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CHEMICAL CONSTITUENTS AND IN-VITRO ANTIMICROBIAL ACTIVITY OF POLYALTHIA STENOPETALA ESSENTIAL OIL

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Graphical abstract

Microorganism	Inhibition zone (mm)	MIC (mg/ml
Escherichia coli	8.33 ± 0.57	1.00
Pseudomonas aeruginosa	8.00 ± 0.00	1.00
Bacillus subtilis	11.00 ± 0.00	0.50
Staphylococcus aereus	10.33 ± 0.57	1.00
Candida albicans	11.00 ± 0.00	1.00
Saccharomyces cerevisiae	9.67 ± 0.57	-

Abstract

Polyalthia stenopetala essential oils were extracted by hydrodistillation from the leaves and were analyzed using gas chromatography-flame ionization detector (GC-FID) and gas chromatography/mass spectrometry (GC/MS) system. Thirty one compounds were identified from the analysis. The most abundant components in the leaves oil are curzerene (37.56%) followed by viridiflorol (11.59%), germacrene B (3.77%) and aromadendrene (4.01%). The antimicrobial activity of the oil essential oils was determined with disk diffusion method and minimum inhibitory concentration (MIC) assay. Four bacteria, Staphylococcus aureus (ATCC 25923), Bacillus subtilis (ATCC 6633), Escherichia coli (ATCC 25922) Psedomonas aeruginosa (ATCC 10145) and two yeasts, Candida albicans (ATCC 10231) and Saccharomyces cerevisiae (ATCC 4098) were selected. The crude oil shows the most reactivity against B. subtilis (ATCC 6633) and C. albicans (ATCC 10231) with an inhibitory zone of 11mm. The minimum inhibitory concentration (MIC) of sample against Staphylococcus aureus (ATCC 25923), Bacillus subtilis (ATCC 6633), Escherichia coli (ATCC 25922) Psedomonas aeruginosa (ATCC 10145) and Candida albicans (ATCC 10231) in range of 0.5 mg/ml – 1.0 mg/ml which can categorized as strong.

Keywords: P. stenopetala, essential oils, disk diffusion method, gas chromatography/mass spectrometry (GC/MS)

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1.0 INTRODUCTION

There are about 128 genera and 2300 species of Annonaceae family [1] In Malaysia, this family is known as Mempisang [2]. It is a family of angiosperm consisting of trees, scrubs or woody lianas. Polyalthia is one of the genus from Annonaceae. Polyalthia species have shown several biological activities, *Polyalthia longifolia* showed antinociceptive [3], *Polyalthia evecta* [4] has anticancer and *Polyalthia suaveolens* [5] observed to have antitrypanosomal. *Polyalthia stenopetala* is a shrub or small slender tree up to 10m tall. It is a lowland

forest species, commonly throughout Peninsular Malaysia except Perlis and Pulau Pinang. *P. stenopetala* is locally called as "jambul cicit". The inner bark is usually brownish with pale streaks or wedges, with ginger-like smell. The flowers grow out of the main trunk, a term known as "cauliflory", salmon pink with narrow linear petals hanging down. The fruit is glossy deep red with one seed, flattened and squarish which will turn black upon maturity. This species can stand full sun with green mature foliage and prominent red young leaf flushes making it suitable for urban site planting [6]. This research is the pioneer in analyzing the essential oil (EOs) of *P. stenopetala*. Moreover, this research is the first one who done the in-vitro antimicrobial activity toward the EOs of *P.stenopetala*. Plant EOs have accompanied the development of human civilization from millennia in different forms and with different uses. The flowers, leaves seeds or other plant parts were extracted and distilled to obtain the plant EOs which is lipid-soluble mixture of volatile compound [7]. Once EOs was exposed to the air at normal temperature, it will substantially evaporate which describe the oils own characteristic odour of a plant [8]. EOs have many therapeutic effects, which include vasodilation, irritation, hypersecretion, hyperperistaltism, the stimulation of heart muscle and they aid the distribution of drug and antiseptic [9]. *P. stenopetala* leaves are used as viand and for curing scorpion stings but no scientific research has been done. Thus, there is a need to analyze the chemical composition and to test its antimicrobial activity.

2.0 MATERIALS AND METHODS

Sample preparation method of EOs extraction and EOs analysis were adapted from Ibrahim (2009)[10]. The method of antimicrobial activity was adopted from Kamazeri (2012) [11] and Jiang (2009) [12]

2.1 Plant Material

Specimen used is *P. stenopetala* from Annonaceae family was collected at Botanic Garden in Forest Research Institute Malaysia (FRIM), Kepong. Fresh specimen was used for essential oil extraction.

2.2 Essential Oils Extraction

The leaves were cut into small pieces to increase the surface area in order to enhance the extraction of essential oils. The sample was placed in a container and filled with distilled water up to the surface of the sample. Then proceed to extraction by hydrodistillation process combined with Clevenger-type apparatus for 8 hours.

2.3 GC-FID Analysis

The analysis was performed on a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector (FID) using fused silica capillary column BP-5 (25m X 0.25mm; 0.25µm film thickness). Helium gas (He) was used as carrier gas with flow rate 1mL/min. The injector and detector temperature were set up at 220°C and 280°C respectively. The oven temperature were programmed from 60°C to 230°C at 3°C/min and the final temperature will be hold at 230°C for 1 min. 1.0 µL of each sample was injected throughout this experiment. The peak areas and retention times were measured by electronic integration.

2.4 GC/MS Analysis

Analysis was carried out on the Agilent 7890A/5975C GC-MSD system operating in the electron ionization (EI) mode at 70 eV which equipped with HP-5MS fused silica capillary column (30m X 0.25mm; 0.25 µm film thickness). The column and injector temperature were same as those for GC-FID analysis.

2.5 Identification of Essential Oil Constituent

The essential oil constituents were identified by comparing their mass spectra with reference spectra in computer library and also by comparing their Kovat's retention indices with reference libraries[13] and from the literature [14]. Each essential oil constituents obtained from GC peaks by applying the correction factor. The retention index for the unknown compound was calculated using the Kovat's equation [15].

2.6 Reference strain

A panel of 6 common pathogenic microorganisms were used in the study, which includes two gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923)and *Bacillus subtilis* (ATCC 6633), two gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 10145) and two types of yeast, *Candida albicans* (ATCC 10231) and *Saccharomyce scerevisiae* (ATCC 4098). Bacterial strains were grown in nutrient agar and incubated at 37°C. Yeast were grown in potato dextrose agar (PDA) and incubated at 25°C.

2.7 Antimicrobial Activity

Antimicrobial activity of essential oils extract was obtained using disk diffusion method. The culture of the reference strain of bacteria were diluted according to McFarland scale with a concentration of 10^5 CFU/mL. The Mueller Hinton (MH) plates of bacterial strains were incubated at 37°C and examined for incubation zone after 24 hours. Chloramphenicol and cyclohexamide were used as positive control for bacteria and fungus respectively. Sterile distilled water was used as negative control. A sterile cotton swab was dipped into the inoculums. After a few seconds the cotton swab was lifted up and rotated firmly against the upper inner wall of the universal bottle in order to squeeze out excess fluid. Then it was spread evenly on Mueller Hinton agar (MHA) plate. This

process was repeated twice by turning the plate 60° at each swab. The lid of the petri dish was replaced and held at room temperature for at least 3 minutes to allow surface moisture to vapourize. Within 15 minutes of swabbing, the positive control disks,
negative control disk and EOs disk were placed onto the surface of the MHA and firmly pressed using a pair of sterile forceps. The disks were arranged so that their centres are at least 24 mm apart from each other and 10mm away from the edge of the agar. Within 15 minutes after the antibiotic disks were placed, the plates were inverted and placed in an incubator at 37°C temperature. The plates were examined after 24-hour incubation. A confluent lawn of growth was observed. The diameters of the zones of inhibition for the EOs disks were measured including the diameter of the discs.

2.8 Minimum Inhibitory Concentration (MIC) Assay

1 mg/ml of essential oil was prepared for this method. About 30 ml of sterile Mueller Hinton broth was poured into a sterile petri dish. A multichannel micropipette was used to dispense 50 µl of the broth into each well of the sterile 96-well microtiter plate. 50 µl of *P.stenopetala* EOs sample was added in the first well. The samples were serially diluted in the first column by mixing and transferring 50 µl to the next column of wells. The process was repeated until eight well. In the ninth well, 50 µl of positive control (chloramphenicol for bacteria and cyclohexamide for fungus) were placed and 50 µl of negative control (sterial distilled water) in the tenth well. In the eleventh well, 50 µl of microorganism was added in order to ensure their growth. Lastly, in the twelfth well, the broth was left empty as an indicator whether the broth is contaminated or not. Six 96-well microtiter plates have been used in this study. Each plate was used for one type of microorganism. The plates then were incubated at 37°C for 24 hours. After 24 hours, the 96-well microtiter plates were examined for the highest concentration with no growth. The MIC will be recorded as the mean concentration of duplicates.

3.0 RESULTS AND DISCUSSION

Hydrodistillation of the leaves of *P. stenopetala* oil gave a yield of 1.01%. The calculations of the yields of oils were based on dry weight basis. The extracted essential oils gave yellowish colour and smell like spices. The samples were washed to reduce the interference, chopped in to small pieces to increase the surface area which can increase the yield of the oils.

GC-FID and GC/MS were used with BF-5 and HP-5MS columns to identify and compare the compounds. As the column used for both instruments are different, the retention time of the compound to be eluted different slightly. A fused silica capillary column BP-5 retained the compounds slightly longer than HP-5MS column. National Institute of Standards and Technology (NIST) standard references database were used in GC/MS.

3.1 Identification of Chemical Constituents

Table 1 shows the retention times of a series of n-alkane saturated hydrocarbons that were used as standards in the calculation of Kovats Retention Index (KI) for every component identified.

Table 1 Retention time of Standard Saturated Hydrocarbon

Number of Carbon	Retention time (min)
C6	1.690
C7	2.027
C8	3.051
C9	5.373
C10	10.794
C11	17.758
C12	23.834
C13	29.328
C14	34.348
C15	38.832
C16	43.079
C17	47.043
C18	50.561
C19	54.095
C20	57.429
C21	60.744
C22	63.894
C23	66.848

Curzerene ($C_{15}H_{20}O$) was the most abundant compound found in leaves of *P. stenopetala* with 73.56% and it was confirmed by gas chromatography-mass spectra. This is followed by viridiflorol ($C_{15}H_{26}O$) 11.59%, aromadendrene ($C_{15}H_{24}$) 4.01%, germacrene B ($C_{15}H_{24}$) 3.77% and β-elemene ($C_{15}H_{24}$) 3.63%. The molecular formulas of aromadendrene, germacrene B and β-elemene show that those three compounds are isomers. Table 2 shows the compounds obtained from the essential oils of *P. stenopetala* by using GC-FID and GC/MS.

Table 2 Most abundance chemical constituents of P. stenopetala Eos

Compound	RI	Composition	Identification
α-Pinene	1043	0.06	RI,MS
β-Elemene	1613	1.72	RI,MS
β-Caryophyllene	1638	0.27	RI,MS
γ-Elemene	1640	0.10	RI,MS
Aromadendrene	1663	1.99	RI,MS
α-Humulene	1673	0.38	RI,MS
α-Gurjunene	1690	0.12	RI,MS
Curzerene	1752	35.40	RI,MS
Germacrene B	1810	1.72	RI,MS
Spathulenol	1826	0.46	RI,MS
Viridiflorol	1845	5.53	RI,MS
Atractylone	1943	0.34	RI,MS

Antimicrobial Activities

The essential oil was evaluated for its inhibitory activity against four bacteria which are *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Pseudomonas* aeruginosa (ATCC 10145) and two types of yeast, namely Candida albicans (ATCC 10231) and Saccharomyces cerevisiae (ATCC 4098) The inhibitory activities of the crude oils (100 µl on each disk) were conducted using disk diffusion method. P. stenopetala oil shows the most inhibitory activies toward B. subtilis and C. albican with 11 mm of inhibitory zone. Generally, chloramphenicol (positive control for bacteria) with a concentration of 250 µg/ml effectively inhibited the four bacteria as compared to cyclohexamide (positive control for yeast) with the same concentration against the two yeast. Table 3 presents the inhibitory activities of the sample against each microorganism.

Table 3 Inhibition zone and MIC of *P. stenopetala* Eos

Microorganism	Inhibition zone (mm)	MIC (mg/ml)
Escherichia coli	8.33 ± 0.57	1.00
Pseudomonas aeruginosa	8.00 ± 0.00	1.00
Bacillus subtilis	11.00 ± 0.00	0.50
Staphylococcus aereus	10.33 ± 0.57	1.00
Candida albicans	11.00 ± 0.00	1.00
Saccharomyces cerevisiae	9.67 ± 0.57	-

Turbidity was taken as indication for the growth of microorganism; thus the lowest concentration which remained clear after macroscopic evaluation was taken as the minimum inhibitory concentration (MIC), The MIC was recorded as the mean concentration of duplicate, The activities were categorized as weak (MIC more or equal than 5 mg/ml), moderate (1.0 mg/ml < MIC < 4.9 mg/ml) and strong (MIC less or equal to1.0 mg/ml). In this method 1 mg/ml of the crude oil was serially diluted. The oils indicate a clear growth of *E. coli*, *P. aeruginosa*, *S. aereus* and *C. albicans* at 1 mg/ml. The oils show no turbidity or clear growth of *B. subtilis* at 0.5 mg/ml and lastly the oils may need more than 1 mg/ml in order to inhibit the growth of *S. cerevisiae* as the first well with 1 mg/ml concentration of the EOs turn cloudy.

4.0 CONCLUSION

Curzerene, viridiflorol, aromadendrene, germacrene B and β-elemene are the major compounds of *P. stenopetala* oil. In average, all essential oils show antimicrobial activities against selected microorganism. *P. stenopetala* showed the most reactivity toward *B. subtilis* and *C. albicans*. The minimum inhibitory concentration of all samples against microorganism (except *S. cerevisiae*) is in a range of 0.5 mg/mL - 1.0 mg/mL which categorized as strong. Isolation of interest compound is suggested for further studies.

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