA. The expression of DNase allows *S. aureus* to escape killer neutrophils' extracellular traps while helping its metastatic infections and tissue deconstruction (Gordon and Lowy, 2008;

Zarringhalam *et al.*, 2013). Polymorphonuclear leukocytes (PMNs or neutrophils) are critical for human innate immunity, and kill most invading bacteria. *S. aureus* avoid destruction by PMNs (neutrophils) to survive, thereby causing human infections (Voyich *et al.*, 2005; Zarringhalam *et al.*, 2013).

High potential in antivirulence is shown by the extracts against α-hemolysin as almost all the tested plants inhibited its production. *S. aureus* has been regarded as a serious threat to human health. (Watkins *et al.*, 2012) due to many cell surface virulence factors and, exotoxins and enzymes that allow strains to cause a multitude of infections (Yarwood and Schlievert, 2003). Once colonization of *S. aureus* occurs, numerous secreted exotoxins, such as hemolysins (α, β, δ and γ), cause serious human disease and are suggested to contribute to significant illnesses (Yarwood and Schlievert, 2003). The best characterized and most potent membrane-damaging toxin is the α-hemolysin toxin which acts on cell membranes causing cell death due to lysis (Foster, 1996; Madigan *et al.*, 2012).

The antivirulence activities may be attributed to the phytochemicals present in the ethnobotanical extractsthat are reported with QSI activities. 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.* (2006), Rezaei *et al.* (2011), Song *et al.* (2012) and Tan *et al.* (2013)among others. The nature and identity of the active compound/s present in the ethanolic extracts of the ethnobotanicals responsible for its quorum sensing inhibition activities is still difficult to indicate 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 (Zahin *et al.*, 2010). 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 to extract the intracellular ingredients (Wang, 2010). The studies of Cowan (1999) and Tiwari *et al.*, (2011) indicated that ethanol is capable of extracting tannins, polyphenols, flavonol, terpenoids, and alkaloids. Moreover, Sultana *et al.* (2009) 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.* (2013). 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 (Sultana *et al.*, 2009).

Ethanolic crude plant extracts are proven to be more effective than individual plant component owing to their synergistic interactions to protect their active component/s and/or help facilitate transport across cell barriers. This may result in higher efficacy of the crude drug compared with the purified components (Ghosh *et al.*, 2014). This supports the current trend of exploring crude extracts as possibly the right strategy in treating multi-drug resistant pathogens e.g. MRSA as compared to the purified compound Isolated from the same extracts.

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 (Quave *et al.*, 2011; Rasmussen and Givskov, 2006 as cited by Yeo and Tham, 2012). This study show that ethnobotanicals of Imugan, Nueva Vizcaya interfere with quorum sensing expression of virulence factors expression in *S. aureus*. All ethanolic extracts of the ethnobotanicals showed, to some degree, quorum sensing inhibition in *S. aureus*, thus, are potential sources of new drugs in this therapeutic direction to combat bacterial infections.

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