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Essential Oil Constituents of *Alpinia scabra* and *Alpinia murdochii*, Two Wild Highland Species from Peninsular Malaysia and Their Anti-Microbial Activity

(Komposisi Minyak Pati *Alpinia scabra* dan *Alpinia murdochii*, Dua Spesies Liar dari Semenanjung Malaysia dan Aktiviti Anti-Mikrobnya)

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ABSTRACT

This study investigates the chemical constituents of essential oils and their anti-microbial activity of Alpinia scabra *and* Alpinia murdochii*, two wild Zingiberaceae species. The essential oils were obtained by hydrodistillation and the chemical components of the oils were determined by GC-FID (retention indices, RI) and GC/MS analysis. The major components of* Alpinia scabra *rhizome oil are γ-selinene (33.45%), α-selinene (3.64%) and α-terpineol (3.55 %) while the major components of the leaves are β-pinene (63.37%), α-pinene (6.58 %) and borneol (3.20 %). The major compounds of* Alpinia murdochii *rhizome oil are γ-selinene (15.51 %), (E,E)-farnesyl acetate (6.56 %), terpinen 4-ol (5.58 %) and α-terpineol (5.04 %). The monoterpenes; β-pinene (23.83 %), sabinene (23.76 %) and terpinene-4-ol (10.49 %) were the major components in the leaf oil of* A. murdochii*. The lowest MIC values were recorded for the rhizome essential oils of both* Alpinia *species against all* Staphylococcus aureus *strains (coded as MSSA, MRSA, Sa7, VISA24, VRSA156) with MIC values ranging from 0.04 mg/mL to 2.50 mg/mL. The rhizome oils of both species also showed a broad spectrum of anti-microbial activity as compared to the leaf oils.*

Keywords: Anti-microbial agent; hydrodistillation; lengkuas*; γ-selinene*

ABSTRAK

Penyelidikan ini mengkaji kandungan kimia minyak pati dan aktiviti anti-mikrob Alpinia sabra *dan* Alpinia murdochii*, dua spesies liar daripada keluarga Zingiberaceae. Minyak pati yang diperoleh melalui penyulingan hidro dan komponen kimia kedua-dua minyak pati tersebut ditentukan menggunakan analisis kromatografi gas (GC-FID) (indeks pengekalan, RI) dan kromatografi gas/spektrometri jisim. Komponen utama dalam minyak pati rizom* A. scabra *adalah γ-selinen (33.45%), α-selinene (3.64%) dan α-terpineol (3.55%) sementara komponen dalam minyak pati daun* A. scabra *adalah β-pinene (63.37%), α-pinene (6.58%) dan borneol (3.20%). Komponen kimia di dalam minyak pati rizom* A. murdochii *pula adalah γ-selinen (15.51%), (E,E)-farnesyl acetate (6.56%), terpinen 4-ol (5.58%) dan α-terpineol (5.04%). Monoterpene, β-pinene (23.83%), sabinene (23.76%) dan terpinene-4-ol (10.49%) merupakan kandungan utama dalam minyak pati daun* A. murdochii*. Bacaan nilai MIC terendah dicatatkan oleh minyak pati rizom kedua-dua spesies* Alpinia *terhadap semua strain* Staphylococcus aureus *(dikodkan sebagai MSSA, MRSA, Sa7, VISA24, VRSA156) dengan nilai MIC antara 0.04 mg/mL ke 2.50 mg/mL. Minyak pati rizom kedua-dua spesies juga menunjukkan spektrum anti-mikrob yang luas berbanding minyak pati daun.*

Kata kunci: Agen anti-mikrob; lengkuas; penyulingan hidro; γ-selinen

Introduction

Alpinia is one of the largest genus of the family Zingiberaceae with more than 250 species in tropical regions. The rhizomes of several species are useful in folk medicine and as alternative treatment for certain illnesses. *Alpinia conchigera,* for example, is traditionally used to treat fungal infection (Ibrahim et al. 2000) and can be consumed as a post-partum medicine (Ibrahim et al. 2009). *Alpinia galanga* is used as treatment for stomach ache, diarrhea and it is a carminative and anti-microbial agent (Oonmetta-Aree et al. 2006). This species is also used in the preparation and flavouring of culinary dishes. In Malaysia it is known by the local name *lengkuas* and it is also used as condiment and spice in many countries of Asia. Several other species of *Alpinia* are valued as ornamentals due to their beautiful orchid like flowers such as *A. purpurata, A. zerumbet* and *A. mutica*.

Alpinia scabra and *Alpinia murdochii* are two wild Zingiberaceae species from the highlands of Pahang, Malaysia. According to Burkill, 1935, a hot water fomentation is made with *A. scabra*, or heated leaves are applied to the abdomen to treat vertigo. Our previous study

on this species showed that the hexane and dichloromethane extracts of the leaves and rhizomes showed very good cytotoxic effect against ovarian cancer cell line (SKOV-3) and the dichloromethane extract of the leaves also showed high inhibitory effect against breast carcinoma cell line (MCF-7) (Ibrahim et al. 2010).

To the best knowledge of our knowledge, no studies have been done on the constituents of the essential oils from both species. Therefore, in this study, we shall communicate the chemical constituents of the essential oils of the two wild *Alpinia* species collected from Genting Highland, Pahang, namely *Alpinia scabra* and *Alpinia murdochii* and their anti-microbial activities.

Materials and Methods

plant material

The fresh leaves and rhizomes of *Alpinia scabra* and *Alpinia murdochii* (herbarium no: HI 1419 and HI 1420, respectively) were collected from Genting Highland, Pahang and authenticated by Professor Dr. Halijah Ibrahim. Voucher specimens were deposited in the herbarium of Institute of Biological Sciences, University of Malaya.

extraction of the essential oils

Fresh samples of leaves and rhizomes were immediately washed, cut into small pieces and air dried for 3 days consecutively. Then, the samples were ground into small pieces and hydrodistilled using a Clevenger type apparatus for 8 h. The oil was dried over sodium sulfate anhydrous and stored in a sealed vial at 4°C until further analysis.

gas chromatography - flame ionization detector (gc-fid)

GC analysis was performed using a Shimadzu GC-2010 capillary chromatograph equipped with a flame ionization detector using fused silica capillary column CBP-5 (25 m × 0.22 mm i.d, 0.25 μm film thickness). The samples were dissolved in hexane and injected in split mode (20:1). The operating parameters were; helium as carrier gas at a linear velocity of 50 cc/min, injector and detector temperature at 250ºC. The column was programmed initially at 60ºC for 10 min, then to 230ºC at 3ºC/min and finally held at 230ºC for 10 min.

gas chromatography-mass spectrometry (gc/ms)

GC/MS analysis was performed using a 6890N Network GC System equipped with a 7683 Series auto-injector coupled to a 5975 Inert Mass Selective Detector and the same capillary GC conditions as described above. Carrier gas used was helium. Significant MS operating parameters: ionization voltage, 70 eV; ion source temperature 230°C; mass range 50-600 amu.

identification of constituents

The constituents of the oils were identified by matching their mass spectra with those from MS Libraries NIST 0.5L, and confirmed by comparison of retention indices (RI) with data in the literature (Adam 2001; Tohar et al. 2006a, 2006b). RI was determined with a standard mixture of homologous series of alkane (C_7-C_{25}) under the same chromatographic conditions used when analyzing the oils. The relative percentage of the oil constituents was expressed as percentage by peak area normalization without the use of correction factors.

anti-microbial activity

The essential oils were individually tested against five strains of *Staphylococcus aureus* namely Methicillin sensitive *Staphylococcus aureus* ATCC 29213 (MSSA), Methicillin resistant *S. aureus* ATCC 33591 (MRSA), Vancomycin intermediate resistant *S. aureus* ATCC 700699 (Sa7), MRSA with intermediate resistance to vancomycin (VISA24) and MRSA with complete resistance to vancomycin (VRSA156), two candida; *Candida albicans* (ATCC 10231) and *Candida glabrata* (ATCC 64677), two dermatophytes, *Microsporum canis* (ATCC 36299) and *Trichophyton rubrum* (ATCC 28188). All isolates were purchased from American Type Culture Collection (ATCC) except two mutant isolates namely VISA24 and VRSA156 that were obtained through a step wise lab-passage procedure as described previously (Mohtar et al. 2009).

Media was sterilized by autoclaving at 120°C for 15 min and all subsequent manipulations were carried out in a class 2 biohazard cabinet. The *Staphylococcus aureus* strains (coded as MSSA, MRSA, Sa7, VISA24 and VRSA156) were cultured in Mueller Hinton Broth (MHB), VISA and VRSA in tryptic soy broth (TSB) overnight (24 h) at 37°C while the candida and dermatophytes were cultured in potato dextrose broth (PDB) overnight at 26°C. The resulting inoculum was further adjusted to obtain turbidity comparable to that of McFarland standard tube No. 0.5 (Vandepitte et al. 1991) prior to use.

The minimum inhibitory concentration (MIC) value determination assay was carried out to evaluate the compounds which maybe potential as inhibitory agents against test isolates using double-broth micro-dilution method involving 96-wells microtitre-plates as described previously (Saiful et al. 2008). Briefly, serial two-fold dilutions of the test compounds dissolved in DMSO were prepared prior to addition of 100 µL overnight microbial suspension (10^8 CFU/mL) followed by incubation at 37°C (bacteria) or 26°C (dermatophytes and candida) for 24 h. The highest concentration of DMSO remaining after dilution (5%, v/v) caused no inhibition of bacterial/candida/dermatophytes growth. Antibiotic cycloheximide was used for anti-candida and anti-dermatophyte comparison while oxacillin was used for anti-bacterial testing. DMSO served as negative control.

Turbidity was taken as indication of growth, thus the lowest concentration which remains clear after macroscopic evaluation was taken as the minimum inhibitory concentration (MIC). For further reconfirmation, 20 µL of MTT reagent (1 mg/mL) was added to the suspension in the selected wells, followed by 20 min incubation at 37°C. The reagent-suspension colour will remain clear/yellowish indicating complete inhibition (cidal) activity as opposed to the dark blue colour for growth (Eloff 1998). The MIC was recorded as the most repeatable minimum concentration of triplicates done in one experiment.

Results and Discussion

Composition of the essential oil

The dried leaves and rhizomes of *Alpinia scabra* and *Alpinia murdochii* were subjected to hydrodistillation using Clevenger-type apparatus. The yield of the oils of *Alpinia scabra* (leaves and rhizomes) are 0.16% and 0.02%, respectively, while the yield of *Alpinia murdochii* oils (leaves and rhizomes) are 0.48% and 0.09%, respectively. Table 1 lists the comparison of the oil components from both species with their retention indices and the relative GC peaks areas of these constituents on the CBP-5 capillary column. Forty components of *Alpinia scabra* leaf oil were identified and comprising of 86.61% of the total oil. This oil could be a good source of β-pinene as it made up to 63.37% of the total oils. Additionally, other major compounds were α-pinene (6.58%), borneol (3.20%), caryophyllene oxide (1.69%), *p*-cymen-8-ol (1.20%), *trans*-pinocarveol (1.15%), myrtenyl acetate (1.03%) and limonene (1.0%). Hydrocarbons formed the most abundant group in this oil with 13 compounds accounting for 73.02% of the total leaf oils. The constituents of the *A. scabra* rhizome oils contained more than fifty compounds; however only 41 components were detected comprising of 70.96% of the total oil. GC and GC/MS analyses showed that the major compounds of the oils were *γ*-selinene (33.45%), *α*-selinene (3.64%), α-terpineol (3.55%), *γ*-muurolene (3.45%), alloaromadendrene (3.32%) and spathulenol (3.25%).

The chemical constituents of the leaf of *Alpinia murdochii* was identified with forty compounds amounting to 94.31% of the total oils. The oils were dominated by 17 hydrocarbons representing 72.56%. Twelve compounds of this group are monoterpenes and five compounds belong to sesquiterpenes. The monoterpenes; *β*-pinene and sabinene were the major components (23.83% and 23.76% respectively). Sabinene was known to impart a woody odour (Moon et al. 2006) and β-pinene was reported to emit turpentine like odour (de Pooter et al. 1985). A total of 37 compounds were identified from the *Alpinia murdochii* rhizome oils which make up to 71.06% of area percent. This oil showed a high content of sesquiterpene hydrocarbons (26.85%) and oxygenated monoterpenes (19.56%). Other compounds are oxygenated non-terpenes (9.57%), monoterpene hydrocarbons (8.56%), oxygenated sesquiterpenes (3.05%), non-terpene hydrocarbons (2.70%)

TABLE 1. Essential oil constituents of the leaves and rhizomes of *Alpinia scabra* and *Alpinia murdochii*

Compounds	RI	Composition (%) [a]			
		Alpinia scabra		*Alpinia murdochii*	
		Leaf	Rhizome	Leaf	Rhizome
Furfural	836	0.12	0.41	-	0.82
3-Hexenol	851	0.55	0.08	0.06	-
α-Thujene	929	-	-	3.54	-
α-Pinene	937	6.58	0.19	8.56	0.76
Camphene	949	0.44	-	0.11	-
Sabinene	974	-	-	23.76	-
β-Pinene	979	63.37	0.21	23.83	2.80
Myrcene	983	-	-	0.16	-
α-Phellandrene	1002	-	-	0.06	-
α-Terpinene	1016	-	-	1.91	1.26
δ-2-Carene	1024	0.10	-	-	-
p-Cymene	1026	-	-	3.83	2.66
Limonene	1028	1.00	0.69	-	0.31
β-Phellandrene	1029	-	-	1.51	-
δ-3-Carene	1030	-	-	1.04	-
1,8-Cineol	1054	0.08	-	-	-
γ-Terpinene	1057	0.12	-	4.25	0.77
cis-Sabinene hydrate	1064	-	-	0.49	-
trans-Sabinene hydrate	1078	-	-	1.11	0.28
3,5,5-trimethyl 2-cyclopenten-1- one	1107	0.06	-	-	-
Exo-fenchol	1123	0.13	1.46	-	-
α-Campholenal	1123	-	-	1.08	0.63
Nopinone	1134	0.20	0.13	1.29	-
trans-Pinocarveol	1140	1.15	0.23	0.41	1.99
Benzene acetaldehyde	1134	-	0.66	-	-
Camphor	1144	0.14	-	-	-
Sabina ketone	1155	-	0.99	0.36	1.07
Isoborneol	1147	0.11	-	-	-
Pinocarvone	1156	0.63	1.16	0.08	0.98
Borneol	1165	3.20	0.14	0.54	0.45

Pinocamphone	1171	0.38	-	-	-
Terpinen- 4-ol	1176	0.07	0.29	10.49	5.58
p-Cymen-8-ol	1183	1.20	0.14	1.56	-
α-Terpineol	1201	0.44	3.55	0.46	5.04
Myrtenal	1201	-	2.30	-	1.99
Myrtenol	1209	0.07	0.45	0.82	0.72
cis-Piperitol	1211	-	-	0.31	-
Verbenone	1216	-	0.1	-	0.33
cis-Carveol	1222	-	-	0.12	-
Cumin aldehyde	1237	-	-	0.27	-
2-Methyl-3-phenyl propanal	1237	-	-	-	0.61
Perilla aldehyde	1275	0.23	-	-	-
Bornyl acetate	1283	-	-	0.35	-
Carvacrol	1284	-	-	-	0.56
Perilla alcohol	1304	-	-	0.28	-
trans-Pinocarvyl acetate	1310	0.23	-	-	-
Myrtenyl acetate	1322	1.03	-	-	-
p-Mentha-1,4-dien7-ol	1331	-	-	-	0.40
α-Copaene	1377	0.07	0.1	0.06	1.48
β-Elemene	1377	-	0.35	-	-
(Z)- Caryophyllene	1407	-	-	0.06	1.64
(E)- Caryophyllene	1418	0.19	0.07	0.41	-
α-Lonone	1423	0.14	-	-	-
β-Selinene	1448	-	-	-	0.32
α-Selinene	1472	-	-	-	1.79
α-Selinene	1471	0.17	3.64	-	2.30
Pentadecane	1481	0.09	-	-	-
β-Bisabolene	1507	0.42	0.39	-	-
δ-Cadinene	1507	-	-	0.12	-
α-Panasinsen	1519	-	2.21	-	1.22
β-Sesquiphellandrene	1521	0.07	-	-	-
α- Maaliene	1527	-	0.55	-	2.59
α-Calacorene	1540	-	0.11	-	1.51
Elemol	1547	-	0.48	-	-
trans-*α*- Bisabolene epoxide	1552	-	0.40	-	
trans-Nerolidol	1553	0.07	0.54	0.13	-
Caryophyllene alcohol	1565	-	0.50	-	-
Caryophyllene oxide	1574	1.69	-	0.27	-
Caryophylladienol I	1638	0.24	-	-	-
α-Eudesmol	1643	-	2.17	0.13	-
β- Eudesmol	1650	-	0.46	0.12	-
γ-Gurjunene	1656	0.40	-	-	-
γ-Selinene	1665	-	33.45	0.08	15.51
Alloaromaadendrene	1668	-	3.32	-	-
Heptadecane	1669	-	-	-	2.70
β-Bisabolol	1671	-	-	-	2.40
α-Bisabolol	1677	0.36	-	-	-
(E, Z) - Farnesol	1750	-	0.33	-	0.65
Benzyl benzoate	1760	-	0.64	-	0.77
(E, E)- Farnesyl acetate	1833	0.13	-	-	6.56
Phytol	1915	0.98	-	0.29	0.77
Total	86.65	70.96	94.31	71.06	

RI; Retention indices calculated on CBP-5 column, [a] Percentage of total FID peak area obtained on CBP-5 column

and oxygenated diterpenes (0.77%). The major compounds of this oil are *γ*-selinene (15.51%), (*E,E*)-farnesyl acetate (6.56%), terpinen 4-ol (5.58%) and α-terpineol (5.04%).

Anti-microbial activity

The leaf and rhizome oils from *Alpinia scabra* and *Alpinia murdochii* were tested against five selected strains of *Staphylococcus aureus* (coded as MSSA, MRSA, Sa7, VISA24, VRSA156) and four selected fungi namely *Candida albican, Candida glabrata*, *Microsporum canis* and *Trycophytum rubrum*. The MIC values of the anti-microbial activities are given in Table 2 with oxacillin and cycloheximide as the positive controls.

The lowest MIC values were recorded from the rhizome oils of both *Alpinia* species against all *S. aureus* strains with MIC values ranging from 0.04 to 2.50 mg/mL. The rhizome oils also showed a broad spectrum of anti-microbial activity as compared to the leaf oils. Furthermore, the rhizome oils of *A. scabra* and *A. murdochii* showed an MIC value equivalent to oxacillin (0.63 mg/mL) against *S. aureus* (Sa7). Rhizome oil of *A. murdochii* exhibited a slightly lower MIC value against VISA24 (0.08 mg/mL) and VRSA156 (0.04 mg/mL) which is also lower compared to oxacillin (0.31 mg/mL). Interestingly, VRSA156 showed high inhibition when treated with all essential oils from both species with an MIC value of 0.04 to 0.63 mg/mL. Meanwhile, in this study, the rhizome and the leaf oils showed the same MIC values (2.50 mg/mL) against all fungi tested.

From our previous study on essential oil of *Alpinia pahangensis* which also contain γ-selinene and *α*-terpineol as the major components, showed that the rhizome oil exhibited lower MIC values and the results were comparable to the activity of oxacillin when tested against the same five strains of *Staphylococcus aureus* used in this study (Awang et al. 2011). The major compound, *α*-terpineol, from rhizomes of *A. scabra* and *A. murdochii* in this study has been reported by Li et al. (2014) to exhibit strong anti-bacterial activity against *Salmonella enteritidis* and *Staphylococcus aureus* with MIC values of 1.56 and 3.13 µg/mL, respectively. Another study by Zhang et al. (2018) showed that terpinen-4-ol exhibit anti-bacterial activity

TABLE 2. Minimum inhibitory concentration (MIC) values (mg/mL) of the essential oils of leaf and rhizome of *Alpinia scabra* and *Alpinia murdochii* against five strains of *Staphylococcus aureus*, two candida and two selected dermatophytes fungi

Microorganisms	MIC (mg/mL)				References antibiotic
	Alpinia scabra		*Alpinia murdochii*		
	Leaf oil	Rhizome oil	Leaf oil	Rhizome oil	
Staphylococcus aureus strains					*Oxacillin*
ATCC 29213 (MSSA)	2.50	0.63	2.50	0.63	<0.02
ATCC 33591 (MRSA)	2.50	1.25	2.50	2.50	0.16
ATCC 700699 (Sa7)	2.50	0.63	2.50	0.63	0.63
VISA24	2.50	0.16	1.25	0.08	0.31
VRSA156	0.63	0.08	0.31	0.04	<0.02
Fungal strains					*Cycloheximide*
Candida albicans (ATCC 10231)	2.50	2.50	2.50	2.50	1.25
Candida glabrata (ATCC 64677)	2.50	2.50	2.50	2.50	1.25
Microsporum canis (ATCC 36299)	2.50	2.50	2.50	2.50	2.18
Trycophyton rubrum (ATCC 28188)	2.50	2.50	2.50	2.50	2.18

Anti-microbial activities were categorized as week (MIC ≥ 5 mg/mL), moderate (1.0 < MIC < 4.9 mg/mL), or strong (MIC ≤ 1 mg/mL); The MIC was recorded as the most repeatable minimum concentration of triplicates done in one experiment

with MIC value of 98 µg/mL against *Staphylococcus agalactiae*. Terpinen-4-ol was present in leaf and rhizome oils of *A. scabra* and *A. murdochii* but in higher quantities in the latter. Other than the major compounds, (*E*)-caryophyllene and *δ*-cadinene, although present in low concentration could account for the anti-microbial activity as they have been associated with anti-bacterial properties (Ali et al. 2017). These findings suggest that the anti-microbial activity exhibited by the rhizome oils of the *Alpinia* species might be due to the major compounds having a synergistic effect with other minor components.

Conclusion

In conclusion, the rhizome oils of *Alpinia scabra* and *Alpinia murdochii* showed comparable inhibition potency as oxacillin which may be due to the presence of known anti-bacterial terpenes such as *α*-terpineol, terpinen-4-ol, (*E*)-caryophyllene and *δ*-cadinene. To the best of our knowledge, this is the first report to confirm anti-microbial properties of the rhizome essential oils derived from *Alpinia scabra* and *Alpinia murdochii* and have potential to be exploited as natural anti-bacterial agents.

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