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Chemical Composition, Antifungal Activity and Toxicity of Essential Oils from the Leaves of *Chimonanthus praecox* Located at Two Different Geographical Origin

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The composition of the essential oils obtained by hydrodistillation of different geographical origin of *Chimonanthus praecox*, including Hangzhou and Wenzhou samples, were investigated by GC/MS. Forty three components comprising 93.05 % of the leave oils from Hangzhou plant, and 32 components comprising 94.26 % of the leave oils from Wenzhou plant were identified. The major components in the leaf oil from Hangzhou samples were (-)-alloisolongifolene (10.20 %), caryophyllene (9.31 %), elixene (8.52 %), germacrene D (7.30 %), germacrene B (7.44 %), δ-cadinene (6.17 %) and β-elemen (4.67 %). While, the oil from Wenzhou samples contained furan, 3-(4,8-dimethyl-3,7-nonadienyl)-, (E)-(21.69 %), eucalyptol (19.02 %), terpilene (12.41 %), *p*-menth-1-en-8-ol (6.65 %) and geraniol (5.29 %) as the major components. The antifungal activity of the oils against 8 phytopathogenic fungi was tested by determining minimum inhibitory concentrations using the microdilution method. Both oils exhibited potent antifungal activities with MIC values of 8-32 μg/mL. Both oils were considered bioactive, showing an LC_{50} value of 30 and 22 μg/mL in the *Artemia salina* lethality test.

Keywords: Essential oils, Composition, Antifungal activity, Toxicity, *Chimonanthus praecox*, Geographical origin.

INTRODUCTION

Since ancient times the crude herbal extracts of aromatic plants have been in use for different purposes, such as food, drugs and perfumery[1]. The essential oils are considered among the most important antimicrobial agents present in these plants, and may also have antioxidant, insecticidal, antifungal, antibacterial, cytotoxic and antiinflammatory activities[2,3].

C. praecox belonging to the Calycanthaceae family are deciduous shrub native to China, which has survived from the tertiary period. They are famous traditional fragrant flower plant with high ornamental value in China. It is also traditional Chinese herbal medicine for the treatment of colds, analgesic, coughs, asthma and other disorders[4]. Therefore, *C. praecox* have been phytochemically and pharmacologically investigated and many molecules have been isolated and identified. In this context, different classes of organic compounds of medicinal interest have been reported, mainly including alkaloids and sesquiterpenoids[5-7].

In recent years, research and development of essential oil products of *Chimonanthus* plants have been more and more attended, due to excellent aroma[8]. Moreover, to the best of our knowledge, the essential oil composition of this medicinal plant has been studied thoroughly and hundreds of components have been identified up to date[9-14]. Generally it is accepted that variability of chemical composition of essential oil of *C. praecox* depends of geographical origin and stage of plant development.

Thus, there is still a considerable research interest in the assay of composition and biological properties of essential oil of *C. praecox* from different geographical origin. In the present work, we have investigated the essential oil composition of *C. praecox* cultivated in Hangzhou and Wenzhou. In addition, the aim of this study was to assess the antifungal activity and toxic activity of the isolated essential oil, which have not been reported to date.

EXPERIMENTAL

The fresh leaves of *C. praecox* were collected in Hangzhou and Wenzhou of Zhejiang province, China. Botanical identification was carried out by Prof. Li Gengyou. Voucher specimens (no. 0270012 and 0270010) of the samples have been deposited with Plant laboratory of Zhejiang A & F University.

Isolation of essential oil: The different original dried leave of *C. praecox* were subjected to hydrodistillation for 5 h and

5 h, resp., using a clevenger-type apparatus. The obtained oils were dried (anhydrous Na_2SO_4) and stored in sealed flasks at 4 °C.

Gas chromatography/mass spectrometry (GC/MS) analysis: Gas chromatography/mass spectrometry analysis was carried out using splitless injection mode on a Varian CP3800/1200L GC-MS instrument with a fused silica capillary DB-5MS column (5 % phenylmethylpolysiloxane, 30 m × 0.25 mm, film thickness 0.25 μm). Helium was used as the carrier gas, at a flow rate of 0.8 mL/min. Oven temperature was programmed at 45 °C for 3 min, then 45-90 °C at 10 °C/min, then 90-180 °C at 6 °C/min, then 180-230 °C at 12 °C/min, then 230-250 °C at 9 °C/min and finally held at 250 °C for 9 min. The injector and detector temperature were set at 250 °C and 280 °C, respectively. The electron impact source was 70 eV, ion source temperature was 200 °C, the mass range 33-450 amu and the scan rate was 0.5 s.

The components of the essential oils were identified by comparison of their RI (retention indices) relative to C_5-C_{24} *n*-alkanes obtained on a nonpolar DB-5MS column, with those provided in the literature, by comparing their mass spectral fragmentation patterns with those of similar compounds from databases (NIST and Wiley Mass Spectral Libraries) and reported in published articles. For each compound on the gas chromatogram, the percentage of peak area relative to the total peak area of all compounds was determined and reported as relative amount of that compound, without using correction factors.

Antifungal bioassay: The test phytopathogenic fungi used in this study were *Botrytis cinerea*, *Fusarium graminearum*, *Fusarium avenaceum*, *Cylindrocarpon destructans*, *Helminthosporium turcicum*, *Colletorichum gloeosporioides*, *Sclerotinia sclerotiorum*, and *Monilinia fructicola*. All the fungi were isolated from infected plant organs at the Zhejiang A&F University.

Antifungal activity was assessed by the microbroth dilution method in 96-well culture plates using a potato dextrose (PD) medium[15]. The serial doubling dilution of the essential oil and its major compound was prepared in DMSO, with concentrations ranging from 0.25 to 32 μg/mL. Final concentration of DMSO never exceeded 2 %. A commercial fungicide carbendazim (Aladdin Chemistry Co. Ltd.) was used as positive control, and the solution of equal concentration of DMSO was used as a negative control. The tested fungi were incubated in the potato dextrose medium for 18 h at 28 ± 0.5 °C at 150 rpm, and spores of different microorganism concentrations were diluted to approximately 1×10^6 CFU with potato dextrose medium. The test oils (10 μL) were added to 96-well microplates, and 90 μL of potato dextrose medium was added. Serial dilutions were made in the 96-well round-bottom sterile plates in triplicate in 50 μL of potato dextrose medium, and then 50 μL of the fungal suspension was added. After incubation for 48 h at 28 ± 0.5 °C, minimum inhibitory concentration (MIC) was taken as the lowest concentration of the test compounds in the wells of the 96-well plate in which no microbial growth could be observed.

Brine shrimp lethality bioassay: Both essential oils were assayed using a modified test of lethality to *A. salina*[16]. The eggs of *A. salina* were incubated in a hatching chamber with sea water and kept at room temperature (average 27 °C) under artificial light around the clock. Larvae after 48 h were extracted and counted using a Pasteur pipette. A standard solution of 1,000 μg/mL was prepared with 100 mg of essential oil diluted in 1 mL of DMSO, and the volume was completed with sea water in a 100 mL volumetric flask. Concentrations of 900, 100, 10 and 1 μg/mL were prepared using standard solution. For each concentration, 10 brine shrimp larvae were used, placed in flasks that were filled with seawater to a total volume of 5 mL. Intermediate concentrations were made to calculate the LC_{50}. For the control group, a solution was prepared with 100 μL of DMSO and 4.9 mL of seawater. After 24 h, the dead larvae were counted and the LC_{50} value was estimated using the Origin 9.0 statistical program.

RESULTS AND DISCUSSION

Gas chromatography-mass spectrometry analysis of essential oil: The detailed composition of the essential oil of *C. praecox* fresh leave from Hangzhou and Wenzhou is reported in Table-1. Overall fifty compounds were identified in the fresh leave of *C. praecox*. For easier comparison of the oils, the components were grouped into five classes: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpenes hydrocarbons, oxygenated sesquiterpenes and others. However, monoterpenes and sesquiterpenes were the main compounds identified. As outlined in Table-1, the GC-MS analysis of the oils in the Hangzhou and Wenzhou branch revealed the identification of 43 and 32 components, respectively, representing 93.05 and 94.26 % of total oils. All oils showed some similarity in the qualitative composition, but they differed significantly from a quantitative point of view, showing some differences in their main constituents (Table-1). The quantity of the chemicals in the oils from Hangzhou was more than the oils from Wenzhou. The major constituents of *C. praecox* essential oil were (-)-alloisolongifolene (10.20 %), caryophyllene (9.31 %), elixene (8.52 %), germacrene D (7.30 %), germacrene B (7.44 %), δ-cadinene (6.17 %), and β-elemen (4.67 %) for the Hangzhou samples, whereas the oil obtained from Wenzhou plants was characterized by a higher level of furan, 3-(4,8-dimethyl-3,7-nonadienyl)-, (E)-(21.69 %), eucalyptol (19.02 %), terpilene (12.41 %), *p*-menth-1-en-8-ol (6.65 %) and geraniol (5.29 %). These results showed that the 2 samples mainly contained large amounts of monoterpene hydrocarbons and sesquiterpene hydrocarbons. But there were some differences in the main compounds between the two samples. The variance observed in the leaves essential oils composition could be related to several factors, including physiological variations, environmental conditions-climate, pollution, diseases and pests, edaphic factors, geographic variation, genetic factors, and amount of plant material/space and manual labor needs[17].

Antifungal activity: The results of the antifungal assays with essential oils of the two samples are summarized in Table-2. Both essential oils obtained from Hangzhou and Wenzhou plants showed varying inhibitory activity on all the microorganisms tested. Whereas, the oils from Wenzhou plants were more active than those of Hangzhou plants in the assays. Among the tested fungi, *Fusarium graminearum*, *Sclerotinia sclerotiorum*, *Colletorichum gloeosporioides* showed significant sensitive to the oils from Wenzhou comparing to the

TABLE-1
PERCENTAGE COMPOSITION OF VOLATILE COMPONENTS OF ESSENTIAL OILS OF *C. praecox* LEAF COLLECTED FROM TWO DIFFERENT LOCATIONS IN CHINA

No.	Components name	RT (min)	RI	Per. (%)	
				1	2
1	1S-α-Pinene	4.7106	931	0.11	1.52
2	β-Phellandrene	5.7494	971	0.33	-
3	β-Pinene	5.8359	974	0.09	0.62
4	β-Myrcene	6.2687	991	0.18	0.52
5	α-Phellandrene	6.6365	1004	0.12	0.36
6	α-Terpinolen	7.0477	1015	-	0.13
7	Eucalyptol	7.5239	1029	0.75	19.02
8	α-Ocimene	8.1513	1046	-	0.49
9	β-Ocimene	8.1514	1046	0.51	-
10	Linalool	10.0773	1101	0.90	2.30
11	Camphor	11.7219	1141	-	0.44
12	Borneol	12.6524	1163	-	1.32
13	2-Methyl-6-methylene-7-octen-4-ol	12.7389	1165	-	0.96
14	4-Terpineol	13.1285	1175	0.44	1.25
15	*p*-menth-1-en-8-ol	13.7128	1189	0.60	6.65
16	Pentanoicacid,3,7-dimethyl-2,6-octadienyl ester, (E)-	16.5260	1255	0.57	-
17	Geraniol	16.6341	1258	-	5.29
18	1,2,5,5,6,7-Hexamethylbicyclo[4.1.0]hept-2-en-4-one	17.3483	1275	0.27	0.24
19	γ-Limonene	19.1010	1316	-	2.12
20	Terpilene	20.5725	1351	-	12.41
21	Nerylacetate	21.1785	1365	0.23	-
22	Copaene	21.4382	1371	0.21	0.07
23	2-Buten-1-one,1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	21.8710	1382	0.55	-
24	Geranyl Acetate	22.0224	1385	0.74	2.13
25	β-Elemen	22.1738	1389	4.67	0.39
26	Caryophyllene	23.3424	1420	9.31	4.53
27	γ-Elemene	24.0782	1440	0.86	-
28	(+)-Cycloisosativene	24.4677	1452	0.46	-
29	α-Caryophyllene	24.7922	1461	2.22	0.96
30	Germacrene D	25.6145	1484	7.30	0.24
31	Eudesma-4(14),11-diene	25.7445	1488	1.04	-
32	Germacrene B	26.0258	1496	7.44	-
33	Bicyclo[3.1.1]hept-2-ene,2,6-dimethyl-6-(4-methyl-3-pentenyl)-	26.0906	1498	-	2.61
34	8-Isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	26.1989	1501	2.61	-
35	α-Farnesene	26.3503	1511	-	0.78
36	4,9-Muuroladiene	26.3937	1513	1.83	-
37	δ-Cadinene	26.5883	1525	6.17	0.70
38	Cadala-1(10),3,8-triene	26.9346	1546	0.54	-
39	Elemol	27.0861	1555	1.30	-
40	Squalene Epoxide	27.1077	1557	-	0.15
41	Elixene	27.1727	1561	8.52	-
42	nerolidol	27.3025	1568	1.77	2.29
43	(-)-Spathulenol	27.4973	1580	2.16	-
44	Furan,3-(4,8-dimethyl-3,7-nonadienyl)-, (E)-	27.5405	1583	-	21.64
45	(+)-γ-Gurjunene	27.5838	1586	2.69	-
46	Ylangene	27.7137	1593	2.31	-
47	Humulene oxide II	27.9301	1609	0.82	-
48	δ-Selinene	28.0599	1621	0.78	-
49	Selinenol	28.2330	1636	2.47	-
50	λ-Cadinol	28.3628	1647	-	0.38
51	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-,(1α,4α,8α)-	28.3629	1647	5.24	-
52	6,6,10-Trimethylundeca-3,8,10-triene-2,7-dione	28.4277	1653	-	0.99
53	(-)-Alloisolongifolene	28.5360	1662	10.20	-
54	juniper camphor	28.9688	1700	0.88	-
55	(2Z, 6E)-Farnesol	29.2067	1726	0.64	0.76
56	*n*-Hexadecanoic acid	31.1760	1967	0.85	-
57	Phytol	32.1931	2118	2.15	-

1, Hangzhou; 2, Wenzhou

TABLE-2
ANTIFUNGAL ACTIVITY OF THE STUDIED ESSENTIAL OILS AGAINST EIGHT PHYTOPATHOGENIC FUNGI STRAINS USING MINIMUM INHIBITORY METHODS

Phytopathogenic fungi	MIC[a]		
	1	2	Carbendazim[b]
Fusarium graminearum	32	8	8
Fusarium avenaceum	32	16	8
Botrytis cinerea	32	16	8
Cylindrocarpon destructans	32	8	4
Monilinia fructicola	> 32	16	8
Sclerotinia sclerotiorum	16	8	8
Helminthosporium turcicum	16	8	4
Colletorichum gloeosporioides	16	8	8

[a]MIC: Minimal inhibitory concentration was determined by a macro-dilution method and expressed in µg/mL (m/v), [b]Reference compound (positive controls). 1, Hangzhou; 2, Wenzhou

commercial fungicide carbendazim as the positive control. The significant difference of the antifungal activity between the two oils could be attributed to the high content of the oils in Wenzhou plant, such as furan, 3-(4,8-dimethyl-3,7-nonadienyl)-, (E)- (21.69 %), eucalyptol (19.02 %), and terpilene (12.41 %). Moreover, regarding their biological properties, it has to be kept in mind that essential oils are complex mixtures of numerous molecules, and one might wonder if their biological effects are the result of a synergism of all molecules or reflect only those of the main molecules present at the highest levels according to gas chromatographic analysis. Thus, synergistic functions of the various molecules contained in an essential oil, in comparison to the action of one or two main components of the oil, seems questionable. However, it is possible that the activity of the main components is modulated by other minor molecules[18].

In conclusion, the examination of the two oils in this study showed promising prospects for the utilization of natural plant essential oils as a potential source of sustainable eco-friendly botanical fungicides, on the basis of their efficacy on different types of plant pathogens and their low cost and easy availability. However, because the *in vitro* effects did not always provide a good criterion for their *in vivo* performances, additional studies are necessary to verify the effectiveness in field conditions.

Brine shrimp bioassay: In the evaluation of plant extract toxicity by the brine shrimp bioassay, an LC_{50} value lower than 1,000 µg/mL is considered bioactive[16]. In this study, the essential oils from of Hangzhou and Wenzhou exerted an LC_{50} values of 30 and 22 µg/mL, respectively, suggesting that the two oils have powerful toxic activities.

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