ORIGINAL ARTICLE

Philippine Ethnobotanicals Inhibit Formation of Coagulase in Staphylococcus aureus

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ABSTRACT

This study screened the quorum sensing inhibition (QSI) activities of ethnobotanicals Cestrum nocturnum, Sarcandra glabra, Derris elliptica, Oreocnide trinervis, Ayapana triplinervis, Alstonia scholaris, Ageratina adenophora collected from the ancestral domain of the Igorot community of Imugan, Nueva Vizcaya, Philippines against Staphylococcus aureus PNRM 1582. Ethanol extracts of Sarcandra glabra, Derris elliptica, Ayapana triplinervis, Ageratina adenophora and Oreocnide trinervis exhibited QSI against Staphylococcus aureus PNRM 1582 through the tube coagulase assay. The results show a considerable potential of the ethnobotanicals as sources of compounds that can inhibit quorum sensing.

Keywords: Quorum sensing inhibition, Coagulase in Staphylococcus aureus

INTRODUCTION

Bacterial cells communicate, regulate and perform cellular activities through quorum sensing (QS) [1]. Through the production and use of signaling molecules called autoinducers (AI), QS controls genes that allow bacterial cells to regulate various processes of bacterial cells such as bioluminescence, sporulation, competence, biofilm formation, antibiotic production, and virulence factor secretion [2] in a synchronized, uniform manner which makes colonization and disease progression much more effective [3, 4].

The continuing increase of highly resistant bacteria has undermined the ability of newer generations of antibiotics [5]. One of these bacteria is Staphylococcus aureus. This bacterium causes superficial skin lesions, osteomyelitis, endocarditis and furunculosis. S. aureus is one of the leading causes of nosocomial infections of surgical wounds in hospitals [6]. Quorum sensing regulates various virulence factors in S. aureus [7] which makes the bacterium exist in two phenotypes, an adhesive colonizer phenotype that is tolerated by the host, and an invasive infective phenotype which damages the tissues of the host and is responsible for the manifestation of disease [8, 9].

A way to stop, control and interfere with bacteria is done through quorum sensing inhibition (QSI) or anti-quorum sensing (AQS). QSI aims to interfere the communication system that bacteria use, which may have been a reason bacteria are developing resistance against antibiotics [10]. Quorum sensing inhibition can be done in a number of ways [11] such as application of compounds that can inhibit quorum sensing. These compounds may come from either synthetic forms or natural extracts from organisms [12]. Plants and phytochemicals are now being studied and screened for their potential to combat quorum sensing in bacteria, especially by the antimicrobial-resistant strains. The search for quorum sensing inhibitors provides new information on the usage of natural compounds for remedy of health conditions, as well as the potential to control and handle diseases caused by pathogens through inhibition or interference of the communication between bacterial cells [13].

The production of secondary metabolites in plants has played a key-role in their survival in environments loaded with bacteria [14]. These phytochemicals synthesized by plants limit the microbes’ abilities to
colonize and spread virulence [15] and has been reported to have disease-controlling potential by altering the genes responsible for the pathogenesis and virulence through interference with QS [16]. Since then, many plants have been tested for their potential as quorum sensing inhibitors. Ethnobotanicals are plants that are part of a particular culture and region used by indigenous people. These plants play roles in local healing processes and the knowledge of these plants are handed from generation to generation [17,18]. Ethnobotanicals have shown therapeutic properties and are being studied for the discovery and development of new drugs [19]. While some ethnobotanicals have shown antibacterial properties [20, 21], the properties of these plants to interfere with QS are yet to be explored because only few studies regarding ethnobotanicals as quorum sensing inhibitors have been published [22]. Ethnobotanicals in the Philippines are not well known because these plants can only be found and are only utilized in the areas of ethnic communities as therapeutic agents for various health conditions. These plants may show potential for the development of new drugs since they are locally used for their medicinal properties, and may also be sources of quorum sensing inhibitors. The ethnobotanicals of the Igorot community of Imugan, Sta. Fe, Nueva Vizcaya have been studied, and has exhibited different medicinal properties such as anti-inflammatory [21], analgesic [21, 23], antibacterial [21], anti-diabetic [24], anti-oxidant [25], anti-gout [26] and antipyretic [23]. Recently, these ethnobotanicals were found to inhibit quorum sensing in two bacteria, Pseudomonas aeruginosa and Staphylococcus aureus through various virulence [27,28,29]. This may contribute in the search of combating diseases against antibacterial-resistant bacteria through quorum sensing inhibition. This study may provide additional information and ways in utilizing plant extracts in the continuous search for the controlling of microbial infections by reducing the risk of developing resistance.

MATERIALS AND METHODS

Collection of Plant Samples
Ethnobotanical plant samples Centrum nocturnum (Dama de Noche), Sarcandra glabra (Hag-ob), Oreocnide trinervis (La-latan), Derris elliptica (Opay), Alstonia scholaris (Palay), Agaratica adenophora (Panawel) and Ayapana triplinervis (Pantealeon) were collected along a trail of Mt. Imanduyan, Brgy. Imugan, Sta. Fe, Nueva Vizcaya, elevated 1092 meters above sea level. Brgy. Imugan is geographically located between the Cordillera Central and Sierra Madre mountain ranges. Leaf samples used in this study were collected at Mount Imanduyan, Brgy. Imugan, Sta. Fe, Nueva Vizcaya. Mature leaf samples were collected by handpicking during the day, placed in clean, sealed plastic bags and transported to the laboratory for processing. The place, time, season and the name of the collector were recorded. Confirmation of the pre-identified collected plants was done by an expert botanist at the National Museum of the Philippines in Manila. Vegetative parts of the specimens were collected and pressed for authentication of the correct species.

Ethanol Extraction Procedure
The collected leaves were washed and rinsed in running tap water for complete elimination of foreign matters on the surface. Second rinsing using distilled water followed next, and then final rinsing with 70% ethanol (v/v) was done afterwards. Dried plant materials were pulverized to fine, sand-like particles using a blender [30]. Fifty (50) grams of finely ground leaves of each plant was soaked in 500 ml of 95% ethanol in a stoppered flask for 72 hours. The resulting mixture was then filtered using Whatman no.1 filter paper and the solvent completely removed using a rotary evaporator [30]. The extracts were then weighed and stored in tightly stoppered sterile amber bottles [31] at temperatures between 0-5 °C.
Sterilization of the extracts was done, first by centrifugation of the mixture at 10,000 x g for 30 minutes, and filtration with a membrane filter 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) from time to time. The sterile extracts was stored at 2-8 °C prior to use [31].

Confirmation of Test Bacterial Characteristics
Staphylococcus aureus stock cultures were revived in brain-heart infusion broth (BHIB), and inoculation in mannitol salt agar (MSA) was performed to check identity. Working stock cultures were grown and maintained in BHIB test tubes at 5°C.

Phenotypic Detection of Quorum Sensing Inhibition in S. aureus PNCM 1582 through Tube Coagulase Assay
The rabbit plasma was rehydrated to reconstitute according to the instructions provided in the kit (Scharlau Lyophilized Rabbit Plasma Art No. 064-PLA-CO). About 260 μl of rabbit plasma was placed in an Eppendorf tube followed by inoculation of 100μl of S. aureus culture. Under aseptic conditions, 40μl of each plant extract was added to the mixture of S. aureus culture and plasma, and was then incubated at
35-37°C during a period between 4 to 24 hours. The formation of a coagulum indicates production of coagulase, and, hence, negative for QSI. QSI is present in the extracts if coagulation did not form in the plasma after 4 to 24 hours.

RESULTS AND DISCUSSION
Phenotypic Detection of Quorum Sensing Inhibition in S. aureus PNCM 1582 through Tube Coagulase Assay

QSI was exhibited by inhibiting the coagulation of the rabbit plasma. Ethanol extracts of D. elliptica, A. trilinervis, O. trinervis, A. adenophora, and S. glabra showed inhibition of the phenotypic expression of coagulase by S. aureus, indicating presence of quorum sensing inhibition activity. Staphylococcus aureus is a deadly pathogen to humans. It affects nearly 30% of the adult population [9], in which resistant strains causes outbreaks in hospitals and can be epidemic [32]. S. aureus expresses many cell-surface associated and extracellular proteins that have potential as virulence factors [6]. Coagulase is an extracellular protein produced by S. aureus, which promotes fibrin formation in human plasma by non-enzymatic activation of prothrombin [33].

Quorum sensing in gram-positive bacteria is encoded by a peptide-based quorum sensing system, specifically by the accessory gene regulator (agr) locus [34]. Pathogenesis of S. aureus has virulence progressing in two discrete stages. Initially, the production of adhesins and surface proteins during exponential growth occurs followed by increased toxin production, which leads to damage of tissues and bacterial spread [35]. One of the virulence factors produced by S. aureus is coagulase. Coagulase is synthesized during exponential growth [36, 37] wherein the gene coa is responsible for coagulase expression in S. aureus [38] wherein the protein’s action is to create a barrier of plasma as a means of protection against phagocytes [6]. After exponential growth, extracellular proteins such as serine protease, nuclease, lipase, fibrinolysin, α-, β-, and δ-hemolysin, are produced, as well as toxins, toxic shock syndrome toxin 1 and enterotoxin [39]. In other words, coagulase creates a defensive mechanism against phagocytes while allowing the bacterial cells to secrete the virulence factors that causes infectious diseases in its host. As coagulase, along with other surface proteins, are secreted during the exponential growth, it could be suggested that the extracts of the ethnobotanicals D. elliptica, A. trilinervis, O. trinervis, A. adenophora, and S. glabra may have affected the pathogenicity of S. aureus by suppressing the phenotypic expression of the gene coa.

Previous studies initially screened the extracts of these ethnobotanicals against Pseudomonas aeruginosa and Staphylococcus aureus virulence factors. Extracts of Oreocnie trinervis, Cestrum nocturnum and Ayapana triplinervis exhibited QSI activity through inhibition of pyocyanin production in P. aeruginosa. Additionally, extracts of Bidens pilosa, C. nocturnum, Sarcandra glabra, O. trinervis, Derris elliptica, Alstonia scholaris, A. triplinervis and Ageratina adenophora interfered with the swarming motility of P. aeruginosa, an activity regulated by quorum sensing. The production of α-hemolysin in S. aureus was also inhibited by the extracts of C. nocturnum, S. glabra, O. trinervis, D. elliptica, A. scholaris, A. adenophora and A. triplinervis. Moreover, DNase assay employed in S. aureus and extracts of C. nocturnum, O. trinervis, and A. triplinervis showed inhibition of QS in the said bacterium [27]. All methanolic extracts of the ethnobotanicals also inhibited swarming motility of P. aeruginosa while extracts of D. elliptica and O. trinervis manifested effect on the production of DNase [28]. N-hexane extracts of the same ethnobotanicals against P. aeruginosa and S. aureus were also subjected to the same assays. In P. aeruginosa, swarming motility was inhibited by P. pentandrum, O. trinervis, D. elliptica, A. scholaris, A. adenophora and A. triplinervis while B. pilosa, C. nocturnumnand S. glabashowed higher decrease in swarming motility. In the α-hemolysin assay, all extracts showed presence of QSI against S. aureus [29].

Some ethnobotanicals used in this study have been reported to contain certain phytochemicals which may have acted on the quorum sensing activity in S. aureus. Most of the compounds found in the ethnobotanicals are among those found to have quorum sensing inhibition in bacteria. S. glabra, D. elliptica, A. adenophora, and A. triplinervis showed anti-QS activities in both assays. S. glabra was found to contain coumarins, flavonoids, rosmarinic acid, and sesquiterpenoids [40, 41]. D. elliptica, on the other hand, was found to have tannins, saponins, terpenoids, and alkaloids [42], while A. adenophora contains sesquiterpenes, chlorogenic acid [43] alkaloids, glycosides, saponins and coumarins [44, 45]. Furthermore, A. triplinervis have been found to have coumarin, tannins, flavonoids, phenols, terpenes and alkaloids [46, 47].

The extracted compounds of plant materials are dependent on the nature of the extracting solvent [48] wherein, ethanol has been proven of extracting tannins, polyphenols, flavonol, terpenoids, and alkaloids [49, 50]. However, the identification of the specific compounds extracted using ethanol in this study is yet to be verified. It is difficult to indicate the nature and identity of the active compound/s present in the
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extracts due to the novelty of some species. The mechanisms of the phytochemicals effective for QSI are yet to be considered [51]. Quorum sensing inhibition activity does not kill or inhibit bacterial growth, rather, it can be able to control and eradicate infections caused by pathogens [52] by targeting quorum sensing in various methods. Most of the ethanolic extracts of the ethnobotanicals have exhibited quorum sensing inhibition activity in both bacteria, thus, these plants can be potential sources of new drugs in this perspective of combating bacterial infections.

The ethnobotanicals utilized in this study are currently in use of the Igorot community of Imugan for its medicinal and toxic properties, and have not yet been widely cultivated and domesticated. Several researches on these ethnobotanicals point out to its remarkable pharmacological potential as quorum sensing inhibitors against *P. aeruginosa* and *S. aureus* virulence factors using different extraction solvents. [27, 28, 29]. The findings of this study can contribute to the development of ways to fight antibiotic-resistant bacteria, hence, decreased use of antibiotics to prevent the growth of resistant strains.

CONCLUSION

Ethanol extracts of *D. elliptica, A. trilinervis, O. trinervis, A. adenophora,* and *G. glabra* show inhibition of the phenotypic expression of coagulase by *S. aureus*, indicating presence of quorum sensing inhibition activity.

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