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QUORUM SENSING-MEDIATED α- HEMOLYSIN INHIBITION OF PHILIPPINE ETHNOBOTANICALS IN *Staphylococcus aureus*

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ABSTRACT

The search for QS inhibitors (QSI) is an emerging strategy aimed at developing new compounds to decrease or eliminate the production of virulence factors by pathogenic, multi-drug resistant bacteria. QSI offers a new perspective on the application of natural or pure compounds as therapeutic agents with the advantage of reducing risks of resistance development.

Philippine ethnobotanicals from the Igorot community of Barangay Imugan, Sta. Fe, Nueva Vizcaya Philippines were tested for its quorum sensing inhibition activity against *Staphylococcus aureus* PNCM 1582 through the α-Hemolysin assay after antibacterial testing. These were *Bidens pilosa, Cestrum nocturnum, Sarcandra glabra, Pittosporum pentandrum, Oreocnide trinervis, Derris elliptica, Alstonia scholaris, Ageratina adenophora, Ayapana triplinervis* and Lipang Daga (no known scientific name). Extraction was done using 95% n-hexane.

All ethnobotanicals showed inhibition of the α-Hemolysin. Results indicate that these ethnobotanicals have pharmacological potential and can be tapped for developing new drugs for therapeutic utilization against *S. aureus* and for future applications in microbial biotechnology. Further screenings on other virulence assays is recommended.

Keywords: quorum sensing inhibition, ethnobotanicals, *Staphylococcus aureus*

INTRODUCTION

With the emergence of infectious diseases, antibiotics rapidly became the treatment of choice for staving off infections and saving lives. However, indiscriminate and constant use of antibiotics develops multi-drug resistance among microorganisms to antimicrobials [1, 2]. This is, at present, a pressing global health problem [3]. Therefore, the development of novel therapeutic medicines to address the control of drug-resistant bacterial pathogens is urgent [4].

One significantly important human bacterial pathogen that is one of the global concerns is *Staphylococcus aureus* [5]. *S. aureus* is a Gram-positive bacterium and causes nosocomial infections [6]. In spite of its widespread incidence in healthy subjects, *S. aureus* is also a very dangerous opportunistic pathogen which has been progressively more associated with antibiotic resistance [7]. While many factors appear to contribute to the success of *S. aureus* as a pathogen, its capability to persist as a commensal, frequent resistance to multiple antimicrobial agents and armory of virulence determinants, often with redundant functions, are among the most important [8]. Infections that are caused by antibiotic-resistant strains of *S. aureus* have reached epidemic proportions worldwide [9]. The overall burden of staphylococcal disease, particularly diseases caused by methicillin-resistant *S. aureus* (MRSA) strains, is increasing in many countries in both health care and community settings [10]. One of the factors which contribute to *S. aureus* virulence is its peptide-based quorum sensing system, encoded by the accessory gene regulator (agr) locus [11]. Because of the significant role of quorum sensing in the regulation of hundreds of virulence factors in *S. aureus*, significant efforts have been made to discover molecules that inhibit quorum sensing in these organisms.

Quorum sensing (QS) is a mechanism in bacteria to monitor cell density by producing certain signal molecules and further regulate the expression of secondary characteristics such as biofilm formation, DNA competence, bioluminescence and virulence [12]. Bacterial virulence is, in many cases, controlled by quorum sensing [13]. This has led to a burst in quorum sensing research and the role of quorum sensing in the virulence of multiple human pathogens.

The search for QS inhibitors (QSI) is an emerging strategy aimed at developing new compounds to decrease or eliminate the production of virulence factors by pathogenic bacteria [14]. Recently, the systems involved in QS of gram-negative

and gram-positive bacteria have been proposed as promising targets for antimicrobial therapy. QSI does not kill or restrain bacterial growth; the inhibition of pathogenesis of bacteria could be accomplished without growth inhibition, thus potentially avoiding selective pressures for drug-resistance [15, 16]. Quorum sensing inhibitors offer a way of controlling microbial infections with the advantage of reducing risks of resistance development [17]. The continuing search for new and novel antimicrobials and anti-pathogenic agents has focused on exploiting the fact that plants surviving in an environment with high bacterial density are known to possess protective means against infections [18]. Search for quorum sensing inhibitors offers a new perspective on the application of natural or pure compounds as therapeutic agents, which by inhibiting this mechanism of cell communication could be used to control bacterial diseases [19].

As QSI does not kill or inhibit bacterial growth, QSI drugs that hinder with signalling may have a benefit because they do not cause strong selective pressure for development of resistance, as would antibiotics [16]. The disturbance of this communication system can attenuate microbial virulence because many important human pathogens depend on QS signalling systems to coordinate expression of virulence genes [20]. Strategies designed to interfere with these signalling systems will likely have broad applicability in the biological control of QS-dependent bacterial infections [21].

Ethnomedicinal plants display great potential for the discovery of drugs for anti-pathogenesis. Most of these plants are endemic and not well studied and mostly unvalidated for their medicinal properties. Indigenous people, who generally live in geographically isolated areas, mostly utilize plants as their sources of medicine or part of their medical treatments [22]. Due to claims of medicinal potential, several folk medicinal plants are now the subjects of screening for their bioactivities [17, 23, 24]. Approximately 3% of the Philippine population is comprised of indigenous, ethnic communities that are known for their utilization of traditional medicine primarily based on plants. The Igorots of the Kalahan community, called Ikalahans, are the indigenous people in the province of Nueva Vizcaya in the northeast of the Philippines. An ethnobotanical survey revealed a diverse selection of ethnomedicinal and ethnotoxic plants used as part of their primary healthcare. Recent biological screenings revealed several pharmacological activities of the Igorot ethnobotanicals [25, 26, 27].

These plants showed biological activities and with further evaluation, can serve as basis on the formulation of anti-pathogenic drugs based on novel compounds and may be used to develop more effective strategies in preventing and managing microbial infections.

MATERIALS AND METHODS

Sample Collection

Plants included in the tests were pre-determined in an ethnobotanical survey [28] 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, Cestrum nocturnum, Sarcandra glabra, Pittosporum pentandrum, Oreocnide trinervis, Derris elliptica, Alstonia scholaris, Ageratina adenophora, Ayapana triplinervis* and Lipang Daga (no known scientific name). Leaves were collected by hand picking 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 followed by second rinse using distilled water and finally with 70% (v/v) ethanol. Plant leaves were dried were ground to fine powder using a blender. Excess ground plant materials were stored in amber bottles or sealed plastic bags in a cool dry place away from sunlight until for use up to six months.

Fifty (50) grams of dried ground plants was soaked with 500 ml of 95% n-hexane and kept in the stoppered flask for 72 hours. Filtration was done using Whatman No. 1 filter paper and was run through rotary evaporator. The resulting extracts were stored in tightly stoppered sterile amber bottles [29] at refrigerated temperatures of 0-5℃. Dimethyl Sulfoxide (DMSO) was used to dissolve crude n-hexane ethnobotanical extracts a final concentration of 20% plant extracts and 80% DMSO (100%).

Sterilization followed by centrifugation of the crude extracts at 10,000 rpm for 30 minutes, then membrane filtration with pore diameter of 0.45 µm [30]. The sterility of the extracts was monitored by inoculating 100 µl in brain heart infusion agar (BHIA) from time to time. The sterile extract was stored at 2-8℃prior to use [30].

Disk-Diffusion Assay for Antibacterial Activity of Plant Extracts in *Staphylococcus aureus* PNCM 1582

Three (3) to five (5) colonies of *S. aureus* PNCM 1582 grown for 16 to 18 hours in BHIA at 35℃were transferred to sterile distilled water and the turbidity was adjusted to McFarland 0.5 standard (~1.5 x 108 CFU/ml) [31]. Inoculation of Mueller Hinton Agar (MHA) plates was done by using a sterile 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 sterile forceps, discs with plant extracts were transferred onto previously inoculated 15-mm MHA plates equidistant to each other. Plates were prepared in triplicates. Erythromycin (15 µg; Hi-Media SD013) served as the positive control while sterile distilled water served as negative control. Presence of a clear or translucent zone of inhibition around the discs show antibacterial activity [32]. To rule out antibacterial-mediated decrease in virulence factor production, which was required for accuracy of the subsequent assays, only plant extracts without antibacterial activity were qualified for the α-Hemolysin Assay.

Evaluation of Quorum Sensing Inhibition in *S. aureus* PNCM 1582 through the α- Hemolysin Assay

Prior to the testing, subcultures of *S. aureus* PNCM 1582 were grown in MSA. Liquefied blood agar (BA, 7 ml) supplemented with plant extracts (3 ml) was poured over pre-solidified BA (10 ml). Overnight culture of *S. aureus* 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. Plates were observed for absence of beta hemolysis produced when blood cells hemolysed. Absence of beta hemolysis in blood agar plate meant suppression of the alpha hemolytic toxin production in *S. aureus*, therefore, presence of QSI mechanism in the extracts.

RESULTS AND DISCUSSION

Antibacterial Activity of Plant Extracts against *S. aureus* PNCM 1582

All ten n-hexane ethnobotanical extracts namely: *B. pilosa, C. nocturnum, S. glabra, P. pentandrum, O. trinervis, D. elliptica, A. scholaris, A. adenophora , A. triplinervis* and Lipang Daga (no known scientific name) did not exhibit antibacterial activity against the test bacteria making them qualified for the succeeding virulence assays.

Plant Extracts Exhibit QSI in α- Hemolysin

B. pilosa, C. nocturnum, S. glabra, P. pentandrum, O. trinervis, D. elliptica, A. scholaris, A. adenophora, A. triplinervis and Lipang Daga (no known scientific name) caused observable absence of beta hemolysis in blood agar plates inoculated with the test bacteria in comparison to the control (sterile distilled water) where hemolysis was apparent. The ten n-hexane ethnobotanical extracts inhibited the production of α-toxin resulting in the absence of beta hemolysis in the plates containing different extracts which indicated presence of QSI.

S. aureus secretes a core set of virulence factors that are significant for pathogenicity including proteases, lipases, elastases, and nucleases as well as a variety of cytolytic toxins such as the hemolysins and leukotoxins [33,34]. One factor which contributes to *S. aureus* virulence is its peptide-based QS system, encoded by the accessory gene regulator (agr) locus [35]. Of the virulence factors regulated by agr, there are two classes: the first class contains virulence factors involved in attachment to the host and immune evasion, while the second class contains genes involved in the production of exoproteins associated with invasion and toxin production [36, 37]. The genetic basis for this temporal gene expression depends on two pleotropic regulatory loci, the agr (accessory gene regulator) [38] and the sar (staphylococcal accessory gene regulator) [39, 40].

Some phytochemicals reported to be present in the plants tested are with proven QS activity. These are flavanones, flavonoids, flavonols [41], curcumin [42], furocoumarins [43], rosmarinic acid, salycilic acid, urolithin, chlorogenic acid, aromatic compounds and furanones, tannic acid (tannins) [44], vannilin, furanones, and ellagitannins [45, 46]. Although no confirmation was done, it is assumed that the phytochemicals in the extracts may have affected the accessory gene regulator (agr) pathway which regulates α-hemolysin biosynthesis in *S. aureus*. Specifically, phytochemicals of the extracts may have inhibited RNAIII which is responsible to the positive regulation of hla gene translation into alpha-toxin. Other plant extracts were reported to inhibit this pathway. The plant extract of goldenseal *(Hydrastis canadensis*) inhibited the agr signalling pathway of methicillin-resistant *S. aureus* (MRSA), and reduce α-toxin production having been found to be rich in alkaloids [47]. On the other hand, a phenolic compound found in *Hamamelis virginiana*, Hamamelitannin, inhibited agr QS regulator RNAIII and δ-hemolysin

production of *S. aureus* (Kiran et al., 2008) [48]. Alkaloids and phenols may be among the phytochemicals educed by n-hexane extraction, and are known to be inhibitory to quorum sensing, hence, could be the reason for the lack of hemolysis in blood agar plates.

It is not clear which level of QS was modulated by these extracts because the potential phytochemicals that were extracted by n-hexane could be competing or disrupting the AHLs binding to the receptors by degradation of AHLs; blocking AHLs from forming AHL-receptor complex or changing the structures of the enzymes that involved AHLs synthesis [49]. Moreover, the agr signalling pathway of *S. aureus* could have also been inhibited, which in turn reduced alpha-toxin production [47]. The phytochemicals may perhaps inhibited agr QS regulator RNAIII responsible for α-toxin production in *S. aureus* [48].

Italian medicinal plants were observed interfering with QS pathways and have QSI activity in delta (δ) hemolysin production of *S. aureus* [15]. Other studies reported QSI activity of plants and their phytochemicals against another virulence factor in *S. aureus*. A medicinal plant, *Rubus ulmifolius*, is shown to inhibit *S. aureus* biofilm formation [50]. Quercetin, a dietary flavonoid, has been reported to be antibacterial and anti-biofilm against *S. aureus* [51]. Terpenoids of *Thymus vulgaris* were shown to downregulate the expression of enterotoxin genes in *S. aureus* [52]. Tannic acid also inhibited *S. aureus* biofilm formation [53].

The results suggest that QS-inhibitory compounds of these plants may inspire the formulation of new generation of antimicrobial agents to control infections exhibited by these pathogens. More important is the potential of these compounds to prevent, weaken, and stop the resistance of *S. aureus*. From this premise, the current study is trending to the recent innovation to stop diseases and infections caused by antibiotic resistant bacteria using ethnobotanicals. The ten ethnobotanicals of Imugan, Nueva Vizcaya are proven to have QSI on alpha hemolysin produced by *S. aureus*.

This research presents an avenue of of increasing evaluation of the potential of plants extracts as sources of new therapeutic and anti-pathogenic agents. These agents are non-toxic inhibitors of quorum sensing, thus controlling infections without encouraging the appearance of resistant bacterial strains. These ethnobotanicals are proven to have pharmacological potential and can be used for developing new drugs for therapeutic utilization and

for future applications in microbial biotechnology.

CONCLUSION

Bidens pilosa, Cestrum nocturnum, Sarcandra glabra, Pittosporum pentandrum, Oreocnide trinervis, Derris elliptica, Alstonia scholaris, Ageratina adenophora, Ayapana triplinervis and Lipang Daga (no known scientific name) show inhibition of the α-Hemolysin, and therefore, have quorum sensing inhibition activity.

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