Antibacterial Activity and Major Constituents of *Polyalthia cinnamomea* Basic Fraction

(Aktiviti Antibakteria dan Juzuk Utama Fraksi Bes *Polyalthia cinnamomea*)

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ABSTRACT

*Polyalthia cinnamomea* Hook.f. & Thomson (Annonaceae) or locally known as ‘Larak Batu Bukit’, ‘Pisang-pisang’, ‘Sugao’ and ‘Sigumpet Hutan’ is a small woody plant found throughout Malaysia and Singapore. In this study, the basic chemical components from the leaves of the plant were fractionated and the antibacterial activity as well as the major constituents of the basic fraction was determined. To the best of our knowledge, this is the first study being carried out on the bioactivity and phytochemistry of *P. cinnamomea*. The basic fraction remarkably inhibited the growth of ten bacteria tested except one. The biggest inhibitory diameter and the lowest minimum inhibitory concentration were 19.0±4.6 mm and 0.125 mg/mL against respective *Bacillus subtilis* and *Bacillus thuringiensis*. Gas chromatography-mass spectrometry (GCMS) analysis of the basic fraction identified four major cyclosiloxanes of octamethylcyclotetrasiloxane (18.1%), decamethylcyclopentasiloxane (16.4%), dodecamethylcyclohexasiloxane (14.1%) and tetradecamethylcycloheptasiloxane (6.0%). The knowledge of the antibacterial potential of *P. cinnamomea* basic fraction and the major constituents present in the fraction can be utilized in the fields of natural products, pharmaceutical, cosmetic and personal care industries.

Keywords: Antibacterial activity; basic fraction; cyclosiloxane; *Polyalthia cinnamomea*

INTRODUCTION

*Polyalthia cinnamomea* Hook.f. & Thomson that belongs to the family Annonaceae is widely found in lowland forest and widespread in Peninsula Malaysia and Singapore (Ridley 1967). Species of the Annonaceae are used all over the tropics in traditional medicine (Chkrabarti & Mukherjee 1968; FRIM 2016; Irausin et al. 2014; Manasa et al. 2004; Supaluk et al. 2009; Sumitra & Rathish 2010; Yamaguchi et al. 1964). The genus *Polyalthia* itself hold the meaning of ‘many to cure’ in Greek (poly-many althea-to cure). In many parts of India, *Polyalthia longifolia* is used in the Indian traditional system of medicine including Ayurveda (Bapuji & Ratnam 2009; Mahajan et al. 2010; Patel 2010; Poornima et al. 2012; Sinhababu & Baherjee 2013; Sugumaran et al. 2010; Sundaresan & Senthil 2013). The stem extract of *P. longifolia* was found to inhibit the growth of gram positive and gram negative bacteria (Faizi et al. 2003) while its methanol extract of leaves and green berries were found to possess promising antibacterial activity (Faizi et al. 2008). *Polyalthia cerasoides* on the other hand, was used as medicinal plant in Thailand. Its roots were made as tonic and febrifuge (Pharmaceutical Sciences 1996). As for Annonaceae plant species, it is well known that the species are active biologically and produced many promising phytocomponents (Faizi et al. 2003; Lertyot et al. 2011; Patoomratna et al. 2000).
Cyclomethicones are group of methyl siloxanes which is a class of liquid silicones that have the characteristics of low viscosity, high volatility and act as cosmetic solvents. They are particularly suited for use with other silicones and as delivery vehicles for a variety of active ingredients (Starch 2016). Cyclomethicone compounds can be found in plant that eventually influenced the potentiality of the plant antibacterial activity. From the previous study, the GC-MS analysis showed that the cyclomethicone components of the olive leaf extract caused the antimicrobial influence against thirteen bacteria whereby the first three major components of the extracts were cyclomethicones (hexamethyldisiloxane 36.98%, octamethylcyclotetrasiloxane 15.18%, decamethylcyclopentasiloxane 14.59%) (Dilek et al. 2012). Besides, the other previous study showed that Toddalia asiatica was used traditionally as medicine to treat various ailments, contained cyclomethicone phytocomponents (Arun & Varsha 2014).

All in all, many studies have been conducted to evaluate the bioactivity of the Polyalthia plant species as they are bioactively potent and consist of various bioactive compounds. Despite of all the studies, there were no study recorded on the specific plant of Polyalthia cinnamomea where its bioactivity and phytocomponents are still remain unknown. Hence, in this study, the leaf extract of the plant was obtained and the antibacterial activity against five gram positive bacteria and five gram negative bacteria were investigated and the major phytocomponents of the leaf extract were analyzed.

PREPARATION OF CULTURE MEDIA FOR ANTIBACTERIAL ASSAY

Stock solution of the test Polyalthia cinnamomea extract was prepared from concentration of 10 mg/mL for disc-diffusion method (methanol as solvent) and 30 mg/mL for minimum inhibitory concentration method (dimethyl sulfoxide, DMSO, as solvent). For disc-diffusion method, blank discs were prepared from the cut out of filter paper Whatman No. 2 and sterilized using autoclave machine at 120-124°C for 1-2 h before being loaded with 10 mg/mL concentration of the test extract and allowed to air dry at room temperature before use.

BACTERIAL STRAINS

The five gram-positive bacterial strains of Staphylococcus aureus ATCC 25923, Staphylococcus epidermidis, Bacillus subtilis ATCC 11774, B. thuringiensis ATCC 10792, Methicillin resistant S. aureus (MRSA) and the five gram-negative bacteria strains of Escherichia coli ATCC 10536, Salmonella typhimurium ATCC 51812, Serratia marcescens ATCC 13880, Vibrio cholera and Vibrio fluvialis were used. They were obtained from the Microbiology Laboratory culture collection, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

EXTRACTION OF LEAVES EXTRACT

The plant leaves were detached and air dried. After the drying, the leaves were grounded into powder by using a mechanical grinder. In an empty, used and cleaned chloroform glass bottle, 300 g of the grounded Polyalthia cinnamomea leaves were soaked in 1.8 L methanol for three days with regular shaking. After that, the methanol extract was filtered using cotton wool and the filtrate was evaporated under vacuum using Buchi rotary evaporator at the temperature of less than 40°C and with 110 rpm rotation until 1/10 of its original volume (Neli et al. 2011). The concentrated extract was used for the extraction of organic base components, most probably of cyclomethicones, following the method of Hadi and Bremner (2001) with some minor modifications and yielded 0.1 g of leaf extract. It was stored under 4°C prior to use.

PREPARATION OF EXTRACT FOR ANTIBACTERIAL ASSAY

To prepare MHA, 800 mL distilled water was added to a Schott bottle (1 L) containing 19 g of MHA powder. Meanwhile, to prepare MHB, 200 mL distilled water was added to a Schott bottle (500 mL) containing 4.4 g of MHB powder. The two mixtures were then shaken thoroughly to dissolve the powder. Then, 10 mL of the MHB solution was poured into 10 universal bottles (20 mL) and 5 mL of the same solution was poured into 10 universal bottles (10 mL). After that, the MHA and MHB solutions were sterilized for 1-2 h at 120-124°C using an autoclave machine. As for minimum inhibitory concentration

MATERIALS AND METHODS

PLANT MATERIAL COLLECTION AND IDENTIFICATION

The part of Polyalthia cinnamomea studied was the leaf. It was collected from Hutan Simpan Universiti Kebangsaan Malaysia Bangi, Selangor. The plant species was identified by Mr. Sani Miran, a botanist of Universiti Kebangsaan Malaysia. For further reference, the plant voucher specimen of 115D was deposited at the Universiti Kebangsaan Malaysia Herbarium (UKMB).
method. Tween 80 solution was used instead of distilled water to prepare the MHB. The sterilized MHB was kept at 4°C until use and the MHA solution was used to prepare agar plates.

PREPARATION OF AGAR PLATE

The laminar hood was first sterilized with 70% ethanol, and all the empty disposable commercial plastic Petri dishes (9 × 9 cm) were assembled in the laminar hood and sterilized using UV light for 15-30 min. Later, the sterilized MHA was poured into each of the disposable commercial plastic Petri dishes under the laminar hood; the Petri dishes were cooled under UV light for 15-30 min. Finally, the plates were covered and stored in the cool room to prevent contamination until use.

PREPARATION OF BACTERIAL INOCULUMS

The sterilized 10 universal bottles with 5 mL MHB solution were seeded with bacteria from the pure culture stock by a sterilized wire loop and incubated at 37°C for 24 h to obtain the culture. After 24 h incubation, 300 μL of the culture were taken and put into the 10 mL sterilized MHB solution that was prepared earlier. The turbidity of culture was measured and standardized against the McFarland 0.5 (optical density of 0.13 at 625 nm).

DISC-DIFFUSION ASSAY

The disc-diffusion assay according to CLSI (2009) and Scorzoni et al. (2007) was carried out to determine antibacterial activity of the leaf extract by measuring the inhibition diameter. The bacteria suspensions were loaded on a sterile cotton swabs and streaked over the dried surface of MHA plates for inoculation. The sterile filter paper discs with 6 mm in diameter were impregnated with the sample (10 mg/mL) and then placed on the inoculated agar. The plates were incubated at 37°C for 24 h. The antibacterial activity was determined by measuring the diameter of inhibition zone against the bacteria. The inhibition zone was measured in millimeter including the disc diameter. The test was carried out in triplicate. Streptomycin (10 μg), Vancomycin (30 μg), Peniciline (30 μg) and Kanamycin (30 μg) were used as the positive controls. For this assay, the extract was dissolved in methanol. A disc which was impregnated with DMSO was used as the negative control.

MINIMUM INHIBITORY CONCENTRATION (MIC) ASSAY

MIC is to determine the lowest concentration of the assayed antibacterial agent that, under defined test conditions, inhibits the visible growth of the bacterium (CLSI 2011). MIC was evaluated on bacteria strain that showed sensitivity to the extracts in the disc-diffusion assay according to CLSI (2011) and Irith et al. (2008). MIC was carried out using broth dilution assay following two-fold serial micro-dilution using 96-well microtiter plates. 100 μL of DMSO solution of the test extract was distributed into the first well and was serially two-fold diluted to obtain the extract concentration ranging from 16 to 0.06 mg/mL. Using a micropipette, 50 μL of the bacteria inoculums were then dispensed into each of the serially diluted extract. Each plate was only used for one bacterium. All tests were carried out in triplicate. For positive controls, Chloramphenicol (128 to 0.25 mg/mL) and Vancomycin (32 to 0.06 mg/mL) were used. The preparation of these antibiotics stock solutions and ranges determination for MIC were according to Andrews (2006). Meanwhile, the last three wells of the line were used as negative control by filling the mixture of 50 μL bacteria inoculums and 50 μL DMSO and as for the growth control, the well was filled with 100 μL of MHB and 50 μL sample. The covered plates were incubated at 37°C for 16-24 h in an incubator.

DETERMINING BACTERIAL GROWTH USING MTT ASSAY

MTT (3-[4,5-dimethylylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a tetrazolium dye which can be reduced by the live bacteria into an insoluble purple formazan. Thus, the reagent therefore can be used to differentiate between the live and the dead bacteria (Stevens & Olsen 1993). MTT was prepared by dissolving in a phosphate-buffered saline (PBS) solution at 0.2 mg/mL. In this study, the MTT assay was incorporated into the MIC method to visually differentiate between the live and the dead bacteria. After 16-24 h incubation in the MIC method, 50 μL of MTT reagent was added into each well and the colour development was observed and recorded after 2 h incubation (Figures 1-9).

GCMS ANALYSIS OF MAJOR CONSTITUENTS

The leaf extract was subjected to Gas Chromatography and Mass Spectroscopy for identification of bioactive volatile major constituents. GC-MS analysis was performed on an Agilent 7890A gas chromatograph (GC) directly coupled to mass-spectrometer (MS) system of an Agilent 5975C inert MSD with triple-axis detector. The column used was polar
gas and the temperature programming was set with initial oven temperature at 50°C and held for 5 min and the final
NIST showed a leaf extract used in being the most inhibited one with the activity of the leaf extract was more pronounced towards the gram positive bacteria strains compared to the negative bacteria strains which have two. Thus, gram positive bacteria strains did not have an effective barrier to the lipophilic solutes while the gram negative bacteria strains have outer phospholipidic membrane that makes the cell wall impermeable to lipophilic solutes, while the porines constitute a selective barrier to hydrophilic solutes with an exclusion limit of about 600 Da (Nikaido & Vaara 1985; Scherrer & Gerhardt 1971). Hence, the observation of the antibacterial activity in this study showed potent antibacterial activity against gram positive bacteria strains. The inhibition zone data was statistically calculated and analyzed through one way analysis of variance (ANOVA) to obtain the F value and P value. Values of p<0.05 were considered statistically significant and the data are presented in Table 2.

The Polyalthia cinnamomea leaf extract used in this study has never been evaluated for its phytochemical components. The major phytocomponents of the leaf extract of P. cinnamomea has been discovered by using the GC-MS. The GC-MS chromatogram of the leaf extract of P. cinnamomea showed four major peaks representing four major phytocomponents (Figure 10). They are cyclosiloxane mixtures of octamethylcyclotetrasiloxane (18.12%), decamethylcyclopentasiloxane (16.41%), dodecamethylcyclohexasiloxane (14.06%) and tetradecamethylcycloheptasiloxane (6.03%) (Table 3).

To the best of our knowledge, there is no report on the chemical composition nor any bioactivities of the P. cinnamomea plant extract. But, previous studies of other plant extracts showed that the isolated octamethylcyclotetrasiloxane, decamethylcyclopentasiloxane and other cyclomethicones are well known antimicrobial and antifungal compounds (Koç et al. 2007; Yusof et al. 2008; Zhen et al. 2008). Other previous study also showed that the antibacterial properties of olive leaf extract were suspected to be associated with the high content of hexamethylocyclotrisiloxane (Hajji et al. 2010). Besides,
TABLE 2. One way ANOVA for the antibacterial activity of Polyalthia cinnamomea leaf extract

<table>
<thead>
<tr>
<th>Bacteria/gram</th>
<th>Inhibition diameter (mm)</th>
<th>Positive control* (mm)</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methicilin Resistant S.aureus (MRSA)/+</td>
<td>10.3±0.6</td>
<td>17±1.0</td>
<td>8.96</td>
<td>0.0005</td>
</tr>
<tr>
<td>Staphylococcus aureus/+</td>
<td>12.3±0.6</td>
<td>14.3±1.2</td>
<td>7.19</td>
<td>0.055</td>
</tr>
<tr>
<td>Staphylococcus epidermidis/+</td>
<td>N.I</td>
<td>15.7±0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis/+</td>
<td>19.0±4.6</td>
<td>34.0±11.0</td>
<td>4.75</td>
<td>0.0947</td>
</tr>
<tr>
<td>Bacillus thuringiensis/+</td>
<td>6.7±1.1</td>
<td>23.3±1.2</td>
<td>312.10</td>
<td>0.0001</td>
</tr>
<tr>
<td>Salmonella typhimurium/-</td>
<td>7.7±2.9</td>
<td>25.0±0.3</td>
<td>108.12</td>
<td>0.0001</td>
</tr>
<tr>
<td>Serratia marcescens/-</td>
<td>6.7±1.2</td>
<td>27.3±1.5</td>
<td>314.57</td>
<td>0.0001</td>
</tr>
<tr>
<td>Escherichia coli/-</td>
<td>7.0±1.7</td>
<td>15.3±2.9</td>
<td>18.38</td>
<td>0.0128</td>
</tr>
<tr>
<td>Vibrio fluvialis/-</td>
<td>12.6±1.2</td>
<td>29.3±1.2</td>
<td>329.52</td>
<td>0.0001</td>
</tr>
<tr>
<td>Vibrio cholera/-</td>
<td>7.6±0.6</td>
<td>10.7±1.2</td>
<td>16.30</td>
<td>0.0156</td>
</tr>
</tbody>
</table>

*Antibiotic Kanamycin for all bacteria except B. subtilis (Peniciline) and MRSA, B. thuringiensis, S. epidermidis, S. typhimurium (Vancomycin). S. aureus (Streptomycin)

FIGURE 10. GC-MS chromatogram of the leaf extract of Polyalthia cinnamomea

in previous study, the oil derived from *Pteroccephalus canus* that has dodecamethylcyclohexasiloxane showed significant antimicrobial activity against *Staphylococcus saprophyticus* and *Escherichia coli* (Moustafa et al. 2013).

Meanwhile, an aqueous extract from the leaves of *Casimiroa edulis* effectively controls *in-vitro* development of the post-harvest fungi *Alternaria* spp., *Fusarium* spp., *Pestalotiopsis* spp. and *Rhizopus* spp (Abou-elela et al. 2009) and GC-MS analysis showed it contains tetracadmethylcycloheptasiloxane (Vahedi et al. 2011). Furthermore, the phytocompounds of the methanolic extract of *Spermacoce articulans* showed the presence of cyclomethicone mixtures of dodecamethylcyclohexasiloxane (5.93%), tetracadmethylcycloheptasiloxane (4.04%) and hexadecamethylcyclooctasiloxane (2.29%) which indicates to be an antimicrobial agent and the plant was used traditionally to treat various ailments (Bautista et al. 2000).

The result presented in this study may suggest that the leaf extract of *Polyalthia cinnamomea* possesses antibacterial properties and it can be a potential source of antibacterial ingredients for the pharmaceutical industry. Furthermore, a recent research has indicated that many chemical compounds, which present in high amounts in several plants exhibited bioactive properties like antimicrobial, antifungal, anti-inflammatory and anti-oxidant (Barakat 2011; Shalini & Rachana 2009). Thus, it is justified that in this study, the major components of cyclosiloxanes in *P. cinnamomea* extract might be responsible for its potent antibacterial activity.
In conclusion, the leaf extract showed sensitivity towards both gram positive and gram negative bacteria but more pronounced towards the gram positive bacteria strains. The phytochemical analysis of the extract showed the presence of four major components of cyclosiloxane mixtures of octamethylcyclotetrasiloxane (18.12%), decamethylcyclopentasiloxane (16.41%), dodecamethylcyclohexasiloxane (14.06%) and tetradecamethylcycloheptasiloxane (6.03%). Thus, from the findings it suggested that the major components of cyclosiloxanes in *P. cinnamomea* leaf extract might be responsible for its potent antibacterial activity as the data obtained from this study mostly showed a statistically significant value with the control (p<0.05). However further study need to be conducted to confirm the correlation between the major components and its antibacterial activity.

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