STUDY ON ANTI-QUORUM SENSING POTENTIAL OF SELECTED LOCAL ULAM IN MALAYSIA

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ABSTRACT

Bacterial intercellular communication, or quorum sensing (QS), controls the pathogenesis of many medically important organisms. Anti-QS compounds have the ability to attenuate bacterial pathogenicity. The current quest for new antimicrobials aimed at discovering non-toxic inhibitors of QS from natural sources which can be used for the treatment of bacterial infections in human. The objective of this research is to study the anti-QS potential in some of local ulam commonly found in Malaysia. In this study, seven types of local ulam namely; Parkia speciosa, Cosmos cardatus, Centella asiatica, Manihot esculenta leaf sprigs, Psophocarpus tetragonolobus, Polygonum minus and Oenanthe javanica were tested on the anti-QS potentials in fresh (edible or macerated) forms and methanol extracts via biomonitor strain Chromabacterium violaceum ATCC 12472. This biomonitor strain has an ability to produce a purple pigment (violacein) under QS-control. The results exhibited the wide variation in the anti-QS activities on selected local ulam in fresh and methanol extract forms. The highest anti-QS activity was recorded by P. minus and C. asiatica extracts as the lowest of minimum QS inhibition concentration value (7.81 mg/ml) was indicated by both extracts respectively. This study introduces not only a new mode of action and possible validation for traditional plant use, but also a potentially new therapeutic direction for the treatment of bacterial infections.

Keywords: Ulam, Chromabacterium violaceum ATCC 12472, anti-quorum sensing.
INTRODUCTION
The emergence of antibiotic resistance begs the need for novel therapeutics [1]. An increasing of antimicrobial resistance presents a major threat to public health since it could reduce the effectiveness of antimicrobial treatment, leading to increased morbidity, mortality, and health care expenditure [2]. Hence, many pharmacological and pharmacognostical investigations are carried out to identify new drugs or to find new lead structures for the development of novel therapeutic agents in the antibiotic treatments [3]. A promising approach to find new lead structure is to target bacterial cell-to-cell communication, known as anti-quorum sensing as the basis of antipathogenic drug treatment [4].

Quorum sensing (QS) is a widespread prokaryotic intercellular communication system based on the signal molecules (auto-inducers) relative to cell density [5]. Many bacterial physiological functions such as luminiscence, virulence, motility, sporulation and biofilm formation are regulated by QS systems. In fact, most pathogens require QS to produce virulence factors, so the signalling pathway can be a target in designing the small-molecule inhibitors [6]. Compounds that interfere with the QS system to attenuate bacterial pathogenicity are termed as anti-QS compounds [7]. Inhibition of QS is therefore being considered as a new target for antimicrobial chemotherapy with the current quest on discovering non-toxic QS inhibitors from natural sources [8].

Ulam is a fresh green salad that may be tossed in a blend of fermented sauces, aromatic herbs or spices eaten by Malays as side dishes with rice. Ulam has been moved out of rural areas and spread to towns where it is embraced by other races as well. An ulam may constitute shoots, leaves, and seeds of certain local plants that are rich in taste and unique in texture. However, ulam are not only flavourful popular side dish; they have been receiving special attention because of their history in folk medicinal uses either for preventative or even curative purposes. Perhaps, more than 120 species of traditional vegetables have been regarded as ulam from various plant families in South East Asia [9]. Ulam species are rich in carbohydrate, protein, mineral and vitamin [10]. In addition, ulam contain dietary phytochemicals (secondary metabolites in plant) which are known to have several health benefits [11].

Several studies suggest that these dietary phytochemicals may give rise to a new category of antimicrobial substances that have a broader spectrum and may overcome the issue of
developing antibiotic resistance [11]. It is possible that several terrestrial plants like *ulam* also produce quorum signal mimics capable of controlling bacterial quorum sensing [12]. Therefore, recent research has focused on the development of natural therapeutic agents from selected local *ulam* namely; *Parkia speciosa*, *Cosmos cardatus*, *Centella asiatica*, *Manihot esculenta* leaf sprigs, *Psophocarpus tetragonolobus*, *Polygonum minus* and *Oenanthe javanica* which prevent bacterial pathogenesis by inhibiting bacterial QS.

**MATERIALS & METHODS**

**Test bacteria**

*Chromobacterium violaceum* ATCC 12472 was used as a biomonitor strain in the anti-quorum sensing assays. The strain was obtained from ATCC: The Global Bioresource Center, Manassas, Virginia (VA), USA. The bacterium was shipped in the freeze dried form (pellet). The pellet was then incubated in Nutrient broth (NB; Merck, Germany) at 30°C for 24 h. The bacterium were further streaked on Luria Bertani agar (LB agar; Sigma-Aldrich, USA) and incubated for overnight at 30°C. The isolated colony from each strain of bacteria was then inoculated in Luria Bertani broth (LB broth; Sigma-Aldrich, USA) at 30°C for overnight with a gentle shaking.

**Plant materials**

In this study, seven selected local *ulam* were performed for anti-QS activity, namely *Parkia speciosa*, *Cosmos cardatus*, *Centella asiatica*, *Manihot esculenta* leaf sprigs, *Psophocarpus tetragonolobus*, *Polygonum minus* and *Oenanthe javanica*. These seven *ulam* were obtained from a local wet market in Nilai 3, Negeri Sembilan, Malaysia. The edible portions of the fresh samples were cleaned and washed either using 70% ethanol and then rinsed with distilled water (washed samples) or just rinsed with distilled water (unwashed samples). These washed and unwashed samples were further used for preliminary screening.

**Plant extraction**

The plant extraction method was implemented based on method [13] with modifications. Fresh selected local *ulam* were washed thoroughly two to three times with running tap water and sterile water, shade-dried for two weeks, powdered and finally used for extraction. About 100 g of each powder plants were soaked with 1000 ml of methanol (extract/solvent ratio = 1:10 w/v) for seven to eight days at room temperature with frequent agitation. Following filtration of the suspension through a Buckner funnel and Whatman filter paper #1, the crude methanol extracts were evaporated in rotary evaporator at 40°C with 65 rpm. The crude
extract obtained was then prepared for stock solution at a concentration of 1000 mg/ml by diluting a 1000 mg of crude extract in 1 ml of 99.5% Dimethylsulfoxide (DMSO). The stock solution was then preserved at 4°C in airtight bottle until further used. This stock solution was further diluted to a series of double dilutions with Mueller-Hinton broth (MHB) to produce several range of concentrations needed for further experiments.

**Preliminary screening of anti-QS from fresh ulam**

Preliminary screening of anti-QS from fresh *ulam* was done according to the methods [14, 15]. The fresh samples were prepared whole washed parts, unwashed macerated parts and washed macerated parts. The preliminary study was carried out to determine which selected *ulam* presence the anti-QS activity as well as to compare the anti-QS activity between whole and macerated *ulam*. Besides, this study was implemented to recognize the effect of surface microbes and epiphylls towards the anti-QS activities. In this study, 5 ml of molten Soft Top Agar (STA) (1.3 g agar, 2.0 g tryptone, 1.0 g sodium chloride, 200 mL deionised water) were seeded with 100 µL of an overnight LB culture of *C. violaceum*. This was gently mixed and poured immediately over the surface of a solidified Luria Bertani agar (LBA; Sigma-Aldrich, USA) plate as an overlay. The whole washed parts (70% ethanol-washed), unwashed macerated and washed macerated parts were directly placed onto the inoculated LBA plates. These agar plates were then incubated at 30°C for overnight. The ability of each *ulam* in inhibiting microbial growth was detected by observing the ring of turbid or creamy ring of viable cells around the samples (known as “halo”). Loss of purple pigment as *C. violaceum* is indicative of QS inhibition

**Bioassays for anti-QS of *ulam* extracts**

Bioassays for anti-QS of *ulam* extracts was done according to [15] with modifications. The *ulam* extracts were screening for anti-QS activities and finally to determine the minimum QS inhibition values. The concentrations of extracts tested ranging from 1000 to 1.89 mg/ml. In this study, 5 ml of molten STA were seeded with 100 µl of an overnight LB culture of *C. violaceum*. This was gently mixed and poured immediately over the surface of a solidified LBA plate as an overlay. Wells of 5 mm diameter and 5 mm depth were made in the solidified agar using a sterile borer. About 10 µl of each test samples at different concentrations was dispensed into each respective well and allowed to stand about 15 minutes for pre-diffusion of samples. The plates were then incubated at 37°C for 24 hours. The sensitivity of the test bacteria to the extracts were determined by measuring the diameters of the zone of QS
inhibition surrounding the wells in millimeter (mm). The values of minimum QS inhibition concentration from each ulam extracts was recorded by monitoring the first concentration tested value that showed no zone of QS inhibition. All the tests were performed in duplicate.

Statistical analysis
There was no statistical test on preliminary screening of anti-QS from fresh parts of E. hirta as the “halo” formation were identified as absent (-), present (+) or highly present (++). The data for bioassay of anti-QS were analyzed by simple arithmetic means and standard deviations (n = 2) of the extracts.

RESULTS
Preliminary screening of anti-QS from fresh ulam
According to the preliminary screening of anti-QS data (Table 1), fresh C. cardatus, C. asiatica, P. minus and O. javanica exhibited the highest anti-QS activities as the ‘halo’ formation present in washed whole samples. In addition, those ulam indicated the highly present of ‘halo’ formation in unwashed and washed samples. Whilst, the others ulam (P. speciosa, M. esculenta leaf sprigs and P. tetragonolobus) showed the moderate of anti-QS activities as the ‘halo’ formation only present in macerated forms (washed and unwashed samples).

Bioassays for anti-QS of ulam extracts
Figure 1 showed the screening data of the anti-QS activities on ulam extracts. The biggest zone of QS inhibition was recorded by P. minus (23.50 ± 0.71 mm). Whilst, P. speciosa exhibited the lowest QS inhibition as the result was 10.50 ± 0.71 mm. Generally, ulam extracts indicated certain values of the minimum QS inhibition concentration (Table 2). The highest anti-QS activity was recorded by P. minus and C. asiatica extracts as the lowest of minimum QS inhibition concentration value (7.81 mg/ml) was indicated by both plant extracts. Meanwhile, the lowest anti-QS activity was indicated by P. speciosa extract with the highest minimum QS inhibition concentration values of 250.00 mg/ml.
Table 1: Preliminary screening of anti-QS for fresh ulam

<table>
<thead>
<tr>
<th>Ulam</th>
<th>Anti quorum sensing effects (a “halo”; clear zone of QS inhibition)</th>
<th>Washed whole samples</th>
<th>Unwashed macerated samples</th>
<th>Washed macerated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. speciosa</strong></td>
<td></td>
<td>-^a</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>C. cardatus</strong></td>
<td></td>
<td>+</td>
<td>++^c</td>
<td>++</td>
</tr>
<tr>
<td><strong>C. asiatica</strong></td>
<td></td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>M. esculenta</strong> leaf sprigs</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>P. tetragonolobus</strong></td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>P. minus</strong></td>
<td></td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>O. javanica</strong></td>
<td></td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

^a“Halo” formation absent; ^b“Halo” formation present; ^c“Halo” formation highly present

Figure 1: Screening on anti-QS activities from ulam extracts

Table 2: Minimum of QS inhibition concentration (mg/ml) values on ulam extracts

<table>
<thead>
<tr>
<th>Local Ulam</th>
<th>Minimum of QS inhibition concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. speciosa</strong></td>
<td>250.00</td>
</tr>
<tr>
<td><strong>C. cardatus</strong></td>
<td>31.25</td>
</tr>
<tr>
<td><strong>C. asiatica</strong></td>
<td>7.81</td>
</tr>
<tr>
<td><strong>M. esculenta</strong> leaf sprigs</td>
<td>125.00</td>
</tr>
<tr>
<td><strong>P. tetragonolobus</strong></td>
<td>125.00</td>
</tr>
<tr>
<td><strong>P. minus</strong></td>
<td>7.81</td>
</tr>
<tr>
<td><strong>O. javanica</strong></td>
<td>15.63</td>
</tr>
</tbody>
</table>
DISCUSSION
Preliminary screening was implemented to determine whether the anti-QS compounds were actually from the plants themselves or from the epiphyll microorganisms. Thus, the ethanol washed plants materials were compared with unwashed, by placing the whole and mashed plants directly onto a prepared C. violaceum. Basically, all the selected ulam have the potential as anti-QS agents based on preliminary screening studies. Among ulam tested, fresh C. cardatus, C. asiatica, P. minus and O. javanica exhibited the highest anti-QS activities as the ‘halo’ formation present in all condition tested (whole washed parts, unwashed macerated parts and washed macerated parts). The presence of anti-QS activity in washed whole samples indicated that the anti-QS compounds also secreted on the ulam surfaces. The high anti-QS activity showed by crush plant material compare to the whole plant samples. Generally, there was no much difference in anti-QS activity was observed between ethanol-washed and unwashed plants materials. This indicated that there is no any effect of surface microbes and epiphylls towards the anti-QS activity showing by fresh ulam tested.

According to the bioassay studies, P. minus and C. asiatica exhibited the highest anti-QS activities as both extracts indicated the lowest of minimum QS inhibition concentration values. Both ulam commonly found to be effective in the antimicrobial treatments and rich with phytochemical constituents [16, 17]. P. minus is an aromatic plant that produces high levels of essential oil containing aliphatic aldehydes, flavonoid and phenolic compounds [17, 18]. Various chemical constituents are reported in C. asiatica like asiaticoside, madecassoside, madecassicacid, asiatic acid, glucose, rhamnose, terpenoids, sitosterol, stigmasterol, fatty oils consist of glycerides of palmitic acid, stearic acid, linoleic acid, linolenic acid vitamins like ascorbic acid [16]. Thus, the present of abundant phytochemicals in this herb with well known antibiotic properties could also potentially posses anti-pathogenic too.

The anti-pathogenic compounds, in contrast to antibacterial compounds, neither kill bacteria nor stop their growth and are not related to the development of resistant strains. Instead, these compounds attenuate the expression of the genes responsible for pathogenesis by interfering with bacterial communication system like the quorum sensing activities [19]. Previous reports indicated the limited study on the specific phytochemicals that responsible as the natural sources of the anti-QS agents. Many plants or herb exhibited the anti-QS activities like Pisum sativum (pea), Allium sativum (garlic) and Daucus carota (carrot). Some fractionation of those crude extracts was attempted; however, no active compounds were elucidated [20].
Generally, most phytochemicals investigated from herbs which act as anti-QS agents like vannilin, furanones, and ellagitannins belong to the phenolic compounds [7, 20]. As *P. minus* and *C. asiatica* rich with phenolic compounds, it might contribute to the highest anti-QS activities as successfully reported throughout this study.

**CONCLUSION**

Local *ulam* has the potential as natural anti-QS agent especially *P. minus* and *C. asiatica*. For future studies, fractionation of the crude extracts need to be carried out to isolate and characterize the anti-QS compounds in order to determine the exact nature of the compounds that contribute to those effect.

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**REFERENCES**


