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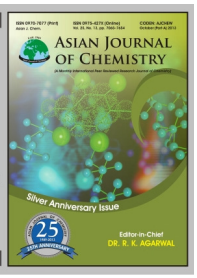

Chemical Composition and Antimicrobial Activities of Essential Oil from *Artemisia integrifolia*

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Hydrodistilled volatile oil obtained from *Artemisia integrifolia*, cultivated in Greater Khingan Mountains, was analyzed by gas chromatography and gas chromatography-mass. Sixty-one compounds were identified, representing 98.86 % of the total oil. The major constituents of essential oil were oxygenated monoterpenes, camphor (12.65 %), artemisia ketone (11.78 %), eucalyptol (6.28 %) and artemisia alcohol (6.5 6%). Antimicrobial potential of oil against different bacterial strains (*Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*), yeast strains (*Hansenula anomala* and *Saccharomy cescerevisiae*) and molds (*Aspergillus niger*, *Chaetomium globosum*, *Mucor racemosus* and *Monascus anka*) was determined by disc diffusion method and broth micro dilution method, respectively. The oil (1000 µg/disc) exhibited promising antibacterial effect as a diameter of zones of inhibition (20.5-26.6 mm). MIC values of oil were ranged from 62.5 to 250 µg/mL.

Key Words: *Artemisia integrifolia*, Essential oil, Chemical composition, Antimicrobial.

INTRODUCTION

Artemisia is a genus of herbs or small shrubs characterized by frequently aromatic, leaves alternate, capitula small, usually racemouse, paniculate or capitate, inflorescence, rarely solitary; involucral bracts in few rows, receptacle flat to hemispherical, without scales and sometimes hirsute; florets all tubular, achenes obovoid, pappus absent or sometimes a small scarious ring[1]. The genus, classified in subtribe Chrysanthemina of Anthemideae, comprises of about 500 species distributed in the temperate zones of Europe, North America, Asia and South Africa, 200 of which are distributed in China[2,3].

This genus includes many important medicinal plants, such as *A. keiskeana*, *A. abrotanum*, *A. herba-alba*, *A. argyi* and *A. annua*, largely used since ancient times for the treatment of a variety of diseases and complaints (malaria, hepatitis, inflammation, diuresis, hypertension, helminthic, allergy, bruising, jaundice, hemostasia, cancer. infections by fungi, bacteria and viruses)[4,5].

Artemisia integrifolia Linn, a perennial herb with a volatile odor, is chiefly distributed in the north of China and has long been collected both as an edible and medicinal plant for inflammation, diuresis, hepatitis and hepatocirrhosis. This study aims to determine the chemical composition of the hydrodistilled essential oil of *A. integrifolia* grown in the Greater Khingan Mountains in Heilongjiang Province, China by gas chromatography-flame ionisation detection (GC-FID) and gas chromatograph-mass spectrometry (GC-MS) and to evaluate its antimicrobial activity against microorganisms.

EXPERIMENTAL

A. integrifolia plants were collected from the Greater Khingan Mountains, Heilongjiang Province, China on June 2010 and identified by A.P. Yin-zhang Zhou. The plants were dried under the shade (at room temperature). The voucher specimen (No. 8717) was deposited in the South China Botanical Garden, Chinese Academy of Sciences.

Isolation of the essential oil: The air-dried plant materials (1000 g) of *A. integrifolia* were chopped and subjected to hydrodistillation for 6 h using a Clevenger type apparatus. The obtained oils were dried over hydrous sodium sulphate for 24 h, filtered and then stored at 4 ºC in brown sealed glass vials until tested.

GC-FID analysis: An Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with an HP-5 5 % phenylmethylsiloxane capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) and equipped with an FID detector was used for the GC-FID analysis. Helium gas at a constant flow rate of 1 mL/min was used as the carrier gas. The injector and mass transfer line temperatures were set at 250 and 280 ºC, respectively. The essential oil solution (1 µL) in hexane was injected and analyzed under the following column conditions: initial column temperature at 40 ºC for 1 min, which was then increased to 250 ºC at a 3 ºC/min heating ramp and then subsequently kept at 250 ºC for 20 min.

GC-MS analysis: Quantitative and qualitative analysis of the essential oil was performed using a GC-MS 6890-5975 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a HP-5 MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). For GC-MS detection, an electron ionisation system with a 70 eV ionization energy was used. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and mass transfer line temperatures were set at 250 and 280 ºC, respectively. Essential oil solution (1 μL) in hexane was injected and then analyzed under the following column conditions: initial column temperature at 40 ºC for 1 min, which was then increased to 250 ºC at a 3 ºC/min heating ramp and then subsequently kept at 250 ºC for 20 min. The Kovats indices were calculated for all volatile components using a homologous series of *n*-alkanes (C_8-C_{25}) on the HP-5 MS column. The major oil components were identified *via* coinjection with standards (whenever possible) and confirmed through the Kovats indices using the Wiley (V.7.0) and National Institute of Standards and Technology (NIST) V.2.0 GC-MS library. The relative concentration of each compound in the essential oil was quantified based on the peak area integrated in the analysis program.

Test microorganisms: The *in vitro* antimicrobial activities of the essential oil were evaluated against a panel that included laboratory control strains obtained from the China Centre for Type Culture Collection (CCTCC). These strains included two gram-negative bacteria (*Pseudomonas aeruginosa* CCTCC AB93066 and *Escherichia coli* CCTCC AB91112), two gram-positive bacteria (*Bacillus subtilis* CCTCC AB92068 and *Staphylococcus aureus* CCTCC AB91053), two yeast strains (*Hansenula anomala* CCTCC AY92046 and *Saccharomy cescerevisiae* CCTCC AY92042) and four molds (*Aspergillus niger* CCTCC AF91004, *Chaetomium globosum* CCTCC AF 200039, *Mucor racemosus* CCTCC AF 93209 and *Monascus anka* CCTCC AF93208). All strains were maintained on an agar slant at 4 ºC. The bacterial strains were cultured in a Muller-Hinton broth at 37 ºC for 24 h, whereas the yeast strains were cultured on a Sabouraud dextrose agar at 28 ºC for 48 h. The fungal strains were cultured on Sabouraud dextrose agar at 28 ºC for 120 h prior to testing.

Inhibitory effect *via* the disc diffusion method: The disc diffusion method was used to determine the antimicrobial activities of the essential oils. Petri plates were prepared by pouring 20 mL of MBH or Sabouraud dextrose agar and allowing the solution to solidify. The plates were then dried and 0.1 mL of the standardized inoculum containing 10^6-10^7 CFU/mL of the bacterial suspension was poured, uniformly spread and allowed to dry for 5 min. A Whatman No. 1 sterile filter paper disc (6 mm diameter) was impregnated with 1000 μg/disc of the essential oils. Negative controls were prepared using the same solvent employed used to dissolve the samples. The standard reference antibiotics, namely, streptomycin and tetracycline (10 μg/disc) were used as the positive controls for the test bacteria. The plates were incubated for bacteria at 37 ºC for 24 h, for yeasts at 28 ºC for 48 h and for molds at 28 ºC for 120 h. The antimicrobial activity was evaluated by measuring the diameter of the zones of inhibition against the test organisms. The experiments were repeated in triplicate and the results were expressed as average values.

Determination of the minimum inhibitory concentration (MIC): The minimum inhibitory concentration (MIC) is the lowest extract concentration at which no visible growth appears during incubation. The MICs of the essential oils against the test microorganisms were determined by the broth micro dilution method. Dilutions of the essential oils, ranging from 0.25-1000 μg/mL, were prepared in Muller-Hinton broth or Sabouraud dextrose agar. Exactly 0.5 MacFarland standard suspensions of the test microorganisms were inoculated in the tubes. A control test was also performed using inoculated broth or agar supplemented only with dimethyl sulphoxide under identical conditions, with streptomycin as the reference. The bacteria were incubated at 37 ºC for 24 h, the yeast strains at 28 ºC for 48 h and molds at 28 ºC for 120 h.

Statistical analysis: All tests were performed in triplicate and the results were calculated as the mean ± SD.

RESULTS AND DISCUSSION

The steam distillation of 1000 g of dried plant material yielded 7.5 mL (0.75 % v/w) of a light-yellow oil with a distinct smell. The oil sample was analyzed by GC-FID and GC-MS and the components were identified on the basis of their retention index (RI) values as well as by comparison of their mass spectra with those reported in literature. The GC-MS analysis of the essential oil of *A. integrifolia* indicated 61 components representing 98.86 % of the oil (Table-1). The composition of the essential oil was as follows: 16.32 % monoterpene hydrocarbon fraction, 12.21 % sesquiterpene hydrocarbon fraction, 50.21 % oxygenated monoterpene fraction, 10.48 % oxygenated sesquiterpenoid fraction and 9.64 % others. The main components of the oil were camphor (12.65 %), artemisia ketone (11.78 %), artemisia alcohol (6.56 %), eucalyptol (6.28 %) and caryophyllene oxide (5.24 %).

The *in vitro* antimicrobial activity of *A. integrifolia* essential oil against the tested microorganisms was assessed by the presence or absence of inhibition zones and MIC values. As shown in Table-2, the essential oil at 1000 μg/disc exhibited potent inhibitory effect against the tested microorganisms. Gram-positive bacteria (*Bacillus subtilis* CCTCC AB92068 and *Staphylococcus aureus* CCTCC AB91053) and moulds (*Aspergillus niger* CCTCC AF91004, *Chaetomium globosum* CCTCC AF 200039, *Mucor racemosus* CCTCC AF 93209 and *Monascus anka* CCTCC AF93208) were found to be the most inhibited bacterial pathogens by the oil, with their respective diameter zones of inhibition ranging from 25.1-26.6 mm. Gram-negative bacteria (*Pseudomonas aeruginosa* CCTCC AB93066 and *Escherichia coli* CCTCC AB91112) and yeast strains (*Hansenula anomala* CCTCC AY92046 and *Saccharomy cescerevisiae* CCTCC AY92042) were inhibited moderately, with diameter of zones of inhibition ranging from 20.5-22.8 mm. Obtained MIC values are summarized in Table-3. The oil exhibited remarkable antimicrobial activity as minimum inhibitory concentration against all gram-positive bacteria and moulds with MIC values of 62.5 μg/mL. The oil also exhibited moderate antimicrobial effect against *Pseudomonas aeruginosa* CCTCC AB93066, *Escherichia coli* CCTCC AB91112, *Hansenula anomala* CCTCC AY92046 and *Saccharomy cescerevisiae* CCTCC AY92042 with MIC values of 125, 125, 125, 250 and 125 μg/mL, respectively.

TABLE-1
CHEMICAL COMPOSITION OF THE ESSENTIAL OIL OF *A. integrifolia*

No.	RI[a]	Components	RA[b] (%)	Identification methods[c]
		Monoterpene hydrocarbons	16.32	
1	922	Artemisia triene	1.56	MS, RI
2	930	α-Thujene	1.76	MS, RI
3	935	α-Pinene	1.36	MS, RI
4	950	Camphene	1.35	MS, RI
5	972	β-Pinene	1.75	MS, RI
6	987	β-Myrcene	3.67	MS, RI, Co
7	1010	α-Terpinene	0.68	MS, RI
8	1038	β-Ocimene	0.58	MS, RI
9	1059	γ-Terpinene	2.34	MS, RI
10	1088	α -Terpinolene	1.27	MS, RI
		Oxygenated monoterpenes	50.21	
11	996	Yomogi alcohol	0.68	MS, RI
12	1033	Eucalyptol	6.78	MS, RI, Co
13	1062	Artemisia ketone	11.78	MS, RI, Co
14	1072	*cis*-Sabinene hydrate	0.24	MS, RI
15	1085	Artemisia alcohol	6.56	MS, RI, Co
16	1096	*cis*-Sabinene hydrate	0.61	MS, RI
17	1102	Linalool	0.34	MS, RI
18	1125	Chrysanthenone	0.26	MS, RI
19	1140	*trans*-Sabinol	0.34	MS, RI
20	1146	Camphor	12.65	MS, RI, Co
21	1162	Pinocarvone	2.45	MS, RI
22	1,166	Borneol	0.27	MS, RI
23	1175	Terpinen-4-ol	3.89	MS, RI, Co
24	1188	α-Terpineol	1.35	MS, RI
25	1193	Myrtenol	0.54	MS, RI
26	1206	Piperitol	0.65	MS, RI
27	1242	Carvone	0.39	MS, RI
28	1265	Furomyrcenol	0.43	MS, RI
		Sesquiterpene hydrocarbons	12.21	
29	1372	α-Copaene	1.28	MS, RI
30	1413	Caryophyllene	1.35	MS, RI
31	1438	*trans*-α-Bergamotene	0.41	MS, RI
32	1442	α-Humulene	0.34	MS, RI
33	1449	*cis*-β-Farnesene	2.34	MS, RI
34	1482	Germacrene D	3.68	MS, RI, Co
35	1487	Selinene	1.34	MS, RI
36	1495	Bicyclogermacrene	0.45	MS, RI
37	1525	δ-Cadinene	1.02	MS, RI
		Oxygenated sesquiterpenes	10.48	
38	1551	Nerolidol	0.68	MS, RI
39	1567	Spathulenol	0.67	MS, RI
40	1578	Globulol	0.35	MS, RI
41	1583	Caryophyllene oxide	5.24	MS, RI, Co
42	1585	Arteannuic alcohol	0.86	MS, RI
43	1605	Cedrol	0.42	MS, RI
44	1640	Cubenol	0.58	MS, RI
45	1645	Aromadendrene epoxide	0.36	MS, RI
46	1682	α-Bisabolol	1.32	MS, RI
		Others	9.64	
47	854	*trans*-2-Hexenal	0.57	MS, RI
48	867	*n*-Hexanol	0.38	MS, RI
49	964	Benzaldehyde	0.26	MS, RI
50	1026	*p*-Cymene	3.42	MS, RI
51	1045	Benzene acetaldehyde	0.43	MS, RI
52	1239	Cuminaldehyde	0.36	MS, RI
53	1297	Thymol	0.57	MS, RI
54	1304	Carvacrol	0.62	MS, RI
55	1356	Eugenol	0.37	MS, RI
56	1381	Isobutyl phenylacetate	0.45	MS, RI
57	1390	*trans*-Jasmone	0.32	MS, RI
58	1517	Calamenene	0.28	MS, RI
59	1834	Hydroxyfarnesyl acetone	0.38	MS, RI
60	1964	*n*-Hexadecanoic acid	0.78	MS, RI
61	2115	Phytol	0.45	MS, RI
Total identified (%)			98.86	–

[a]Retention index (RI) relative to n-alkanes on the HP-5 MS capillary column. [b]Relative area (peak area relative to the total peak area). [c]MS = mass spectrum, Co = co-injection with standard compound.

TABLE-2
ANTIMICROBIAL ACTIVITY OF THE *A. integrifolia* ESSENTIAL OIL AGAINST THE GROWTH OF MICROORGANISMS

Microorganism	Diameter of the inhibition zones		
	Essential oil[a]	Standard[b]	
		SM	TC
Pseudomonas aeruginosa	22.3 ± 0.9	20.8 ± 0.6	20.6 ± 0.6
Escherichia coli	22.8 ± 0.7	21.0 ± 0.5	20.4 ± 0.6
Bacillus subtilis	25.3 ± 0.8	20.8 ± 0.6	21.3 ± 0.6
Staphylococcus aureus	25.1 ± 0.6	20.9 ± 0.6	21.3 ± 0.5
Hansenula anomala	20.5 ± 0.5	20.5± 0.6	21.2 ± 0.5
Saccharomy cescerevisiae	21.7 ± 0.7	20.7 ± 0.6	20.3 ± 0.4
Aspergillus niger	25.4 ± 0.8	21.6 ± 0.7	21.2 ± 0.6
Chaetomium globosum	26.3 ± 0.7	20.5 ± 0.5	20.7 ± 0.5
Mucor racemosus	26.2 ± 0.8	20.3 ± 0.7	21.0 ± 0.6
Monascus anka	26.6 ± 0.7	20.3 ± 0.6	21.2 ± 0.7

Diameter of the inhibition zones (mm), including the disc diameter (6 mm), are given as the mean ± SD of triplicate experiments. [a]Diameter of the inhibition zones of the essential oil (tested volume, 1000 µg/disc). [b]Standard antibiotics: SM, streptomycin; TC, tetracycline (tested volume, 10 µg/disc).

TABLE-3
MINIMUM INHIBITORY CONCENTRATIONS (MIC) OF THE *A. integrifolia* ESSENTIAL OIL AGAINST THE GROWTH OF MICROORGANISMS

Microorganism	[a]MICs	
	Essential oil	Streptomycin
Pseudomonas aeruginosa	125	–
Escherichia coli	125	–
Bacillus subtilis	62.5	–
Staphylococcus aureus	62.5	–
Hansenula anomala	250	–
Saccharomy cescerevisiae	125	–
Aspergillus niger	62.5	–
Chaetomium globosum	62.5	–
Mucor racemosus	62.5	–
Monascus anka	62.5	–

[a]MIC, minimum inhibitory concentration (values in µg/mL).

Literature survey reveals that antimicrobial activity of other *Artemisia* species essential oil has been well demonstrated. The major constituents of *A. annua* essential oil were artemisia ketone (30.7 %), camphor (15.8 %) and eucalyptol (4.8 %). The oil exhibited antimicrobial activity against *Haemophilus influenzae*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Micrococcus luteus* and *Candida krusei*[6]. Hydrodistilled volatile oils from the aerial parts of *Artemisia afra*, consisting of camphor (46.2 %), α-thujone (15.2 %), artemisia ketone (7.4 %) and eucalyptol (4.2 %), had a moderate antimicrobial activity against gram-negative bacteria and oral pathogens[7]. The essential oil of *A. annua*, consisting of camphor (31.7 %), artemisia ketone (22.3 %), eucalyptol (10.1 %),

caryophyllene oxide (7.1 %), α-copaene (3.4 %) and camphene (3.3 %), remarkably inhibited the growth of fungal pathogens of tomato, including *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Phytophthora infestans* and *Verticillium dahliae*[8]. The essential oil of the aerial parts of *A. lobelia* showed strong activity against *P. aeruginosa*. The major constituents of the oil were camphor (33.2-36.8 %), eucalyptol (15.2-21.1 %) and artemisia ketone (6.0-24.2 %) during four stages of the growing cycle[9].

The current study and above-cited literatures shows that the essential oil from *A. integrifolia* share some relatively similar main constituents, such as artemisia ketone, camphor, eucalyptol, caryophyllene oxide, terpine-4-ol and germacrene D with other several species of *Artemisia* and serve as chemosystematic markers of *A. integrifolia*. Further, the results shows that the antimicrobial activity of the oils from some *Artemisia* species, could, in part, be associated with its major components such as artemisia ketone, camphor, eucalyptol, artemisia alcohol, terpine-4-ol, germacrene D and caryophyllene oxide. The antimicrobial activity of these compounds were reported by others[10-13]. Although expression of antimicrobial activity is often very clear, the mechanism of action of the essential oils is incompletely understood. It is difficult to attribute the activity of a complex mixture to a single or particular constituent. It is possible that the activity of the main components is modulated by other minor molecules[14]. Although present in low concentrations, the components, such as pinene, terpinene, terpineol, caryophyllene, farnesene, selinene, bisabolol could have imparted a significant effect on the antimicrobial activities of the oils *via* a synergistic or antagonistic effect.

Conclusion

The essential oil of *A. integrifolia* is described by codominance of main components such camphor, artemisia ketone, artemisia alcohol, eucalyptol and caryophyllene oxide that had described in the literature and seems characteristic of *Artemisia* species essential oil. Antimicrobial assays of the oils has demonstrated that the gram-positive bacteria and moulds in this study were more sensitive to the oil compared to the gram-negative bacteria and yeast strains. The sensitivity of the test microorganisms toward essential oil of *A. integrifolia* would suggest that this oil may be promising natural preservatives in the food industry.

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