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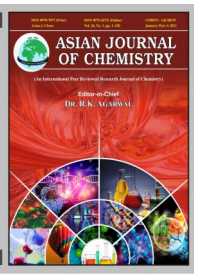

# Chemical Composition, Antifungal Activity and Toxicity of Essential Oils from Leaves of *Chimonanthus praecox* and *Chimonanthus zhejiangensis*

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Essential oils of two Calycanthaceae species, including *Chimonanthus praecox* and *Chimonanthus zhejiangensis*, harvested in Hangzhou, Zhejiang province, extracted by hydrodistillation, were analyzed by GC/MS. The antifungal activity of the oils against eight phytopathogenic fungi (*Botrytis cinerea*, *Fusarium graminearum*, *Fusarium graminearum*, *Cylindrocarpon destructans*, *Helminthosporium turcicum*, *Colletorichum gloeosporioides, Sclerotinia sclerotiorum* and *Monilinia fructicola*) was tested by determining minimum inhibitory concentrations using the microdilution method. The two oils exhibited potent antifungal activities with MIC values of 8-32 µg/mL. The two oils were considered bioactive, showing an $LC_{50}$ value of 32 and 38 µg/mL in the *Artemia salina* lethality test.

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

## INTRODUCTION

Extracts of aromatic plants, including essential oils, have been used in food, medicine and perfumery for decades, due to their special flavours and functions. Among them, the essential oils are considered to be the most important antimicrobial agents and are suspected to have antioxidant, insecticidal, cytotoxic and antiinflammatory activities as well[1].

*C. praecox* and *C. zhejiangensis*, belong to the Calycanthaceae family, are deciduous Chinese shrub that has survived since the tertiary period. They are both famous traditional fragrant flower plant with high ornamental value in China. *Chimonanthus* plants are also traditional Chinese herbal medicine for the treatment of colds, analgesic, coughs, asthma and other disorders[2]. In recent years, more attention has been paid to research and development of the essential oil products of *Chimonanthus* plants. A number of literatures have reported the composition of essential oil extracted from the leaves, flowers and seeds of *Chimonanthus* plants, such as *C. nitens*[3], *C. zhejiangensis*[4] and *C. praecox*[5].

In the present work, essential oils were extracted from the fresh leaves of *C. praecox* and *C. zhejiangensi,* followed by composition analysis using GC-MS. The antifungal and toxic activities of the extracted oils were especially evaluated. It is noteworthy that the biological activities of the extract of the fresh leaves of *C. praecox* and *C. zhejiangensi* are reported for the first time.

## EXPERIMENTAL

The fresh leaves of *C. praecox* and *C. zhejiangensis* were collected on Hangzhou of Zhejiang province, China. Botanical identification was carried out by Prof. Li Gengyou. Voucher specimens (No. 0270010 and 0270013) of the samples have been deposited with Plant laboratory of Zhejiang A & F University.

**Isolation of essential oil:** Dried leaves of *C. praecox* and *C. zhejiangensis* were subjected to hydrodistillation for 5 h and 4 h, resp., using a clevenger-type apparatus. The obtained oils were dried (anh. $Na_2SO_4$) and stored in sealed flasks at 4 °C.

**Gas chromatography/mass spectrometry (GC/MS) analysis:** GC/MS 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 KI (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 graminearum*, *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 medium[6].The serial doubling dilution of the essential oil and its major compound was prepared in dimethyl sulfoxide, 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

TABLE-1
CHEMICAL COMPOSITION OF THE LEAVE ESSENTIAL OILS OF *C. praecox* AND *C. zhejiangensis*

| Compounds | m.f. | RT | RI | Relative content (%) | |
|---|---|---|---|---|---|
| | | | | *C. praecox* | *C. zhejiangensi* |
| 1S-α-Pinene | $C_{10}H_{16}$ | 4.7105 | 931 | - | 1.25 |
| 1R-α-Pinene | $C_{10}H_{16}$ | 4.7321 | 932 | 8.01 | - |
| Camphene | $C_{10}H_{16}$ | 5.1000 | 946 | 0.10 | 4.70 |
| β-Phellandrene | $C_{10}H_{16}$ | 5.7492 | 971 | 1.43 | |
| β-Pinene | $C_{10}H_{16}$ | 5.8358 | 974 | 3.02 | 0.16 |
| β-Myrcene | $C_{10}H_{16}$ | 6.2686 | 991 | 2.04 | 1.41 |
| α-Phellandrene | $C_{10}H_{16}$ | 6.658 | 1004 | 2.30 | - |
| Eucalyptol | $C_{10}H_{18}O$ | 7.5237 | 1029 | 39.44 | 2.16 |
| β-Ocimene | $C_{10}H_{16}$ | 8.1512 | 1046 | 0.96 | 0.39 |
| γ-Terpinene | $C_{10}H_{16}$ | 8.4974 | 1056 | 0.25 | - |
| (+)-4-Carene | $C_{10}H_{16}$ | 9.5794 | 1087 | 0.15 | 0.07 |
| Linalool | $C_{10}H_{18}O$ | 10.0772 | 1101 | 0.26 | 0.36 |
| Borneol | $C_{10}H_{17}OH$ | 12.7172 | 1165 | - | 5.43 |
| 4-Terpineol | $C_{10}H_{18}O$ | 13.1283 | 1175 | 0.86 | 0.15 |
| *p*-Menth-1-en-8-ol | $C_{10}H_{18}O$ | 13.7558 | 1190 | 4.82 | - |
| Geraniol | $C_{10}H_{18}O$ | 16.5257 | 1255 | 1.36 | 0.91 |
| Bornyl acetate | $C_{12}H_{20}O_2$ | 17.9540 | 1289 | - | 23.29 |
| γ-Limonene | $C_{10}H_{16}$ | 19.0792 | 1315 | 1.54 | 0.06 |
| Terpilene | $C_{10}H_{16}$ | 20.4641 | 1348 | 4.43 | - |
| Geranyl Acetate | $C_{12}H_{20}O_2$ | 22.0006 | 1385 | 0.64 | 4.17 |
| β-Elemen | $C_{15}H_{24}$ | 22.1520 | 1388 | 0.20 | 0.13 |
| Caryophyllene | $C_{15}H_{24}$ | 23.2989 | 1418 | 3.92 | 1.70 |
| α-Caryophyllene | $C_{15}H_{24}$ | 24.7704 | 1460 | 1.28 | 0.70 |
| (+)-Epi-bicyclosesquiphellandrene | $C_{15}H_{24}$ | 25.4413 | 1479 | 0.09 | 0.34 |
| Germacrene D | $C_{15}H_{24}$ | 25.6360 | 1485 | 2.42 | 1.00 |
| γ-Elemene | $C_{15}H_{24}$ | 26.0039 | 1495 | 1.76 | - |
| α-Muurolene | $C_{15}H_{24}$ | 26.0905 | 1498 | 1.18 | - |
| Longifolenaldehyde | $C_{15}H_{24}O$ | 26.1338 | 1499 | - | 1.08 |
| α-Farnesene | $C_{15}H_{24}$ | 26.3501 | 1511 | 0.89 | - |
| δ-Cadinene | $C_{15}H_{26}$ | 26.5882 | 1525 | 2.30 | - |
| Isoledene | $C_{15}H_{24}$ | 26.6099 | 1526 | - | 1.21 |
| 1,4-Cadinadiene | $C_{15}H_{24}$ | 26.7396 | 1534 | 0.11 | 0.48 |
| Aromadendrene oxide-(1) | $C_{15}H_{24}O$ | 26.9561 | 1547 | - | 0..47 |
| Alloaromadendrene oxide-(1) | $C_{15}H_{24}O$ | 27.1292 | 1558 | - | 0.31 |
| (-)-Alloaromadendrene | $C_{15}H_{24}$ | 27.1508 | 1559 | 0.34 | - |
| 4-Hexadecen-6-yne, (E)- | $C_{15}H_{24}O$ | 27.3240 | 1570 | | 4.87 |
| (-)-Spathulenol | $C_{15}H_{24}O$ | 27.4754 | 1579 | 0.60 | |
| Furan, 3-(4,8-dimethyl-3,7-nonadienyl)-, (E)- | $C_{15}H_{22}O$ | 27.5404 | 1583 | - | 18.21 |
| (+)-γ-Gurjunene | $C_{15}H_{24}$ | 27.5620 | 1584 | 0.46 | - |
| Carotol | $C_{15}H_{26}O$ | 27.8217 | 1600 | - | 1.55 |
| α-Cedrene | $C_{15}H_{24}$ | 28.1679 | 1630 | 0.55 | 0.68 |
| λ-Muurolol | $C_{15}H_{26}O$ | 28.3626 | 1647 | 3.01 | - |
| 6,6,10-Trimethylundeca-3,8,10-triene-2,7-dione | $C_{14}H_{20}O_2$ | 28.4276 | 1653 | - | 4.25 |
| γ-Cadinene | $C_{15}H_{26}$ | 28.5141 | 1660 | 4.48 | - |
| Calarene epoxide | $C_{15}H_{24}O$ | 28.6873 | 1675 | - | 2.44 |
| 1,4,7,-Cycloundecatriene,1,5,9,9-tetramethyl-, Z,Z,Z- | $C_{15}H_{24}$ | 29.0768 | 1712 | - | 3.05 |
| 2Z,6E-Farnesol | $C_{15}H_{26}O$ | 29.2066 | 1726 | 0.44 | 1.86 |
| Santalol | $C_{15}H_{24}O$ | 29.4230 | 1750 | - | 0.32 |
| (-)-Isolongifolol, methyl ether | $C_{15}H_{24}O$ | 29.5312 | 1762 | - | 1.31 |
| (+)-α-Elemene | $C_{15}H_{24}$ | 29.7476 | 1786 | 0.30 | - |
| 1,6-Octadien-3-ol, 4,7-dimethyl-, isovalerate | $C_{10}H_{18}O$ | 30.0073 | 1816 | - | 0.95 |

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 $1 \times 10^6$ 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:** The essential oils of *C. praecox* and *C. zhejiangensis* were assayed using a modified test of lethality to *A. salina*[7]. The eggs of *A. salina* were incubated in a hatching chamber with seawater 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 seawater 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 7.0 statistical program.

## RESULTS AND DISCUSSION

The oil yield for *C. praecox* was 1.5% (v/w), with 35 compounds identified that represented 95.94 % of the oil content. The main components were eucalyptol (39.44 %), 1R-α-pinene (8.01 %), *p*-menth-1-en-8-ol (4.82 %), γ-cadinene (4.48 %) and terpilene (4.43 %; Table-1). A number of studies on essential oil content and constituents from the flowers and seeds of *C. praecox* have been performed. However, these results differ from our findings. Si *et al.*[5] showed that supercritical carbon dioxide extraction of the essential oil detected 57 compounds in the flowers from cultivated sources, with benzyl alcohol (28.48 %), cadinol (14.61 %) and bornyl acetate (6.52 %) as the major constituents. Thus, essential-oil-bearing plants usually show a variable chemical composition due to both intrinsic (sexual, seasonal, ontogenetic and genetic variations) and extrinsic (ecological and environmental aspects) factors.

The yield of oil from *C. zhejiangensis* was 2.5 % (v/w), higher that the yield obtained from *C. praecox*. GC/MS identified 35 compounds, representing 91.42 % of the oil content. Bornyl acetate and 3-(4,8-dimethyl-3, 7-nonadienyl)-furan, (E)- were the main monoterpene hydrocarbons accounting for 23.29 and 18.21 %, respectively of the chromatograms (Table-1). Interestingly, 1,4-cineole, trioctylamine, caryophyllene and α-terpinylpropionate which were described in an earlier report to be the major constituents of *C. zhejiangensis* oil[4] were either not detected or present at trace levels in our analysis.

Evaluation of MIC values of the oils showed a variability of inhibition among all the fungi tested (8-32 µg/mL) (Table-2). According to these activity ranks, the essential oils of *C. praecox* and *C. zhejiangensis* showed a effective antifungal activity against most of the tested strains, which could be attributed to the high content of the oils, of compounds with known antimicrobial activity, such as linalool, (-)-spathulenol and caryophyllene. Against *F. graminearum*, *C. destructans and M. fructicola*, the *C. zhejiangensi* oil was more active than the *C. praecox* oil. The fungistatic properties of the oils are suspected to be associated with the high content of terpenes type components[8]. On the other hand, it is also possible that the minor components might be involved in some type of synergism with the other active compounds. The observed antifungal activity is of importance, because fungal infections have increased considerably, attributed to its intrinsic resistance to commercial fungicides.

TABLE-2
ANTIFUNGAL AACTIVITY OF THE ESSENTIAL OILS OF *C. praecox* AND *C. zhejiangensis* AGAINST EIGHT PHYTOPATHOGENIC FUNGI STRAINS

| Phytopathogenic fungi | MIC (µg/mL (m/v)) | | |
|---|---|---|---|
| | *C. praecox* | *C. zhejiangensi* | Carbendazim |
| *Fusarium graminearum* | 32 | 8 | 8 |
| *Fusarium graminearum* | 32 | 16 | 8 |
| *Botrytis cinerea* | 32 | 16 | 8 |
| *Cylindrocarpon destructans* | 32 | 8 | 4 |
| *Monilinia fructicola* | > 32 | 16 | 8 |
| *Sclerotinia sclerotiorum* | 16 | 32 | 8 |
| *Helminthosporium turcicum* | 16 | 32 | 4 |
| *Colletorichum gloeosporioides* | 16 | 32 | 8 |

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[7]. In this study, the essential oils from of *C. praecox* and *C. zhejiangensi* exerted an $LC_{50}$ values of 32 and 38 µg/mL, respectively, suggesting that the two oils have powerful toxic activities.

## ACKNOWLEDGEMENTS

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