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ORIGINAL RESEARCH

Essential Oil Compositions and Antimicrobial Activity of *Piper arborescens* Roxb.

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ABSTRACT

The essential oils obtained from the leaves and stems of *Piper arborescens* by hydrodistillation were analyzed by GC and GC–MS. Fifty components have been identified from the leaves and stems oils, comprise of thirty six (97.5%) and forty six (90.5%) components, respectively. The most abundant components in the leaves oil were β-phellandrene (24.3%), sabinene (16.3%), α-pinene (10.4%) and terpinen-4-ol (7.2%), while β-phellandrene (20.4%), methyl eugenol (11.0%) and β-caryophyllene (9.0%) were the main components in the stems oil. The essential oils were tested for antimicrobial activity by using disc diffusion and micro dilution methods for the minimum inhibitory concentration (MIC). The results showed that the leaves oil exhibited significant antimicrobial activity towards *Staphylococcus aureus* with MIC value 250 µg/mL, while stems oil showed the activity against Gram positive bacteria, *Pseudomonas aeruginosa* and fungal, *Aspergillus niger* each with MIC value 500 µg/mL.

Keywords: Essential oil, Piperaceae, *Piper arborescens*, antimicrobial activity

INTRODUCTION

The genus *Piper* has a large number of species and has been of worldwide interest due to their wide utilization as aromatic species and their use in the traditional medicine. Analysis of volatile constituents from Piperaceae species has revealed the presence of monoterpenes, sesquiterpenes and arylpropanoids that have shown interesting biological properties including cytotoxic, fungistatic, insecticide, molluscicidal, antioxidant and antimicrobial activities (1-5). As part of an exhaustive research of the composition of the essential oils of the aromatic and medicinal plants from Malaysia, we report herein the results of the investigation from *Piper arborescens* Roxb. *P. arborescens* is a climber and rarely shrub, distributed in Peninsular Malaysia, Philippines and Taiwan. It is also used in traditional medicine for treating rheumatism (6). Phytochemical studies on this plant have led to the isolation of pyridone alkaloids, cyclobutanoid alkaloids and lignans (7-9). The methanolic stem extract of *P. arborescens* showed significant antiplatelet aggregation activity and cytotoxicity activities (9), while chloroform stems extract was found to display significant activity against a KB cell culture system and a P-388 lymphocytic leukemia system in cell culture (10).

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A literature survey revealed no report on the antimicrobial activity of the essential oil of this plant, so this paper reports the essential oil components of the leaves and stems of *P. arborescens* together with their antimicrobial activity for the first time.

MATERIAL AND METHODS

Plant materials

Samples of *P. arborescens* were collected from Sarawak, Malaysia, in July 2010. This species was identified by Mrs. Mohizar Mohamad from the Forest Research Centre, Kuching, Sarawak and the voucher specimen (UiTMKS3001) was deposited at Natural Products Research & Development Centre (NPRDC), UiTM Sarawak.

Extraction of essential oils

The fresh leaves and stems were subjected to hydrodistillation in an all glass Dean-stark apparatus for 5 hours. The oils were dried over anhydrous magnesium sulfate and stored at 4-6°C. The oil yields (w/w) were 0.24% and 0.16% for leaves and stems, respectively based on the fresh weight.

Gas chromatography (GC)

GC analyses were performed on a Hewlett Packard 6890 series II A gas chromatograph equipped with an *Ultra-1* column (100% polymethylsiloxanes) (25 m long, 0.33 µm thickness and 0.20 mm inner diameter). Helium was used as a carrier gas at flow rate of 0.7 mL/min. Injector and detector temperature were set at 250°C and 280°C, respectively. Oven temperature was kept at 50°C, then gradually raised to 280°C at 5°C/min and finally kept isothermally for 15 min. Diluted samples (1/100 in diethyl ether, v/v) of 1.0 µL were injected manually (split ratio 50:1). The injection was repeated three times and the peak area percentages were reported as means ±SD of triplicates. Calculation of peak area percentage was carried out by using the GC HP Chemstation Software (Agilent Technologies).

Gas chromatography-mass spectrometry (GC-MS)

GC-MS chromatograms were recorded using a Hewlett Packard Model 5890A gas chromatograph and a Hewlett Packard Model 5989A mass spectrometer. The GC was equipped with *Ultra-1* column (25 m long, 0.33 µm thickness and 0.20 mm inner diameter). Helium was used as a carrier gas at a flow rate of 1 mL/min. Injector temperature was 250°C. Oven temperature was programmed from 50°C (5 min hold) at 10°C/min to 250°C and finally kept isothermally for 15 min. For GC-MS detection, an electron ionization system, with ionization energy of 70 eV was used. A scan rate of 0.5 s (cycle time: 0.2 s) was applied, covering a mass range from 50-400 amu.

Identification of components

The chemical components of the essential oils were identified by comparing their MS to the reference spectra in the computer library (Wiley) and also by comparing their retention indices and Kovats index in the literature data (11). The quantitative data were obtained electronically from FID area percentage without the use of correction factor.

Antimicrobial activity

The tested microorganism, *Staphylococcus aureus (ATCC29737), Bacillus subtilis (ATCC6633), Pseudomonas aeruginosa (ATCC9027), Pseudomonas putida (ATCC49128), Escherichia coli (ATCC10536), Candida albicans (ATCC10231) and Aspergillus niger (ATCC16888)* were purchased from Mutiara Scientific, Cheras, Kuala Lumpur, Malaysia. The bacteria were cultured overnight at 35°C in Nutrient broth (NB) and fungi were cultured overnight at 30°C in Potato dextrose broth (PDB) which were further adjusted to obtain a turbidity comparable to that of Mc Farland standard tube No. 0.5 for further use.

Antimicrobial activity of the essential oils of *P. arborescens* was determined by agar disc diffusion method (12). The essential oils were dissolved in DMSO (4 mg/mL). Antimicrobial test were carried out by using 400 µL of suspension containing 10^8 CFU/mL of bacteria and 10^6 CFU/mL of fungi, spread on the Nutrient agar (NA) and Potato dextrose agar (PDA) media, respectively. The disc (6 mm diameter) impregnated with 10 µL of the essential oils and DMSO (negative control) was placed on the inoculated agar, and was incubated for 24 h at 35°C (bacteria) and 48 h at 30°C (fungi). Streptomycin sulfate (10 µg/mL) and nystatin (100 IU) were used as positive controls for bacteria and fungi, respectively. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. All tests and analysis were carried out in triplicates.

Minimum inhibitory concentration (MIC) was determined by broth micro dilution method using 96-well microplates (12). Each test sample (1 mg) was dissolved in DMSO (1 mL) to get 1000 µg/mL stock solution. A number of wells were reserved in each plate for positive and negative controls. Sterile broth (100 µL) was added to well from row B to H. The stock solutions of samples (100 µL) were added to wells at row A and B. Then, the mixture of samples and sterile broth (100 µL) at row B were transferred to each well in order to obtain a twofold serial dilution of the stock samples (concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 µg/mL). The inoculated bacteria (100 µL) were added to each well. After an incubation period at 37°C for 16-20 h, turbidity was taken as indication of growth, thus the lowest concentration which

remained clear was taken as the MIC value. The MIC was recorded as the mean concentration of triplicates.

Statistical analysis

Data obtained from essential oil analysis and antimicrobial activities was expressed as mean values. The statistical analyses were carried out by employing one way ANOVA ($p>0.05$). A statistical package (SPSS version 11.0) was used for the data analysis.

RESULTS AND DISCUSSION

The chemical composition of the leaves and stems oil of *P. arborescens* is listed in **Table 1**. A total of fifty components were identified from both oils. Thirty six components, contributing 97.5% of the leaves oil had been identified. Monoterpene hydrocarbon (71.0%) was the most dominant group in the leaves oil with β-phellandrene (24.3%), sabinene (16.3%) and α-pinene (10.4%) being the major components. Other significant components which gave more than 3% were terpinen-4-ol (7.2%), limonene (5.4%), γ-terpinene (3.6%), α-terpinene (3.4%), β-caryophyllene (3.4%) and bicyclogermacrene (3.3%). The stems oil yielded forty six components constituting 90.5% of the total oil. In contrast to the leaves oil, the stems oil was rich in sesquiterpene hydrocarbon (38.8%). They comprised of β-phellandrene (20.4%), β-caryophyllene (9.0%), bicyclogermacrene (4.5%), α-amorphene (3.1%), β-selinene (3.0%), α-gurjunene (2.9%), sabinene (2.9%) and α-humulene (2.9%). Monoterpene hydrocarbon only contributed 31.9% compared to 71.0% of the components in the leaves oil. Most of the compounds found in the stems oil are also present in the leaves oil. The major differences found is the percentage of methyl eugenol which was found at 11.0% in the stems but 0.3% in the stems oil. α-Pinene existed in 10.4% in the leaves but 2.1% in the stems oil. Sabinene dominated 16.3% in the leaves and detected only 2.9% in the stems while terpinen-4-ol constituted 7.2% in the leaves oil and present in 1.4% in the stems oil. In addition, α-thujene, α-terpinene, (*E*)-β-ocimene and α-guaiene were absent in the stems oil.

β-Phellandrene the major component for both oils, is known to be used in fragrances due to the pleasing aroma (13). Previous study on the essential oil from leaves of *P. arborescens* detected α-eudesmol, α-caryophyllene, globulol, and (*Z*)-nerolidol as the major components (14). In comparison, all these components were not detected in this study was probably due to the different environmental and genetic factors, chemotypes and nutritional status of the plants, which may influence the essential oil composition (15).

Table 1. Constituents identified in the essential oils of *P. arborescens*

Components	KI[a]	KI[b]	Percentage (%) Leaf	Stem
α-Thujene	924	925	3.4 ± 0.1	-
α-Pinene	932	932	10.4 ± 0.1	2.1 ± 0.1
Camphene	946	946	0.5 ± 0.2	0.4 ± 0.1
Sabinene	970	969	16.3 ± 0.2	2.9 ± 0.1
Myrcene	988	985	2.11 ± 0.1	0.9 ± 0.1
β-Phellandrene	1002	1005	24.3 ± 0.1	20.4 ± 0.1
δ-3-Carene	1008	1008	-	0.2 ± 0.2
α-Terpinene	1014	1014	3.4 ± 0.1	-
Limonene	1024	1024	5.4 ± 0.2	2.5 ± 0.1
(*Z*)-β-Ocimene	1032	1030	0.3 ± 0.2	0.4 ± 0.2
(*E*)-β-Ocimene	1044	1045	0.4 ± 0.2	-
γ-Terpinene	1055	1054	3.6 ± 0.3	1.6 ± 0.1
α-Terpinolene	1086	1086	0.9 ± 0.3	0.5 ± 0.2
Linalool	1092	1092	1.5 ± 0.1	1.5 ± 0.3
Camphor	1142	1142	0.1 ± 0.3	0.2 ± 0.2
Terpinen-4-ol	1175	1175	7.2 ± 0.3	1.4 ± 0.3
α-Terpineol	1186	1186	0.4 ± 0.1	0.1 ± 0.1
α-Cubebene	1346	1345	0.6 ± 0.3	0.7 ± 0.1
α-Copaene	1374	1374	0.3 ± 0.2	2.3 ± 0.1
β-Bourbonene	1387	1386	-	0.1 ± 0.1
β-Cubebene	1388	1386	-	0.2 ± 0.2
β-Elemene	1390	1389	-	1.8 ± 0.1
Methyl eugenol	1404	1405	0.3 ± 0.3	11.0 ± 0.1
α-Gurjunene	1406	1405	1.0 ± 0.2	2.9 ± 0.2
α-Cedrene	1410	1410	0.4 ± 0.3	0.1 ± 0.3
β-Caryophyllene	1418	1417	3.4 ± 0.3	9.0 ± 0.2
α-Guaiene	1437	1435	0.3 ± 0.2	-
Aromadendrene	1440	1440	0.2 ± 0.1	0.4 ± 0.3
α-Humulene	1452	1452	-	2.9 ± 0.1
Dehydroaromadendrene	1460	1460	-	0.6 ± 0.1
α-Amorphene	1483	1482	1.2 ± 0.1	3.1 ± 0.3
Germacrene D	1485	1485	0.1 ± 0.2	0.3 ± 0.2
β-Selinene	1488	1487	1.1 ± 0.2	3.0 ± 0.1
Cadina-1,4-diene	1495	1495	-	0.3 ± 0.3
α-Selinene	1498	1498	-	1.9 ± 0.3
α-Muurolene	1502	1502	0.3 ± 0.1	0.2 ± 0.1
Bicyclogermacrene	1503	1502	3.3 ± 0.2	4.5 ± 0.2
(*E*,*E*)-α-Farnesene	1505	1505	0.6 ± 0.2	0.4 ± 0.1
δ-Cadinene	1520	1520	0.6 ± 0.1	1.5 ± 0.1
β-Sesquiphellandrene	1522	1520	2.2 ± 0.2	2.1 ± 0.1
cis-Calamenene	1528	1528	-	0.1 ± 0.1
Germacrene B	1560	1560	0.4 ± 0.3	0.4 ± 0.1
Spathulenol	1578	1578	0.1 ± 0.1	0.1 ± 0.1
Caryophyllene oxide	1582	1585	0.1 ± 0.2	0.4 ± 0.2
Globulol	1592	1590	-	1.1 ± 0.1
Viridiflorol	1595	1595	-	0.9 ± 0.1
Ledol	1602	1600	0.8 ± 0.2	0.6 ± 0.2
t-Muurolol	1645	1645	-	0.7 ± 0.3
α-Cadinol	1650	1652	-	1.4 ± 0.2
α-Bisabolol	1685	1685	-	0.4 ± 0.1
Group components				
Phenylpropanoids			0..3	11.0
Monoterpene hydrocarbons			71.0	31.9
Oxygenated monoterpenes			9.2	3.2
Sesquiterpene hydrocarbons			16.0	38.8
Oxygenated sesquiterpenes			1.0	5.6
Identified components (%)			97.5	90.5

[a]Kovat indices (KI) experimental; [b]KI literature (11)

Antimicrobial activity of *P. arborescens* was determined by using disc diffusion and MIC methods against bacteria, yeast and fungi as shown in **Table 2**. The inhibition zones of disc and MIC values were in the range 7.0-12.2 mm and 250-1000 µg/mL, which signified that both oils possessed moderate antibacterial activity against the tested microorganisms. The leaves oil showed the activity against Gram-positive bacteria, *B. subtilis* and *S. aureus* with MIC values 250 µg/mL each, while the stems oil was only active towards *P. putida* with MIC values 500 µg/mL. The essential oils also exhibited moderate activity against *A. niger* with MIC value 500 µg/mL. The leaves and stems oils of *P. arborescens* appeared to be more active against the bacteria. Gram-positive bacteria seemed to be more sensitive to the observe essential oils than Gram-negative bacteria due to the differences in the cell membrane of these bacterial groups (16). The antimicrobial properties of the essential oils are assumed to be associated with their oxygenated compounds (17). However, small amounts of the oxygenated components in both oils resulted in moderate antimicrobial activity.

Table 2. Antimicrobial activity of the essential oils of *P. arborescens*

Test microbes		Leaf oil	Stem oil	SS	NYS
Bacillus subtilis	DD[a]	8.2 ± 0.2	7.4 ± 0.3	17.2 ± 0.4	-
	MIC[b]	500	1000	7.8	-
Staphylococcus aureus	DD	11.0 ± 0.3	7.5 ± 0.2	17.8 ± 0.5	-
	MIC	250	1000	7.8	-
Pseudomonas aeruginosa	DD	NI	NI	17.2 ± 0.2	-
	MIC	> 1000	> 1000	7.8	-
Pseudomonas putida	DD	7.2 ± 0.2	12.2 ± 0.4	17.3 ± 0.5	-
	MIC	>1000	500	7.8	-
Escherichia coli	DD	NI	NI	17.5 ± 0.4	-
	MIC	> 1000	> 1000	7.8	-
Candida albicans	DD	7.2 ± 0.3	7.0 ± 0.2	-	15.2 ± 0.2
	MIC	>1000	>1000	-	7.8
Aspergillus niger	DD	7.8 ± 0.2	8.2 ± 0.4	-	15.3 ± 0.2
	MIC	500	500	-	7.8

Data represent mean±standard deviation of three independent experiments; [a]Zone of inhibition including the diameter of disc (6 mm); [b]Mininum inhibitory concentration (µg/mL); SS - streptomycin sulphate; NYS – nystatin; NI - no inhibition

***Piper arborescens* Roxb.'in Uçucu Yağ Bileşenlerinin Antimikrobiyel Etkileri**

ÖZ

Piper arboresanın'un yaprak ve saplarından hidrodistilasyonla elde edilen uçucu yağlar GC ve GC-MS ile analiz edildi. Yaprak ve sap uçucu yağlarında sırasıyla otuzaltı bileşik (% 97.5) ve kırkaltı bileşik (% 90.5) olmak üzere elli bileşik tanımlanmıştır.. Yaprak uçucu yağı içinde en bol bulunan bileşenler β-fellandren (% 24.3), sabinen (% 16.3), α-pinen (% 10.4) ve terpinen-4-ol (7.2%) iken sap uçucu yağında ise β-fellandren (% 20.4), metil öjenol (% 11.0) ve β-karyofillen (% 9.0) bulunmuştur. Uçucu yağlar, minimum inhibe edici konsantrasyon (MIC) belirlemek için disk difüzyon ve mikro seyreltme yöntemleri kullanılarak, antimikrobiyal aktivite bakımından test edilmiştir. Yaprak yağı *Staphylococcus aureus*'a karşı 250 ug/mL MIC değeri ile kayda değer aktivite gösterirken, sap yağı ise *Pseudomonas aeruginosa* Gram pozitif bakterilere ve *Aspergillus niger* mantara karşı, her biri için 500 ug/mL MIC değerinde aktivite göstermiştir.

Anahtar kelimeler: Uçucu yağ, Karabibergiller, *Piper arborescens*, antimikrobiyal aktivite

CONCLUSION

The result demonstrated that β-phellandrene, sabinene, α-pinene, terpinen-4-ol, methyl eugenol and β-caryophyllene were the most abundant components in *P. arborescens* oils. In the case of *P. arborescens* oils, although there is no striking on their antimicrobial activity in the leaf and stem oils, it is still worthwhile to investigate the other parts of the plant as a natural source for essential oil composition or phytochemical studies. In conclusion, the essential oils and their components generally displayed significant bioactivity properties, which are useful in daily life in foods and as preventive agents from various diseases. However, further investigation should be carried out on their activities against other foodborne pathogens.

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