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Chapter 5

Screening of Indonesian Medicinal Plants for Quorum Sensing Inhibitory Compounds

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ABSTRACT

Targeting the quorum sensing system and interrupting bacterial communication, is an example of an anti-pathogenic effect and may provide a solution to increasing antibiotic resistance. The use of plants as medicine in Indonesia has always been a tradition. Previous anti-infective studies on Indonesian medicinal plants have focused mainly on anti-microbial drug discovery perspectives. However, no systemic effort has been made to explore the anti-quorum sensing activity of these plants. Our objective is to investigate several Indonesian medicinal plants’ ethanol extracts and essential oils on anti-quorum sensing activity using a quorum sensing violacein biosensor, Chromobacterium violaceum CV026. Also, the effect of extracts and essential oils on the motility of Pseudomonas aeruginosa PAO1 is of interest, since bacterial motility has been shown to be associated with its virulence. Of the 54 plant ethanol extracts and 30 essential oils screened, Nymphaea nouchali ethanol extract, Syzygium aromaticum essential oil and Massoia aromatica essential oil demonstrated violacein production inhibition in the reporter strain. A significant reduction in quorum sensing related motility of P. aeruginosa PAO1 was also observed. This screening system forms an interesting potential for the selection of active principles from plant ethanol extracts and essential oils.

Keywords: Quorum sensing, Chromobacterium violaceum CV026, motility, Pseudomonas aeruginosa PAO1, Nymphaea nouchali, Syzygium aromaticum, Massoia aromatica.
INTRODUCTION

Quorum sensing is a process of cell to cell communication in bacteria mediated by a small diffusible molecule called autoinducers (oligopeptides in Gram positive and N-acyl homoserine lactone (AHL) in Gram negative bacteria). These autoinducers diffuse freely from the bacterial cell and accumulate in the surrounding environment. When a threshold concentration (quorum) has been reached they diffuse back into the cell and regulate transcription of specific genes as in a response to their changing environmental conditions (Waters and Bassler, 2005). Fungi, like bacteria, also use quorum sensing to affect population-level behaviors such as biofilm formation and pathogenesis (Hogan, 2006; Nickerson et al., 2006; Albuquerque and Casadevall, 2012). In the human commensal and pathogenic fungus Candida albicans, two quorum sensing molecules have been described: farnesol and tyrosol (Chen et al., 2004; Hornby et al., 2001). In Candida albicans, accumulated farnesol and tyrosol affect both dimorphism and biofilm formation. As well as in C. albicans, another QS molecules in fungi, phenylalanine and tryptophan, were found in Saccharomyces cerevisiae (Albuquerque and Casadevall, 2012).

Examples of cellular processes modulated by quorum sensing are biofilm formation (McClean et al., 1997), bioluminescence (Nealson and Hasstings, 2006) and motility (Eberl et al., 1999). These traits have also shown to be involved in the pathogenicity of bacteria (Choo et al., 2006). It has been suggested that targeting the quorum sensing system by interruption of bacterial communication, instead of killing bacteria, is an example of an antipathogenic effect and may give a solution to antibiotics resistance (Hentzer and Givskov, 2003). Therefore, anti-quorum sensing (anti-QS) compounds can be of great interest in the treatment of bacterial infections (Fuqua et al., 2001; Rice et al., 2005).

Anti-QS compounds like halogenated furanones produced by the marine alga Delisea pulchra (Manefield et al., 1999) have limited or no therapeutic application due to their toxicity and high reactivity on the host. Other anti-QS molecules have been found from garlic extract and Penicillium species. During screening of 100 extracts from 50 Penicillium species, Rasmussen et al. (2005) found patulin and penicillic acid as biologically active QS inhibitor (QSI) compounds. However, patulin is found to be toxic and today it is belongs to a list of mycotoxins that level in food is regulated. The US Food and Drug Administration (FDA) limits patulin to 50 µg/L (Puel et al., 2010). As well as patulin, penicillin acid also belongs to mycotoxin group. It found in many fruits and vegetables, and toxic effects may result from the accidental ingestion of the material. Animal experiments indicate that ingestion of less than 40 gram
may be fatal or may produce serious damage to the health of the individual (Anonym, 2010). By bioassay-guided fractionation of garlic extracts, Jakobsen et al. (2012) determined the primary QS inhibitor present in garlic to be ajoene, and the toxicity investigation revealed that ajoene has a very low cytotoxicity effects on human epithelium cells. Therefore, the discovery of non-toxic, broad spectrum quorum sensing inhibitors is still needed for successful exploitation in inhibiting bacterial communication (Choo et al., 2006).

Plants have long been a source of medicines and continues to contribute significantly to the development of today’s pharmaceuticals for therapeutics and source of new bioactive compounds (Cragg et al., 1997). Indonesia harbors a very high flora of diverse species used in traditional ways as medicine (Damayanti et al., 2001). Previous anti-infective studies on Indonesian medicinal plants have focused mainly on antimicrobial drug discovery perspectives. However, no systemic effort has been made to explore its anti-QS activity. Furthermore, shifting the focus from antibacterial activity to anti-QS properties may disclose new quorum quenching compounds (Adonizio et al., 2006). For this reason, research in determining anti-QS activity of a compound is generating potential for development of a new therapeutic.

Motility of \textit{P. aeruginosa} in aqueous and dry environments has been shown to be associated with its virulence. In the presence of a quorum sensing inhibitory compound, the motility and therefore the virulence will be limited (Drake and Montie, 1988; Häse, 2001; Rasmussen et al., 2011; Majik et al., 2013). Six different forms of bacterial movement have been described including swimming, swarming, twitching, gliding, sliding and darting (Henrichsen, 1972). These various forms of surface motility enable bacteria to increase their efficiency of nutrient uptake, avoid toxic substances, move to preferred hosts and provide access to optimal colonization sites within, and spread themselves into the environment (Rashid and Kornberg, 2000). Bacterial motility plays a different role in biofilms. They can promote adhesion of the cell to the surface for biofilm maturation process and/or in be involved in the dispersal process (Marchal et al., 2010). However, motility is not critical to biofilm formation, which was shown for biofilms produced by \textit{P. aeruginosa} PAO1 mutant strain which is lack flagella and type IV pili (Chow et al., 2011).

In this study, we provide the screening result of some commonly used Indonesian medicinal plants for anti-QS activity using the quorum sensing violacein biosensor, \textit{Chromobacterium violaceum} 31532 wild
type (WT) strain, *C. violaceum* CV026 mutant strain, and *P. aeruginosa* PAO1. The wild type strain *C. violaceum* ATCC 31532 produces a purple pigment, violacein, when AHL molecules reach a threshold level, whereas the mutant strain CV026 lacks the ability to produce violacein unless exogenous AHLs are detected. We also investigated the correlation of the quorum sensing inhibition in reducing quorum sensing related motility of *P. aeruginosa* PAO1. Swimming, swarming and twitching motility have been studied in this research. We have found anti-QS activity in 10 out of 54 plant ethanol extracts and 12 out of 29 plant essential oils, with 1 extract and 2 essential oils demonstrating a high anti-quorum sensing activity.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions**

The bacterial strains used in this study were *Chromobacterium violaceum* ATCC 31532 wild type (WT) strain, *C. violaceum* CV026 mutant strain and *P. aeruginosa* PAO1 strain. The C6-homoserine lactone (HHL) (Sigma Aldrich, Germany) was dissolved in dimethylsulfoxide (DMSO) and used as autoinducer for violacein pigment production in *C. violaceum* CV026. The bacterial strains were grown on Luria-Bertani (LB) Agar and incubated for 24 hours at 30⁰C. Following the incubation on agar plate, the colonies were transferred to LB broth and incubated for another 24 hours at 30⁰C with shaking (250 rpm). Cell density was adjusted to 10⁸ CFU/mL by altering the optical density of the suspension to 0.1 at 600 nm.

**Indonesian medicinal plants**

Fifty four commonly used medicinal plants were collected from Yogyakarta, and its surroundings, and the bark of *Massoia aromaticia* Becc. was collected from Nabire district, West Papua, Indonesia, on the basis of ethnopharmacological information. Crude ethanol extracts were prepared by washing and cutting the plant samples into small pieces followed by oven drying (40⁰C) for 48-72 hours. The dried plant materials were grind into a fine powder. The pulverized materials were soaked in Petroleum Ether (PE) in a ratio of 1 g (plant material) : 10 ml PE to remove the lipids. Secondly, plant materials were also extracted with 70% ethanol (EtOH) using a ratio of 1 g (plant material) : 10 mL (EtOH) to obtain crude ethanol extract. Extracts were dried and concentrated under reduced pressure using a rotary evaporator (Ika RV 10 Basic, Ika HB 10, Heidolph). Stock solutions (100 mg/mL) of crude ethanol extracts in the excipient DMSO were prepared, filter-sterilized (0.2 µm) and stored at 4°C. A total of 29 essential oils were obtained from water-steam distillation process. The oils obtained were dried over anhydrous sodium sulphate (Na₂SO₄), filtered using a Whatman filter paper no. 40, and stored in sealed dark glass
vial at 4°C. Stock solutions of 50% (v/v) essential oils were prepared in methanol (MeOH), for the following dilution to obtain essential oil concentration ranging from 1 to 0.01 % v/v.

**Screening for anti-quorum sensing assay using disc diffusion method**

Standard disc diffusion assay was used to detect anti-quorum sensing activity of the plant ethanol extracts and essential oils. 100 µL of *C. violaceum* WT strain adjusted to OD$_{600}$ nm = 0.1 (approximately 1 x $10^8$ CFU/mL) was spread on LB agar plates. A cork-borer (6 mm in diameter) was used to make wells on the agar plates. Each plant ethanol extract (1 mg/mL) in DMSO and essential oil 1% v/v in MeOH was loaded in the wells. DMSO concentrations of 1 % v/v and methanol concentration of 1 % v/v were used as the background control. Streptomycin at a concentration of 100 µg/mL was used as positive (growth inhibition) control. Bacterial growth inhibition by the plant extracts and essential oils was measured the radius (r1) in mm from the obtained halo, while plant extracts and essential oils showing both growth and pigment inhibition was measured as radius (r2) in mm. The pigment inhibition was determined by subtracting bacterial growth inhibition radius (r1) from the total radius (r2) thereby, quorum sensing inhibition is r2-r1 in mm, as illustrated in Figure 1 (Zahin *et al*., 2010).

![Fig. 1: Illustration of quorum sensing inhibition diameter measurement on *C. violaceum* growth on a petridish.](image)

**Planktonic Minimal Inhibitory Concentration (PMIC)**

Planktonic minimal inhibitory concentrations (PMIC) of plant ethanol extracts and essential oils which showed anti-QS activity against *C. violaceum* WT strain were determined by the broth microdilution test. Briefly, LB broth containing two-fold increments of plant extracts and essential oils was added to a 96-wells plate micro dilution tray. The bacterial suspension equal to a 0.5 McFarland standard was further
diluted and added to the tray to achieve a final inoculum of $5 \times 10^5$ CFU/mL per well. Inoculated 96-well plates were incubated for 18–24 h at 30 °C. The PMIC$_{50}$ concentration, i.e. the extract or oil concentration showing 50% growth inhibition on the bacterial tested, was calculated using probit analysis.

**Quantification of violacein production**

Properly labeled tubes containing: (i) LB broth and HHL; (ii)LB broth, HHL and methanol; (iii) LB broth, HHL and different dilutions of essential oils; were used to measure the amount of violacein production. 100 µL of a bacterial suspension ($10^8$ CFU/mL) was added to each tube. Tubes were incubated for 24 hours at 30°C (Table 1).

**Table 1:** Composition of the tubes for determining cell growth inhibition and antiquorum sensing activity.

<table>
<thead>
<tr>
<th>Content</th>
<th>Tube</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td></td>
<td>895 µL</td>
<td>895 µL</td>
<td>895 µL</td>
<td></td>
</tr>
<tr>
<td>CV026</td>
<td></td>
<td>50 µL</td>
<td>50 µL</td>
<td>50 µL</td>
<td>$1 \times 10^8$ CFU/mL</td>
</tr>
<tr>
<td>HHL</td>
<td></td>
<td>5 µL</td>
<td>5 µL</td>
<td>5 µL</td>
<td>15 µmol/mL</td>
</tr>
<tr>
<td>Ethanol extracts/Essential oils</td>
<td></td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
<td>½ PMIC</td>
</tr>
<tr>
<td>DMSO/Methanol</td>
<td></td>
<td>50 µL</td>
<td>50 µL</td>
<td>-</td>
<td>≤ 1% (v/v)</td>
</tr>
<tr>
<td>Total Volume</td>
<td></td>
<td>1000 µL</td>
<td>1000 µL</td>
<td>1000 µL</td>
<td></td>
</tr>
</tbody>
</table>

After incubation, tubes were centrifuged at 13,000 rpm for 10 minutes to precipitate the insoluble violacein. The culture supernatant was discarded and 1 mL of DMSO or MeOH was added to the pellet. The solution was vortexed vigorously for 30 seconds to completely solubilize violacein and centrifuged at 13,000 rpm for 10 minutes to remove the residue of cells. 200 µL of the violacein containing supernatant was added to a 96-well flat bottomed microplate, four wells per each sample and the absorbance was measured at 595 nm with a microplate reader (Perkin Elmer’s Enspire® 2300 multimode plate reader) (Choo et al., 2006).
**Pseudomonas aeruginosa PAO1 motility test**

The plant ethanol extract and essential oils which inhibited violacein production were further tested to explore their effects on quorum sensing related swarming, swimming and twitching motility of *P. aeruginosa* PAO1. Swimming, swarming and twitching motility assays were performed by the method of Rashid and Kornberg (2000). Briefly, LB agar (0.3%) plates (for swimming motility), and LB agar (0.5%) plates (for swarming motility) containing sub-inhibitory concentrations of ethanol extract or essential oils were prepared and allowed to dry for 3-4 h at 30°C. Plates were point inoculated with freshly grown culture cells using a blunt ended sterile toothpick. For twitching motility, LB agar (1%) plates were used bacterial cells were inoculated by using a sharp end toothpick and stabbing through the agar to the bottom of the petridish. After 24 hours of incubation in upright position at 30°C, the extent of motility was determined by measuring the diameter of the bacterial colony.

**Statistical analysis**

All data were initially analyzed by a normal distribution using the one-sample Kolmogorov-Smirnov test. Following the confirmation of normal distribution, differences for individual parameters between treated groups and control were tested by one way ANOVA, followed by Dunnett’s test. Differences were considered significant with P-values of 0.05 or less.

**RESULT**

**Samples preparations**

A list of the plant extracts studied, including the botanical name, voucher specimen and data related to traditional use is listed in Table 1 Chapter 2, whereas the list of essential oils is indicated in Table 1 Chapter 3. Fifty four plants ethanol extracts and 29 essential oils were obtained as described in Material and Methods of Chapter 2 and 3, this thesis. The yields of plant oils are presented in Table 2 Chapter 3, this thesis.

**Anti-quorum sensing activity**

The inhibition in quorum sensing activity of the evaluated plant ethanol extract and essential oils are shown in figure 2 and 3. The disappearance of the violet colored (violacein) pigment in *C. violaceum* is an indication of anti-QS activity by the added plant ethanol extracts and essential oils. From out of 54 plant ethanolis extracts screened for anti-quorum sensing activity, 10 plants (*Cinnamomum burmannii*,...
Zingiber officinale, Terminalia catappa, Sesbania grandiflora, C. sintoc, Piper bettle, Caesalpinia sappan, Kaempferia rotunda, Nymphaea nouchali and Syzygium aromaticum) showed anti-QS activity, 12 out of 29 plants essential oil extracts (Massoia aromatica, Ocimum basilicum, Z. purpureum, Litsea cubeba (leaves and bark part), C. sintoc, S. polyanthum, S. aromaticum, Myristica fragrans, Z. officinale var rubrum, Coriandrum sativum, K. rotunda, and K. galanga) proved to be effective. Strong anti-QS activity was observed in essential oils from M. aromatica (Table 2).

Fig. 2: Inhibition in violacein production and cell growth in Chromobacterium violaceum ATCC 31532 (wild type strain) by ethanol extracts. Anti-QS activity and growth inhibition was demonstrated for (a) Cinnamomum burmannii, (e) Zingiber officinale, (f) Syzigium aromaticum, (i) Sesbania grandiflora, (l) Cinnamomum sintoc, (m) Piper bettle, (n) Caesalpinia sappan, (o) Kaempferia rotunda, (p) Nymphaea nauchalli, and (v) Terminalia catappa ethanol extracts. As a positive control (t), Streptomycin 50 µg/mL was used as antibiotic control, and (u) DMSO was used as a solvent control.
Fig. 3: Inhibition in violacein production and cell growth in *Chromobacterium violaceum* ATCC 31532 (wild type strain) by essential oils: (1) *Massoia aromatica*, (2) *Ocimum basilicum*, (3) *Cinnamomum burmannii*, (4) *Zingiber purpureum*, (5) *Litsea cubeba* (leaves), (6) *L. cubeba* (seeds), (7) *L. cubeba* (bark), (8) *Cinnamomum sintoc*, (9) *Elettaria cardamomum*, (10) *Citrus aurantifolia*, (12) *Z. officinale var rubrum*, (13) *Syzigium aromaticum*, (14) *S. polyanthum*, (15) *Coriandrum sativum*, (16) *Piper nigrum*, (17) *Caesalpinia sappan*, (20) *Myristica fragrans*, (21) *Kaempferia rotunda*, (27) *K. galanga*. As a positive control (S), Streptomycin 100 µg/mL was used.

Fig. 4: Anti-QS activity versus antibacterial activity. (a) no anti-QS nor antibacterial activity, (b) anti-QS activity, (c) anti-QS and antibacterial activity, (d) antibacterial activity.
Table 2: Antiquorum sensing activity of Indonesian medicinal plants ethanol extracts by agar well diffusion method (Values are mean ±SD of 3 experiments).

<table>
<thead>
<tr>
<th>No</th>
<th>Plants</th>
<th>Sample form</th>
<th>Part used</th>
<th>Zone of inhibition (mm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total radius (r1)*</td>
<td>Growth Inhibition (r2)*</td>
</tr>
<tr>
<td>1</td>
<td><em>C. burmannii</em></td>
<td>Extract</td>
<td>Bark</td>
<td>13.0±0.0</td>
<td>11.5±0.0</td>
</tr>
<tr>
<td>2</td>
<td><em>Z. officinale</em></td>
<td>Extract</td>
<td>Rhizome</td>
<td>13.0±0.0</td>
<td>9.5±0.0</td>
</tr>
<tr>
<td>3</td>
<td><em>S. aromaticum</em></td>
<td>Extract</td>
<td>Flower</td>
<td>21.5±0.0</td>
<td>9.5±0.0</td>
</tr>
<tr>
<td>4</td>
<td><em>Sesbania grandiflora</em></td>
<td>Extract</td>
<td>Leaves</td>
<td>13.0±0.0</td>
<td>8.0±0.0</td>
</tr>
<tr>
<td>5</td>
<td><em>C. sintoc</em></td>
<td>Extract</td>
<td>Bark</td>
<td>13.5±0.0</td>
<td>9.0±0.0</td>
</tr>
<tr>
<td>6</td>
<td><em>P. betle</em></td>
<td>Extract</td>
<td>Leaves</td>
<td>12.0±0.0</td>
<td>9.5±0.0</td>
</tr>
<tr>
<td>7</td>
<td><em>C. sappan</em></td>
<td>Extract</td>
<td>Bark</td>
<td>12.5±0.0</td>
<td>8.0±0.0</td>
</tr>
<tr>
<td>8</td>
<td><em>K. rotunda</em></td>
<td>Extract</td>
<td>Rhizome</td>
<td>11.5±0.0</td>
<td>8.0±0.0</td>
</tr>
<tr>
<td>9</td>
<td><em>Nymphaea nouchali</em></td>
<td>Extract</td>
<td>Flower</td>
<td>24.5±0.0</td>
<td>11.5±0.0</td>
</tr>
<tr>
<td>10</td>
<td><em>Terminalia catappa</em></td>
<td>Extract</td>
<td>Leaves</td>
<td>16.0±0.0</td>
<td>9.2±0.0</td>
</tr>
<tr>
<td>11</td>
<td><em>Massoia aromatica</em></td>
<td>Oil</td>
<td>Bark</td>
<td>24.2±0.3</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td><em>Syzygium aromaticum</em></td>
<td>Oil</td>
<td>Flower</td>
<td>23.7±0.6</td>
<td>8.3±0.56</td>
</tr>
<tr>
<td>13</td>
<td><em>Ocimum basilicum</em></td>
<td>Oil</td>
<td>Leaves</td>
<td>9.7±0.6</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td><em>Zingibier purpureum</em></td>
<td>Oil</td>
<td>Rhizome</td>
<td>12.2±0.3</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td><em>Z. officinale var rubrum</em></td>
<td>Oil</td>
<td>Rhizome</td>
<td>10.0±0.00</td>
<td>8.0±0.0</td>
</tr>
<tr>
<td>16</td>
<td><em>Litsea cubeba</em></td>
<td>Oil</td>
<td>Leaves</td>
<td>12.2±0.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bark</td>
<td>12.0±0.0</td>
<td>9.3±0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Seeds</td>
<td>8.0±0.0</td>
<td>8.0±0.0</td>
</tr>
<tr>
<td>17</td>
<td><em>S. polyanthum</em></td>
<td>Oil</td>
<td>Leaves</td>
<td>12.0±0.0</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td><em>Myristica fragrans</em></td>
<td>Oil</td>
<td>Seeds</td>
<td>10.2±0.3</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td><em>Kaempferia rotunda</em></td>
<td>Oil</td>
<td>Rhizome</td>
<td>10.0±0.0</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td><em>K. galanga</em></td>
<td>Oil</td>
<td>Rhizome</td>
<td>10.0±0.0</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td><em>Coriadrum sativum</em></td>
<td>Oil</td>
<td>Seeds</td>
<td>9.5±0.5</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td><em>Cinnamomum burmannii</em></td>
<td>Oil</td>
<td>Bark</td>
<td>18.0±0.0</td>
<td>18.0±0.0</td>
</tr>
<tr>
<td>23</td>
<td><em>C. sintoc</em></td>
<td>Oil</td>
<td>Bark</td>
<td>8.2±0.3</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td><em>Elettaria cardamomum</em></td>
<td>Oil</td>
<td>Bark</td>
<td>12.0±0.0</td>
<td>12.0±0.0</td>
</tr>
<tr>
<td>25</td>
<td><em>Piper nigrum</em></td>
<td>Oil</td>
<td>Fruit</td>
<td>9.2±0.3</td>
<td>9.2±0.3</td>
</tr>
<tr>
<td>26</td>
<td><em>Caesalpinia sappan</em></td>
<td>Oil</td>
<td>Bark</td>
<td>9.2±0.2</td>
<td>9.2±0.3</td>
</tr>
<tr>
<td>27</td>
<td><em>Citrus aurantifolia</em></td>
<td>Oil</td>
<td>Leaves</td>
<td>9.3±0.6</td>
<td>9.3±0.6</td>
</tr>
<tr>
<td>28</td>
<td>Control (Streptomycin 50 µg/mL)</td>
<td></td>
<td></td>
<td>24.8±0.0</td>
<td>24.8±0.0</td>
</tr>
<tr>
<td>29</td>
<td>Control (Streptomycin 100 µg/mL)</td>
<td></td>
<td></td>
<td>36.2±0.3</td>
<td>36.2±0.3</td>
</tr>
</tbody>
</table>

r1= radius of growth + pigment inhibition (mm); r2= radius of growth inhibition (mm); (r1-r2)= radius of pigment inhibition (mm).
*including 6mm wells diameter on agar plate
PMIC determination

The antibacterial activity of plant ethanol extracts and essential oils against *C. violaceum* CV026 was determined using the microbroth dilution method. The results obtained from this assay are presented in Table 3.

*S. aromaticum* and *N. nouchali* ethanol extracts showed the capability to inhibit 50% of *C. violaceum* CV026 growth (PMIC$_{50}$) at a concentration of 0.12 mg/mL and 0.25 mg/mL. Higher concentrations to inhibit CV026 growth were needed for the other ethanol extracts. A total of four essential oils (*M. aromatica*, *O. basilicum*, *K. rotunda*, and *C. sativum*) had a PMIC$_{50}$ at a concentration of 0.06% (v/v). Essential oils from *C. burmannii* showed a PMIC$_{50}$ at 0.03% (v/v).

Violacein quantification

Quantification of violacein production by *C. violaceum* CV026 was used to measure the inhibition quantity in quorum sensing activity. *N. nouchali* ethanol extract, *S. aromaticum* essential oil and *M. aromatica* essential oil showed the highest quorum sensing inhibition activity by using the agar well diffusion method. Figure 5 shows the result of reduction in violacein production by *N. nouchali* ethanol extracts, *S. aromaticum* essential oil and *M. aromatica* essential oil.

*Nymphaea nouchali* ethanol extracts, *S. aromaticum* essential oil and *M. aromatica* essential oil showed a concentration dependent anti-quorum sensing activity derived from the amount of violacein produced. At elevated concentrations, both the ethanol extract and essential oils tested demonstrated antibacterial activity besides the anti-QS activity (Figure 5).

At the lowest concentration tested (0.06% v/v) of *M. aromatica* oil, the amount of violacein pigment produced by *C. violaceum* was 38.9±0.6% (**P<0.01), whereas the percentage of cell growth on that concentration was 72.0±0.5% (*P<0.05). In the presence of concentration of 0.06% v/v of *S. aromaticum* oil, violacein production of *C. violaceum* was 43.7±0.4% (**P<0.01), and the percentage of total cell growth was around 64.4±0.6% (**P<0.05), respectively. This result indicates that the anti-quorum sensing activity of *M. aromatica* and *S. aromaticum* oil is independent of cell growth (Figure 5).

The violacein production of *C. violaceum* CV026 impregnated with HHL was respectively decreased by the ethanol extract of *N. nouchali* at a concentration of 0.25 mg/mL, with total amount of violacein
produced as much as 49.2±0.9% (**P<0.01). However, at the same concentration the percentage of cell growth was found to be 51.2±0.8% (**P<0.01) indicates that for *N. nouchali* extract, the violacein production inhibition is basically dependent on the cell growth of bacterial reporter strain (Figure 5).

The ethanol extracts and essential oils which showed anti-QS activity by inhibition of violacein production were further tested to explore their effect on quorum sensing related swimming, swarming and twitching motility for *P. aeruginosa* PAO1.

**Table 3: Antiquorum sensing activity of Indonesian medicinal plants essential oils by microdilution method.**

<table>
<thead>
<tr>
<th>No</th>
<th>Plants</th>
<th>Materials</th>
<th>PMIC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>N. nouchali</em></td>
<td>Extract</td>
<td>0.25 mg/mL</td>
</tr>
<tr>
<td>2</td>
<td><em>Syzygium aromaticum</em></td>
<td>Extract</td>
<td>0.12 mg/mL</td>
</tr>
<tr>
<td>3</td>
<td><em>Massoia aromatica</em></td>
<td>Oil</td>
<td>0.06% (v/v)</td>
</tr>
<tr>
<td>4</td>
<td><em>Syzygium aromaticum</em></td>
<td>Oil</td>
<td>&gt;1.00% (v/v)</td>
</tr>
<tr>
<td>5</td>
<td><em>Ocimum basilicum</em></td>
<td>Oil</td>
<td>0.06% (v/v)</td>
</tr>
<tr>
<td>6</td>
<td><em>Zingiber purpureum</em></td>
<td>Oil</td>
<td>0.12% (v/v)</td>
</tr>
<tr>
<td>7</td>
<td><em>Z. officinale var rubrum</em></td>
<td>Oil</td>
<td>0.25% (v/v)</td>
</tr>
<tr>
<td>8</td>
<td><em>Litsea cubeba</em> (bark)</td>
<td>Oil</td>
<td>0.25% (v/v)</td>
</tr>
<tr>
<td></td>
<td><em>L. cubeba</em> (leaves)</td>
<td>Oil</td>
<td>0.25% (v/v)</td>
</tr>
<tr>
<td></td>
<td><em>L. cubeba</em> (seeds)</td>
<td>Oil</td>
<td>0.12% (v/v)</td>
</tr>
<tr>
<td>9</td>
<td><em>S. polyanthum</em></td>
<td>Oil</td>
<td>0.25% (v/v)</td>
</tr>
<tr>
<td>10</td>
<td><em>Myristica fragrans</em></td>
<td>Oil</td>
<td>0.12% (v/v)</td>
</tr>
<tr>
<td>11</td>
<td><em>Kaempferia rotunda</em></td>
<td>Oil</td>
<td>0.06% (v/v)</td>
</tr>
<tr>
<td>12</td>
<td><em>K. galanga</em></td>
<td>Oil</td>
<td>0.12% (v/v)</td>
</tr>
<tr>
<td>13</td>
<td><em>Coriandrum sativum</em></td>
<td>Oil</td>
<td>0.06% (v/v)</td>
</tr>
<tr>
<td>14</td>
<td><em>Cinnamomum burmannii</em></td>
<td>Oil</td>
<td>0.03% (v/v)</td>
</tr>
<tr>
<td>15</td>
<td><em>C. sintoc</em></td>
<td>Oil</td>
<td>0.12% (v/v)</td>
</tr>
<tr>
<td>16</td>
<td><em>Elettaria cardamomum</em></td>
<td>Oil</td>
<td>0.50% (v/v)</td>
</tr>
<tr>
<td>17</td>
<td><em>Piper nigrum</em></td>
<td>Oil</td>
<td>0.25% (v/v)</td>
</tr>
<tr>
<td>18</td>
<td><em>Caesalpinia sappan</em></td>
<td>Oil</td>
<td>0.12% (v/v)</td>
</tr>
<tr>
<td>19</td>
<td><em>Citrus aurantifolia</em></td>
<td>Oil</td>
<td>0.50% (v/v)</td>
</tr>
</tbody>
</table>

<sup>a</sup> PMIC<sub>50</sub>: plant ethanol extract concentration (mg/mL) or essential oil concentration (% v/v) at which 50% respectively of *C. violaceum* CV026 planktonic growth is inhibited.
Fig. 5: Inhibition of *Chromobacterium violaceum* CV026 growth and its violacein production by: (a) *Massoia aromatica* essential oil, (b) *Syzygium aromaticum* essential oil, and (c) *Nymphaea nouchali* ethanol extract. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.
Inhibition of bacterial motility

We investigated if the extracts and essential oils which inhibited quorum sensing had any effect on quorum sensing related motility in the human opportunistic pathogen *P. aeruginosa* PAO1. Our result, shown in figure 6, indicated that ethanol extracts of *N. nouchali* at a concentration of 1 and 0.5 mg/mL significantly reduced the swimming motility of *P. aeruginosa* PAO1 by 74.7±0.9 % (**P<0.001) and 54.4±0.6 % (**P<0.01), respectively. The swarming motility of *P. aeruginosa* PAO1 was also reduced as much as 67.8±0.3 % (**P<0.001) and 53.4±0.3 % (**P<0.01) when concentrations of 1 mg/mL 0.5 mg/mL were applied. The same concentrations of the extract also showed a decrease in twitching motility namely, 75.8±0.0 % (**P<0.001) and 62.1±0.3 % (**P<0.001) respectively.

These results are in accordance with our previous study (chapter 2 this thesis) which showed that ethanol extracts from *N. nouchali* have inhibitory effects on the biofilm of *P. aeruginosa* PAO1 at the concentrations of 0.06 mg/mL. According to O’Toole and Kolter (2002), twitching motility of the type IV pili has been shown to be important for the adherence adhesion to eukaryotic cell surfaces and is necessary for biofilm development. At the microscopic level, the edge of the colonies in twitching motility is highly irregular and this is thought to be a consequence of the surface movement associated with type IV pili (Figure 7).

Both concentrations of essential oils from *M. aromatica* (1% (v/v) and 0.5% (v/v)) that were tested significantly decreased the swarming motility of *P. aeruginosa* PAO1 by 29.57± 0.57 % (***P<0.001), and 51.4 ± 0.0 % (**P<0.01) respectively. As much as 100±0.0 % and 50.9 ± 0.6 % inhibition in twitching motility for *P. aeruginosa* PAO1 was demonstrated by these essential oils with concentrations ranging from 1 to 0.06 % (v/v) (Figure 8). The capability of essential oils from *M. aromatica* in reducing *P. aeruginosa* PAO1 twitching motility correlated significantly with a decrease in *P. aeruginosa* PAO1 biofilm formation when these cells were incubated with the same essential oil concentration of 0.06 % (v/v) and higher (data chapter 3 this thesis). However, at concentrations lower than 0.5 % (v/v) essential oils from *M. aromatica* failed to reduce more than 50% of the swimming motility of *P. aeruginosa* PAO1.
Fig. 6: Effect of *Nymphaea nouchali* ethanol extracts on swimming, swarming and twitching motility of *Pseudomonas aeruginosa* PAO1. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

![Graphs showing the effect of Nymphaea nouchali ethanol extracts on swimming, swarming, and twitching motility of Pseudomonas aeruginosa PAO1.](image)

Fig. 7: *Pseudomonas aeruginosa* PAO1 twitching motility. (a) Arrow shows twitching motility of *P. aeruginosa* PAO1 on 1% LB agar, (b) Twitching motility of *P. aeruginosa* PAO1 on 1% LB agar stained with crystal violet, (c) Direct visual inspection of the colony edges by microscopy and (d) colony edges stained with crystal violet. The edge is highly irregular. Micrographs were taken at 100x (b) and 400x (c) magnification using a Leica Light microscope.

![Images showing twitching motility of Pseudomonas aeruginosa PAO1](image)
Fig. 8: Effect of Massoia aromatica essential oils on swimming, swarming and twitching motility of Pseudomonas aeruginosa PAO1. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.
At concentrations ranging from $1 - 0.25\%$ (v/v), essential oils from *Syzygium aromaticum* significantly ($**P<0.01$) decreased the swimming motility of *P. aeruginosa* PA01 from $60.5\pm0.3\%$ to $50.5\pm0.6\%$, respectively. However this essential oil failed to reduce the swarming motility of *P. aeruginosa* PA01 at concentrations lower than $1\%$ (v/v) (Figure 9). As much as $53.2\pm0.8$ to $50.0\pm 0.6\%$ ($**P<0.01$) inhibition in twitching motility of *P. aeruginosa* PA01 was shown by this essential oil with a concentration range from $1$ to $0.5\%$ (v/v). The capability of the essential oil from *S.*
aromaticum in reducing P. aeruginosa PAO1 twitching motility correlated with a significant decrease in P. aeruginosa PAO1 biofilm formation when incubated with the same essential oil concentration of 0.5 and 1 % (v/v) (chapter 3, this thesis).

**DISCUSSION**

The aim of this study was to determine the anti-QS potential of ethanol extracts and essential oils from Indonesian medicinal plants with a potential to a possible use in controlling detrimental infections. Quorum sensing in P. aeruginosa has been well studied. This organism has two QS systems, LasR/I and RhlR/I systems. The lasI produces diffusible extracellular signal, N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) which binds with LasR to activate a number of virulence genes and biofilm maturation, and to regulate the expression of LasI. In the Rhl system, the RhlI synthase produces N-butyryl-L-homoserine lactone (C4-HSL), which interacts with RhlR protein and activated C4-HSL-RhlR complex, further stimulates the expression of RhlI, virulence genes and biofilm associated genes and biofilm associated genes (Whiteley *et al*., 1999; De Kievit, 2009; Desai and Gala, 2014).

*P. aeruginosa* produces a third signaling molecule, 2-heptyl-3-hydroxy-4(1H)-quinoline, called Pseudomonas quinolone signal (PQS). It is produced by the *pqs* (*pqsABCDE*) operon and *pqsH* gene, wherein the *pqsABCDE* operon products synthesize 2-heptyl-4-hydroxyquinoline (HHQ), and the PqsH converts HHQ into PQS. PQS diffuses in and out of the cell, accumulates in the environment, and when a threshold concentration reached, it binds to the regulator protein, PqsR, which modulates genes encoding virulence factors and the synthesis of PQS itself, resulting in autoinduction (Whiteley *et al*., 1999; Desai *et al*., 2014).

There are several reports in literature that propose different mechanisms of quorum sensing inhibition by natural products, either by inhibition of the signal molecule biosynthesis (Vattem *et al*., 2007), preventing the binding of the AHL molecules to its receptors (Hentzer and Givskov, 2003), or enzymatic inactivation and biodegradation of the quorum sensing signaling molecules (Defoirdt *et al*., 2004).

Plants are the sources of varied bioactive metabolites which useful for the development of successful and effective drugs. Various plants have demonstrated the ability to interfere with microbial QS systems that further and control its virulence. Vanilla (*Vanilla planifolia*), garlic (*Allium sativum*), weeping bottlebrush (*Callistemon viminalis*), Zaragoza mangrove (*Conocarpus erectus*), graceful sandmat (*Chamaesyce hypericifolia*), black olive (*Bucida buceras*), Florida clover ash
(Tetrazygia bicolor), and southern live oak (Quercus virginiana), showed quorum sensing inhibitory properties against C. violaceum and Agrobacterium tumefaciens (Choo et al., 2006; Adonizio et al., 2006). Study from Zahin et al., (2010), Chong et al., (2011) and Priya et al., (2013) also revealed quorum sensing property of Mangifera indica, Punica granatum, Myristica cinnamomea and Phyllanthus amarus, which showed efficacy in regulating violacein production of C. violaceum and inhibiting motility of P. aeruginosa. The result in our study have revealed that the ethanol extract of N. nouchali, M. aromatica and the essential oils from S. aromaticum significantly inhibit the quorum sensing mechanism of P. aeruginosa PAO1 as indicated by a reduction in violacein production of the reporter strain C. violaceum. They are also able to inhibit the motility of P. aeruginosa PAO1 which is an important trait for its pathogenicity.

To interfere with signal reception, plants produce molecules that structurally mimic the AHLs, and the competitive binding is effective to block activation of AHL-mediated QS (Koh et al., 2013). Halogenated furanones isolated from D. pulchra were found to inhibit QS regulated behaviors by competitively bind to the LuxR type proteins, thus, promote their rate of proteolytic degradation without killing the bacteria (Manefield et al., 1999). Chong et al., (2011) extracted malabaricone C from nutmeg Myristica cinnamomea, whose structure is not similar to AHL but possesses anti-QS activity by inhibits both lasR and rhlR systems in P. aeruginosa, CviR system in C. violaceum. However, that compound does not inhibit AHL production in P. aeruginosa.

S. aromaticum essential oil also known as clove oil has a long history of use in traditional medicine, and is a proven antimicrobial (Indonesian National Health Department, INHD, 1985). The oil contains majority of eugenol, which comprises around 72-90% of the oil. Other important consituents of the oil include tannins, flavonoids, and several sesquiterpenes (Joseph and Sujatha, 2011). Eugenol found to be toxic in relatively small quantities, as low as 5 mL (Jirovetz et al., 2006). Krishnan et al., (2012) discovered that hexane and methanol extract of S. aromaticum bud inhibited QS-controlled virulence production in P. aeruginosa, including reducing the bioluminescence of P. aeruginosa PAO1 lecA::lux (by hexane extract), swarming (maximum inhibition by methanol extract), pyocyanin (maximum inhibition by hexane extract). Inhibition of P. aeruginosa lecA::lux fusion suggests that S. aromaticum extracts might interfere with lux, rhl and las QS-regulated system. Flavonoids contains in S. aromaticum is thought to be the compound responsible for the anti-QS mechanism. Flavonoids such as catechin, naringenin and taxifolin were found to have capability to reduce the expression of QS genes: lasI, lasR, rhlI and rhlR, and attenuate virulence factors such as pyocyanin, elastase and biofilm formation in P. aeruginosa (Vandeputte et al., 2010; Sarabhai et al., 2013).
Beside used as spices, massoia oil from *M. aromatica* bark traditionally used in Indonesia to cure diarrhea, sore throat and stomach ache. Balinese people use this oil in the massage therapy (Hartnoll *et al.*, 1993). From GC-MS analysis we found out that massoia oil contains massoia lactone (77.06%) (Chapter 4, this thesis). Amaya *et al.*, (2012) discovered anti-QS activity of six sesquiterpenes lactones isolated from *Centratherum punctatum*. At low concentration, those lactones showed capability to prevent biofilm formation, elastase activity controlled by AHL, and production of AHLs. Similar to Amaya *et al.*, Cartagena *et al.*, (2007) studies acetogenin and squamocin, a plant sesquiterpene lactones isolated from Asteraceae and Hepaticae, which found to be able to inhibit *P. aeruginosa* QS-regulated biofilm formation.

Similar to study from Krishnan *et al.*, (2012), the result from this study showed that *S. aromaticum* oil possess antibacterial as well as anti-quorum sensing activity. The fact that at elevated concentrations, *N. nouchali* extract, *M. aromatica* and *S. aromaticum* essential oils also demonstrated antibacterial activity along with anti-QS activity raised a question on whether the antibacterial and anti-QS effect in the ethanol extract and essential oils are from the same or distinct chemical compounds.

Bacterial motility plays a pivotal role in microbial surface colonization and the spreading of bacteria across the surface. These motilities contribute to the formation of structured surface-associated communities of bacteria called biofilms. The gram-negative bacterium, *P. aeruginosa*, can undergo the flagellum-mediated swimming motility and the surface-associated swarming and twitching motilities (Chow *et al.*, 2011).

Swimming and swarming motility are dependent on flagella, whereas twitching has been shown to require type IV pili (Harshey, 2003). Swimming motility of bacteria formed on the plates with a low concentration of agar (0.3%) is not a social event unlike swarming, swimming motility. This motility represents individual cell movement and bacteria are fully immersed within the semisolid medium. The cells move separately in an unorganized manner resulting in an unorganized pattern, and this does not involve differentiation into polar hyperflagellated cells (Rashid and Kornberg, 2000; Inoue *et al.*, 2008). Flagellum-mediated swimming motility is not required for *P. aeruginosa* biofilm structure development (Wu *et al.*, 2011) however, this type of motility increases initial attachment to surfaces during initial biofilm development (O'Toole and Kolter, 2002).

Swarming is a cell density dependent way of movement and is considered as a model of bacterial social behavior. This motility is assessed on more solidified media (0.5% agar). This multicellular migration, generally dependent on flagella, takes place when the fluid layer on a surface is relatively
thin and involves a complex process of cell differentiation (elongated and hyperflagellated) leading to co-ordinated movement (Soto et al., 2002). In addition to flagella, swarmer cells require an increased production of certain extracellular components known as wetting agents that reduce surface frictions and enable the smooth migration of a group of cells on viscous surfaces (Rashid and Kornberg, 2000; Inoue et al., 2008).

Type IV pili extend and retract from the poles of the cell to mediate a form of surface translocation termed twitching motility, which is critical for host infection. The cells move in short, intermittent jerk of up to several micrometers and movement only appears when cells are within several micrometers of each other. The morphological pattern of twitching is less organized than in swarming motility (Rashid and Kornberg, 2000).

According to Shrout et al., (2006), swarming motility promotes cell movement on the surface and results in the formation of a flat, uniform biofilm. Limitations in swarming motility resulted in biofilms containing cell aggregates, whereas twitching motility has been shown to be important in later steps in the formation of a structured biofilm. Klausen et al., (2003) demonstrated that the bacterial migration which forms biofilm cap is mediated by type-IV pili. Cap formation is a process during biofilm maturation, by which a motile subpopulation in older biofilms moves across the surface and accumulates on an immobile subpopulation of cells. This immobile subpopulation is an aggregate of cells and has been termed “the stalk”.

The capability of ethanol extract from N. nouchali to inhibit swarming and twitching motility more severe than swimming motility of P. aeruginosa suggest that these extracts probably contain compound(s) that are capable to reduce the amount of quorum sensing signal in P. aeruginosa PAO1. Subsequently they also showed a reduction in violacein production by C. violaceum CV026 impregnated with HHL (Figure 3).

It is also still not clear why essential oils from M. aromatica at a low concentration (0.06% v/v) show a larger inhibition on swarming than swimming motility of P. aeruginosa. A possible explanation can be that essential oils from M. aromatica contain compounds capable to reduce the amount of quorum sensing signal in P. aeruginosa, as again demonstrated by the reduction in violacein production, Such low concentrations showed less activity in inhibition of single cell swimming motility, followed by no planktonic growth inhibition at a concentration of 0.06 % (v/v) of M. aromatica oil (chapter 3 this thesis).

The inhibition of quorum sensing signals could block cell-to-cell communication but fail to interfere with surface colonization by swimming motility. The blocking of cell-to-cell communication reduced
the production of several virulence factors and cytotoxic compounds such as elastase, rhamnolipids, cyanide and pycocyanine, which in turn strongly reduced the swarming motility (which also requires rhamnolipids (RLs) and 3-(3-hydroxylalkanoyloxy) alkanoic acids (HAAs) (Reimmann et al., 2002; Tremblay and Déziel, 2010).

The capability of *S. aromaticum* in reducing the swimming motility to a larger extend than reducing the swarming and twitching motility of *P. aeruginosa* PAO1 could be due to the antibacterial compounds present in the essential oil. These antibacterial compounds might disturb the single cell movement as in swimming instead of groups of cells movement in swarming motility.

In order to have a better insight whether the antibacterial and anti-QS effect from essential oils from *M. aromatica* are from the same or distinct chemical compounds, the bioautography test using *C. violaceum* WT strain should be performed. Further work will focus on chemical identification of the anti-quorum sensing compounds using chromatographic methods and mass spectrometry.

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