Chemical Composition and Antimicrobial Activity of the Essential Oils from *Chloranthus japonicus* Sieb. and *Chloranthus multistachys* Pei

Jie-fang Kang, Yuan Zhang, Yong-liang Du, and Zhe-zhi Wang*

Key Laboratory of the Ministry of Education for Medicinal Resources and Natural Pharmaceutical Chemistry, National Engineering Laboratory for Resource Development of Endangered Crude Drugs in Northwest of China, Shaanxi Normal University, Xi'an, 710062, P. R. China. Fax: +86 29 85310546. E-mail: zzwang@snnu.edu.cn

* Author for correspondence and reprint requests

Z. Naturforsch. **65 c**, 660–666 (2010); received March 23/July 14, 2010

We examined the composition and antimicrobial activity of two essential oils from *Chloranthus japonicus* Sieb. and *Chloranthus multistachys* Pei. GC-FID and GC-MS analyses identified 48 and 39 compounds, which represented 95.56% and 94.58%, respectively, of all components in these oils. Of these, 28 compounds were common to both, with a relatively high amount of oxygenated monoterpenes (50.95% and 39.97%). Antimicrobial properties were evaluated *in vitro* via disc diffusion and microbroth dilution assays. Activities were strong against most tested microorganisms, with inhibition zones ranging from 8.1 to 22.2 mm. For both species, minimum values for inhibitory and bactericidal concentrations were 0.39 to 12.50 mg/mL and 0.78 to 50.00 mg/mL, respectively. These results suggest that these essential oils are potent natural sources of antimicrobial agents for the medicinal and pharmaceutical industries.

Key words: Chloranthus japonicus Sieb., *Chloranthus multistachys* Pei, Essential Oil

Introduction

Chloranthus, within the Chloranthaceae family, comprises thirteen species in Asia. In China, nine species of this perennial herb have already been recorded (Chen *et al*., 1982). *Chloranthus japonicus* Sieb. and *Chloranthus multistachys* Pei are folklore medicines native to the Qinling Mountains of China. Whole plants have been traditionally used for hundreds of years to treat boils, dermatopathia, enteric fever, detumescence, snake bite, bone fractures, cough, and rheumatic pain. Their various bioactivities have now gained attention by researchers because of their antifungal, antitumour, and cytotoxic activities (Kuang *et al*., 2009). *Chloranthus* species are also popular for their aromatic qualities, and volatile constituents are believed to contribute to those bioactivities. Analyses have been performed on the volatile composition of various organs and origins of *Chloranthus* (Li *et al*., 1992; Li and Yao, 2005; Wang *et al*., 1987; Yu *et al*., 2002; Tesso *et al*., 2006; Kuang *et al*., 2007; Xia *et al*., 2009). However, little focus has been fixed on the chemical composition of the volatile metabolites that result from differences in the natural habitats of *C. japonicus* and *C. multistachys* in China, and no studies have compared their antimicrobial activities.

As part of our on-going research on essential oil-bearing herbs from the Qinling Mountains, our objectives here were to (1) examine the chemical composition and potential biochemical activity of the essential oils from *C. japonicus* and *C. multistachys*, and (2) deduce which of these components was likely to contribute to such antimicrobial and antibacterial traits.

Material and Methods

Plant materials

Plants of *Chloranthus japonicus* Sieb. were collected from the North Slope of Taibai Mountain (N 34°05′, E 107°42′, at an altitude of 1200 m), at the peak of the Qinling Mountains, China, while those of *C. multistachys* Pei were sampled from Ningshan County (N 33°16′, E 108°21′, at an altitude of 1350 m), in the middle of the Qinling Mountains, Shaanxi Province, China. All of the plant materials were collected during July 2007. Voucher specimens were deposited in the Key

0939–5075/2010/1100–0660 $ 06.00

Laboratory of the Ministry of Education for Medicinal Resources and Natural Pharmaceutical Chemistry, Shaanxi Normal University, Xi'an, Shaanxi, China.

Essential oil extraction

Air-dried whole plants were hydrodistilled for 4 h in a Clevenger-type apparatus. Their essential oils were then dried with anhydrous sodium sulfate and stored in the dark at 4 °C.

Essential oil analysis

Essential oils were analysed by both gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). The former was conducted on a gas chromatograph equipped with an FID detector (Model 6890N; Agilent Technology, Palo Alto, CA, USA). A fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Model HP-5MS, Agilent) with 5% phenyl methyl siloxane was used for the separations. Injector and FID detector temperatures were set at 250 °C and 200 °C, respectively. The column temperature was programmed to increase initially from 50 °C to 125 °C, at 5 °C/min, then from 125 °C to 200 °C, at 3 °C /min, where it was held for 3 min before finally rising to 260 °C at 10 °C/min. Samples (1.0 μL) were injected using the splitless mode, and the column flow rate of the carrier gas was 1.5 mL/min.

GC-MS was performed with a Shimadzu QP2010 GC-MS apparatus and Shimadzu ChemStation software (Shimadzu Corporation, Analytical and Measuring Instruments Division, Kyoto, Japan). A fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Shimadzu RTX-5 ms) with 5% phenyl methyl siloxane was used for the separations. Injector and ion source temperatures were set at 250 °C and 200 °C, respectively. A 2-μL aliquot of oil was injected into the column with a 10:1 split. The other operating conditions were as described for GC-FID. The carrier gas was helium at a flow rate of 1.3 mL/min. The mass spectrometer was operated in the electron-impact ionization (EI) mode, with 70 eV energy, and the scan range was 40 to 600 amu.

Identification of components

Linear retention indices for all compounds were obtained by co-injecting the samples with the homologous series of $C_{10}-C_{18}$, C_{20}, C_{24}, C_{28}, C_{32}, C_{36}, C_{40}, and C_{44} *n*-alkanes. Individual components were identified by matching their recorded mass spectra with those of data from NIST05.LIB and NIST05s.LIB (National Institute of Standards and Technology) libraries, as provided by the GC-MS software. We further confirmed these results by comparing the retention indices with those reported previously (Adams, 1995; Zhang and Wang, 2007).

Antimicrobial activity

Microbial strains

Strains included *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megatherium*, *Bacillus coagulans*, *Sarcina lutea*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, *Rhodotorula glutinis*, *Candida lipolytica*, *Candida tropicalis*, *Saccharomyces cerevisiae* (Shaanxi Sanitation and Antiepidemic Station, China), *Enterobacter cloacae*, *Streptococcus pneumoniae*, and *Klebsiella pneumoniae* subsp. *pneumoniae* (Institute of Microbiology, Chinese Academy of Sciences). These were conserved at the College of Life Sciences, Shaanxi Normal University, Xi'an, China.

Antimicrobial screening

The agar disc diffusion method was employed for determining the antimicrobial activities of our essential oils (Yu *et al.*, 2007). A suspension of one microorganism (0.1 mL of 10^8 cfu/mL) was spread on each solid media plate. Filter paper discs (6 mm i.d.) were individually impregnated with 15 μL of the oil and placed on the inoculated plates for 2 h at 4 °C. Afterwards, they were incubated at either 37 °C for 24 h (bacteria) or 30 °C for 48 h (yeasts). Diameters of the inhibition zones were measured in millimeters, and all tests were carried out in triplicate.

Determinations of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A broth microdilution assay was used for measuring the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Yu *et al.*, 2007). Tests were generally performed in Mueller Hinton broth (MHB) supplemented with 0.5% (v/v) Tween 80; the exception was for yeasts, which required Sabouraud dextrose broth (SDB) plus Tween 80. Overnight culturing was done at 37 °C with MHB for

bacteria and at 30 °C in SDB with yeasts. Test strains were suspended in MHB to give a final density of $5 \cdot 10^5$ cfu/mL, and were confirmed by viable counts. Geometric dilutions ranging from 0.03 mg/mL to 50.00 mg/mL of these oils were prepared in 96-well microtiter plates, and they included one growth control (MHB + Tween 80) and one sterility control (MHB + Tween 80 + test oil). Plates were incubated under normal atmospheric conditions at 37 °C with 24 h for bacteria and at 30 °C for 48 h with yeasts. MIC was defined as the lowest concentration of oil at which a tested microorganism did not demonstrate visible growth, as indicated by turbidity. To determine MBC, broth was taken from each well and inoculated in Mueller Hinton agar for 24 h at 37 °C (bacteria) or in Sabouraud dextrose agar for 48 h at 30 °C (yeasts). MBC was defined as the lowest concentration of oil at which an inoculated microorganism was killed. Each test was duplicated and then repeated three times. Positive controls were tetracycline for the bacteria and nystatin for the yeasts.

Results and Discussion

Chemical composition

The distilled essential oils of *C. japonicus* and *C. multistachys* were yellow and brown liquids, with respective yields of (0.20 ± 0.01)% (w/w) and (0.29 ± 0.01)% (w/w), on a dry-weight basis. In all, 48 and 39 compounds were identified via GC-FID and GC-MS, accounting for 95.56% and 94.58% of all constituents in the oil from *C. japonicus* and *C. multistachys*, respectively (Table I). Of these, 28 compounds were common to both. *C. japonicus* had a relatively higher amount (50.95%) of oxygenated monoterpenes than did *C. multistachys* (39.97%). Our analysis demonstrated that these essential oils were rich in monoterpenes (77.04% for *C. japonicus* and 46.64% for *C. multistachys*). The content of bornyl acetate, the most abundant monoterpene in these oils, was 30.98% and 35.99%, which is consistent with other *Chloranthus* species, such as *C. fortunei, C. holostegius*, and *C. henryi* (Li *et al*., 1992; Kuang *et al*., 2007). Minor qualitative and major quantitative variations were found for some compounds. For example, the oil from *C. japonicus* was characterized by higher portions of thymol methyl ether (10.06%), camphene (9.03%), *β*-phellandrene (5.09%), and *ρ*-cymene (4.19%). In contrast, myristicin (16.08%), 3-octanol acetate (7.52%), thymol methyl ether (5.91%), and eudesma-4(14),11-diene (4.60%) were more prevalent in *C. multistachys*. Some compounds were present in only one of the species, *e.g*. myristicin in *C. multistachys*, and *α*-bulnesene and *δ*-selinene in *C. japonicus*.

Only a few chemically oriented analyses of the essential oils from *Chloranthus* of diverse origins have been published previously (Li *et al*., 1992; Li and Yao, 2005; Wang *et al*., 1987; Yu *et al*., 2002;

Table I. Qualitative and quantitative compositions of essential oils from *Chloranthus japonicus* Sieb. and *Chloranthus multistachys* Pei.

No.	R.I.[a]	Compound[b]	*C. japonicus* (%)[c]	*C. multistachys* (%)[c]
1	802	Hexanal	0.32 ± 0.03	0.15 ± 0.01
2	936	*α*-Pinene	3.38 ± 0.39	0.29 ± 0.04
3	952	Camphene	9.03 ± 0.42	1.79 ± 0.22
4	976	*β*-Phellandrene	5.09 ± 0.23	0.36 ± 0.02
5	981	*β*-Pinene	1.94 ± 0.16	0.23 ± 0.03
6	988	3-Octanone	0.80 ± 0.06	0.34 ± 0.04
7	994	2-Pentylfuran	1.32 ± 0.16	0.19 ± 0.02
8	1028	*ρ*-Cymene	4.19 ± 0.32	0.46 ± 0.02
9	1032	Limonene	1.56 ± 0.18	0.18 ± 0.02
10	1035	1,8-Cineole	0.32 ± 0.03	-
11	1062	*γ*-Terpinene	0.73 ± 0.08	0.57 ± 0.07
12	1090	Camphenilone	0.14 ± 0.01	-
13	1092	Terpinolene	0.17 ± 0.02	-
14	1113	Octen-1-ol acetate	-	1.54 ± 0.16
15	1125	3-Octanol acetate	2.28 ± 0.29	7.52 ± 0.62
16	1132	*α*-Campholenal	0.77 ± 0.05	1.14 ± 0.12

Table I continued.

17	1152	Camphor	0.32 ± 0.05	-
18	1163	Phellandral	0.22 ± 0.03	-
19	1165	1-Nonyl-1-cyclohexene	0.38 ± 0.03	-
20	1167	Pinocamphone	-	0.10 ± 0.01
21	1170	Pinocarvone	0.36 ± 0.04	0.26 ± 0.03
22	1175	*trans*-3(10)-Caren-2-ol	0.67 ± 0.04	1.46 ± 0.15
23	1182	3-Pinanone	-	0.17 ± 0.02
24	1204	Myrtenal	0.28 ± 0.02	0.41 ± 0.05
25	1217	Verbenone	0.14 ± 0.02	0.25 ± 0.02
26	1239	Thymol methyl ether	10.06 ± 1.01	5.91 ± 0.60
27	1248	Cumaldehyde	0.18 ± 0.01	0.17 ± 0.02
28	1251	Carvone	0.22 ± 0.01	0.22 ± 0.01
29	1257	Methyl camphenoate	0.15 ± 0.01	0.25 ± 0.03
30	1292	Bornyl acetate	30.98 ± 2.89	35.99 ± 2.64
31	1296	Safrole	-	2.79 ± 0.41
32	1306	*trans*-Pinocarvyl acetate	0.13 ± 0.01	0.17 ± 0.01
33	1351	Terpinyl acetate	0.65 ± 0.07	-
34	1354	α-Cubebene	-	1.75 ± 0.23
35	1373	Cyclosativene	0.37 ± 0.03	-
36	1376	β-Terpinyl acetate	-	0.34 ± 0.05
37	1377	Tetradecene	0.35 ± 0.02	-
38	1382	Copaene	0.44 ± 0.04	0.88 ± 0.11
39	1389	α-Cedrene	-	0.24 ± 0.02
40	1398	β-Elemene	0.72 ± 0.07	-
41	1409	δ-Selinene	2.20 ± 0.18	-
42	1430	α-Ionone	0.18 ± 0.02	0.18 ± 0.04
43	1439	Germacrene D	0.82 ± 0.07	-
44	1455	Geranyl acetone	0.14 ± 0.01	-
45	1459	β-Farnesene	0.16 ± 0.01	-
46	1467	α-Caryophyllene	0.84 ± 0.08	-
47	1477	α-Amorphene	0.15 ± 0.03	-
48	1481	Cadina-1(10),4-diene	-	0.94 ± 0.12
49	1491	β-Ionone	0.90 ± 0.07	1.27 ± 0.12
50	1495	Eudesma-4(14),11-diene	3.16 ± 0.22	4.60 ± 0.68
51	1501	γ-Muurolene	0.25 ± 0.03	1.23 ± 0.27
52	1505	α-Muurolene	-	1.16 ± 0.22
53	1505	α-Bulnesene	3.51 ± 0.25	-
54	1509	*trans*-β-Guaiene	1.28 ± 0.29	-
55	1522	γ-Cadinene	0.83 ± 0.09	-
56	1530	Myristicin	-	16.08 ± 1.83
57	1551	α-Calacorene	-	0.27 ± 0.02
58	1566	Germacrene B	0.70 ± 0.06	-
59	1593	Caryophyllene oxide	1.78 ± 0.32	2.73 ± 0.33
Total identified			95.56 ± 7.26	94.58 ± 4.81
Monoterpene hydrocarbons			26.09 ± 2.99	6.67 ± 0.71
Oxygenated monoterpenes			50.95 ± 4.41	39.97 ± 2.19
Sesquiterpene hydrocarbons			14.59 ± 1.87	11.07 ± 1.03
Oxygenated sesquiterpenes			1.78 ± 0.11	3.13 ± 0.29
Phenylpropanoids			-	16.08 ± 1.24
Others			5.32 ± 0.42	17.66 ± 1.51

[a] Retention indices on an HP-5MS capillary column, as calculated by the following equation: R.I. = $100 \cdot n + 100 \cdot (t_x - t_n)/(t_{n+1} - t_n)$, where t_x, t_n, and t_{n+1} are the retention times for compound x and *n*-alkanes, where n is the number of carbon atoms found in the molecule ($t_n < t_x < t_{n+1}$).

[b] Compounds listed in order of elution from the column.

[c] Relative percentage of the identified volatiles based on GC-FID; mean values of three determinations (three replicates) calculated from FID areas, data are presented as means ± SD.

Table II. Antimicrobial activities of essential oils from *Chloranthus japonicus* Sieb. and *Chloranthus multistachys* Pei.

Test strain	*C. japonicus*			*C. multistachys*			Tetracycline/Nystatin		
	DD[a]	MIC[b]	MBC[b]	DD[a]	MIC[b]	MBC[b]	DD[c]	MIC[d]	MBC[d]
Gram-positive bacteria									
Bacillus cereus	22.2 ± 1.1	0.39	0.78	18.8 ± 0.8	0.78	1.56	21.0 ± 0.4	2.0	2.0
Bacillus subtilis	12.8 ± 0.6	3.13	12.50	12.5 ± 0.4	3.13	12.50	18.9 ± 0.3	3.9	7.8
Bacillus megatherium	12.2 ± 0.4	3.13	6.25	12.1 ± 0.6	3.13	6.25	19.6 ± 0.2	2.0	3.9
Bacillus coagulans	15.4 ± 0.7	0.78	1.56	13.5 ± 0.5	3.13	6.25	22.7 ± 0.3	2.0	2.0
Sarcina lutea	12.6 ± 0.5	3.13	12.50	11.4 ± 0.6	6.25	25.00	21.2 ± 0.2	2.0	2.0
Staphylococcus epidermidis	12.4 ± 0.7	3.13	12.50	12.6 ± 0.5	3.13	12.50	19.4 ± 0.4	2.0	3.9
Staphylococcus aureus	11.9 ± 0.8	3.13	6.25	12.2 ± 0.4	3.13	6.25	18.8 ± 0.2	3.9	3.9
Streptococcus pneumoniae	13.0 ± 0.4	3.13	6.25	12.8 ± 0.4	3.13	6.25	20.2 ± 0.2	2.0	2.0
Gram-negative bacteria									
Enterobacter cloacae	8.1 ± 0.4	12.50	50.00	8.5 ± 0.5	12.50	50.00	21.4 ± 0.3	2.0	3.9
Escherichia coli	9.5 ± 0.7	12.50	50.00	8.8 ± 0.5	12.50	50.00	23.1 ± 0.2	2.0	2.0
Klebsiella pneumoniae subsp. *pneumoniae*	8.4 ± 0.6	12.50	50.00	8.7 ± 0.8	12.50	50.00	18.6 ± 0.3	3.9	7.8
Yeasts									
Rhodotorula glutinis	11.9 ± 0.6	6.25	12.50	14.7 ± 0.8	3.13	6.25	20.8 ± 0.2	1.0	1.0
Candida tropicalis	12.7 ± 0.5	6.25	12.50	12.2 ± 0.6	6.25	12.50	19.5 ± 0.1	1.0	1.9
Candida lipolytica	15.6 ± 0.8	0.78	1.56	17.8 ± 0.7	0.78	1.56	16.9 ± 0.3	1.0	1.9
Saccharomyces cerevisiae	11.1 ± 0.6	6.25	25.00	11.6 ± 0.5	6.25	25.00	17.4 ± 0.2	1.0	1.9

DD, diameter of zone of inhibition (mm) including disc diameter of 6 mm, data are presented as mean ± SD. MIC, minimum inhibitory concentration. MBC, minimum bactericidal concentration.
[a] Tested at 10.0 mg/disc.
[b] Values are given as mg/mL.
[c] Tested at 5 μg/disc.
[d] Values are given as μg/mL.

Tesso *et al*., 2006; Kuang *et al*., 2007; Xia *et al*., 2009). Some differences are rather obvious. For instance, oil obtained by supercritical fluid extraction from the aerial portions of *C. japonicus* plants collected in Heilongjiang Province, China, contained shizukanolide, 9,12,15-octadecatrien-1-ol, and 11,14-eicosadienoic acid methyl ester (Xia *et al*., 2009). Yu *et al*. (2002) have reported that α-terpineol, geraniol, limonene, and aromadendrene are the major components of *C. multistachys* from JingGang Mountain in China. Oils from some *Chloranthus* species (*C. fortunei*, *C. henryi*, *C. holostegius*, and *C. spicatus*) are rich in 3-octanol acetate, cadina-3,9-diene, aromadendrene, bornyl acetate, shizukanolide A, furanodienone, α-terpineol, geraniol, α-elemol, and farnesol (Li *et al*., 1992; Li and Yao, 2005; Yu *et al*., 2002; Kuang *et al*., 2007), whereas the essential oil of flowers of *Chloranthus spicatus* (Thunb.) Makino from Phu Tho Province, Vietnam, have abundant (*Z*)-β-ocimene, allo-aromadendrene, and selina-4(15),7(11)-diene (Tesso *et al*., 2006). In general, such fluctuations in chemical composition result from several factors, including climatic conditions, geographical origin, season when collected, plant nutritional status, and the occurrence of chemotypes.

Antimicrobial activity

Our essential oils were measured for their diameters of inhibition zones (DDs), minimum inhibitory concentrations (MICs), and minimum bactericidal concentrations (MBCs) (Table II). Both exhibited antimicrobial activity against all microorganisms tested. Based on data we obtained from the disc diffusion method, *Bacillus cereus* was the most sensitive, being associated with the largest inhibition zone (22.2 and 18.8 mm) compared with the weakest zone for *Enterobacter cloacae* (8.1 and 8.5 mm). Oil from *C. japonicus* was generally more efficient in inhibiting the bacterial growth. MIC and MBC determinations indicated that Gram-positive bacteria and yeasts were more sensitive to both oils than were Gram-negative bacteria. The oil of *C.*

japonicus showed the strongest bactericidal activity against *B. cereus*, as was evidenced by the lowest values for MIC (0.39 mg/mL) and MBC (0.78 mg/mL). In contrast, oil from *C. multistachys* exerted the strongest bactericidal activity against *B. cereus* and *Candida lipolytica*, with the lowest MIC (0.78 mg/mL) and MBC (1.56 mg/mL), respectively. Activity was weakest against *E. cloacae*, *Escherichia coli*, and *Klebsiella pneumoniae* subsp. *pneumoniae*, with the highest MIC, being 12.50 mg/mL, and MBC, being 50.00 mg/mL. *C. japonicus* oil was performing better against *B. cereus*, *B. coagulans*, and *Sarcina lutea* than was *C. multistachys*, but the former oil was weaker against *Rhodotorula glutinis*. Antimicrobial activities of both essential oils markedly inhibited the development of *B. cereus* and *C. lipolytica*, proving to be as effective as the positive references tetracycline and nystatin.

The chemical structures of its most abundant compounds are related to the antimicrobial activity of an essential oil. For example, monoterpenes have antibacterial properties that destroy the cellular integrity by inhibiting respiration in microbial cells (Helander *et al.*, 1998). Essential oils with a high portion of oxygenated monoterpenes exhibit stronger antifungal activities than do those that are rich in monoterpene hydrocarbons or sesquiterpenes (Kotan *et al.*, 2008). Here, the antibacterial activity of the essential oils from both species may have been due to the presence of high contents of oxygenated monoterpenes (39.97% and 50.95%). Bornyl acetate, thymol methyl ether, and myristicin might have been responsible for suppressing the microbial growth; the first two have been shown to have bacteriostatic activity in several microorganisms (Runyoro *et al.*, 2010). Against the larvae of *Brontispa longissima*, the phenylpropanoid myristicin has strong antifeedant, contact-toxic, and growth-retardation effects, while also blocking the activities of AChE, CarE, GSTs, and Na^+,K^+-ATPase (Qin *et al.*, 2010). In addition, lower contents of *a*-pinene, *β*-pinene, limonene, 1,8-cineole, camphor, caryophyllene, and caryophyllene oxide might contribute to such antimicrobial activity. Both caryophyllene and caryophyllene oxide are reportedly antibacterial (Azaz *et al.*, 2002), as are enatiomers of *a*-pinene, *β*-pinene, and limonene (Dorman and Deans, 2000). These components can exert their toxic effects by disrupting the integrity of bacterial or fungal membranes (Knoblock *et al.*, 1989). Synergism has been demonstrated between carvacrol and *p*-cymene against *Bacillus cereus* vegetative cells. *p*-Cymene is one of the major constituents of the investigated oils but it has an ineffective antibacterial function when used alone (Dorman and Deans, 2000). However, when combined with carvacrol, the two are effective against *B. cereus in vitro* and in rice plants (Ultee *et al.*, 2000). In fact, the synergistic effects between major and minor components of essential oils should be considered when assessing their entire antimicrobial properties.

Conclusion

The essential oils of *C. japonicus* and *C. multistachys* show antimicrobial activity, having particularly strong bactericidal action against both *Bacillus cereus* and *Candida lipolytica*. Our results suggest that these oils can be utilized as natural food preservatives, as well as possible sources of antimicrobial ingredients for the food and pharmaceutical industries. Analyses by GC-FID and GC-MS and the calculations of retention indices demonstrated that both oils contain primarily terpenoid compounds and are exceptionally rich in monoterpenes. Quantitative differences in the components of the two oils might explain variations in their bioactivities. Therefore, we conclude that these two species warrant further investigation for their potential therapeutic efficacy.

Acknowledgements

This work benefitted from financial support from the "Technology Innovation Support Project" (CXY1018-3) of Xi'an and "Foundation for Excellent Doctor Degree Dissertation" (S2008YB04) of Shaanxi Normal University, P. R. China.

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