Natural Product Sciences
8(4) : 133-136 (2002)

Composition of the Essential Oil of *Chrysanthemum sibiricum*, and Cytotoxic Properties

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Abstract – GC-MS data on the volatile oil (CS-oil) of *Chrysanthemum sibiricum* herbs led to the identification of 2-methoxythioanisol, (+)-camphor, geraniol, citral, thymol, eugenol, β-caryophyllene oxide, β-caryophyllene, β-eudesmol, juniper camphor together with an unknown substance using the mass spectral library and literature data. CS-oil exhibited significant cytotoxicities on HL-60 (IC_{50} 12.5 μg/ml) cell and mild on HepG-2 cell (IC_{50} 102.4 μg/ml), though the antioxidant ability was found not to be potent (IC_{50} 97.2 μg/ml). However, the component eugenol showed potent antioxidant ability but mild cytotoxicity. Methyleugenol with no phenolic OH showed less potent cytotoxic and antioxidative properties than eugenol suggesting that phenolic OH plays an important role for the cytotoxic and antioxidant abilities. The oil-pretreatment prevented lipid peroxidation induced by bromobenzene in the rat. Therefore, it was demonstrated that CS-oil could be a cytotoxic agent with antioxidant properties.
Keywords – *Chrysantemum sibiricum*, Compositae, essential oil, eugenol, cytotoxicity, antioxidative.

Introduction

Chrysanthemum sibiricum (Compositae) is a perrenial herb wildly distributed in Korea. This whole plant and buds have been used to treat pneumonia, bronchitis, cough, and common cold in addition to cystitis and menstrual irregularity (Yun, 1995). This plant has strong flavor but the essential oil has not been so far analyzed. It has been reported that the essential oils with flavor exhibit the activities of anti-inflammation (Martin, *et al.*, 1993), anticarcinogen (Zheng *et al.*, 1992), antibacteria (Scaltsa *et al.*, 1999) and local anesthetics (Ghelardini *et al.*, 1999).

Eugenol, phytochemically classified into phenylpropenes, structurally has phenol and an allyl group attached to arenes. Based on its structure, it was assumed to have both free radical-scavenging activity due to phenolic OH and cytotoxicity due to its electrophilicity. Therefore, the eugenol, the constituent of *C. sibiricum* was tested on DPPH, MTT assay *in vitro* and *in vivo* anti-lipid peroxidative ability. For further elucidation of structure-activity relationship, eugenol was methylated in this experiment and tested also on DPPH and MTT assays.

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Materials and Methods

Plant material – The herbs of *Crysanthemum sibiicum* was collected in Wonju, Korea. The plant origin was identified as *C. sibiicum* by Dr. G.T. Kim (Department of Forestry, Sangji University, Wonju, Korea) and the voucher specimen (#NATCHEM 25) is deposited in the hebarium of Division of Applied Plant Sciences, Sangji University, Korea.

Steam-distillation extraction – This plant material (1.5 kg) was extracted under steam distillation apparatus for 4 h. The distillated liquid was extracted with diethyl ether and dehydrated with anhydrous sodium sulfate followed by being evaporated on a rotatory evaporator at 40°C. The residual oil (9 g) was obtained and used for chemical analysis, further purification and biological assays.

GC-Analysis – Column {DB-1 (length 30 meters, i.d. 0.25 mm, film thickness 0.25 μm, J&W scientific, USA)}; Column temp. program {init. temp. 50°C (3 min), temp. increase velocity (8°C/min), final temp. 250°C (10 min)}; solvent cut (3 min); temp. program {injector (250°C), transfer line (250°C), ion source (150°C), manifold (70°C)}; detector {Electron Impact-Quadrupole 1 (EI energy 70 eV); carrier gas {He (99.99%), flow rate (1.5 ml/min)}.

Isolation of eugenol and preparation of methyleugenol – Extracted material was subjected to silica gel column chromatography (Art No. 7734, Merck, Germany, 3×21 cm, 60 g) with the eluent of *n*-hexane-ethyl acetate (10:1). The eluate was collected by each 10 ml. Thirty fractions were collected and checked by spraying vanillin-sulfuric acid reagent. The fractions showing similar TLC pattern were combined and dried *in vacuo*. Fractions over the retention volume 160-280 ml was dried in *vacuo* and yielded colorless oil (compound **1**, Rf 0.34, 2.2 g). This compound was identified as eugenol on the basis of NMR and MS data (Kwon *et al.*, 2001). Methyleugenol (**2**) was prepared by methylation of eugenol under a usual method using dimethylsulfate and K_2CO_3. Methyleugenol (**2**). Colorless oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.35 (2H, d, J=6.7 Hz, H-1), 3.88, 3.87 (2×OCH_3), 5.09 (1H, dm, J=16.7 Hz, H-3_{trans}), 5.11 (1H, dm, J=10.0 Hz, H-3_{cis}), 5.98 (2H, ddt, J=16.7, 10.1, 6.7 Hz, H-2), 6.75 (1H, dd, J=8.0, 1.8 Hz, H-6), 6.75 (1H, d, 1.8 Hz, H-2), 6.82 (1H, d, J=8.0 Hz, H-5); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 40.5 (C-1), 56.5, 56.6 (OCH_3), 112.1 (C-5), 112.7 (C-3), 116.2 (C-2), 121.9 (C-6), 133.4 (C-2), 138.3 (C-1), 148.2 (C-4), 149.7 (C-3).

DPPH radical scavenging effect – The scavenging effect corresponded to the intensity of quenching DPPH radical was determined as described by Xiong *et al.* (1996). The effects were expressed by the percent scavenging (%) of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical *vs.* that of control. The IC_{50} values were calculated from regression lines where the abscissa represented the concentration of tested compound and the ordinate the average percent reduction of DPPH radical from three separate tests. Tocopherol was used as a positive control.

MTT assay – The *in vitro* tests against 3LL cell (human lung carcinoma) was carried out essentially according to the method described previously (Denizot *et al.*). Cells (1×10^4) were seeded in each well containing 100 μl of RPMI medium supplemented with 10% FBS in a 96-well microtiter plate and incubated overnight. The test samples, CC-oil, CNA, and compound **2**, were dissolved in dimethylsulfoxide (DMSO) and were added in serial dilution (the final DMSO concentrations in all assays did not exceed 0.01%). Twenty-four hours after seeding, 100 μl new media or test samples were added, and the plates were incubated for 48 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue which formed in the cells was replaced by adding 50 μl DMSO. Optical density was measured at 540 nm. Cisplatin was used as a positive control.

Anti-lipid peroxidation assay in the rat – Male Sprague-Dawley rats (weighting 150-200 g) were fed *ad libitum* with commercial standard rat diet and water, and maintained on 202°C on a 12 hr light/dark cycle. The animals were intraperitoneally administered daily at a dose of 10 mg/kg of *C. sibiricum* oil for a period of one week. After this, bromobenzene (480 mg/kg) was intraperitoneally injected twice a day for two days and the animals were decapitated 24 h after the final injection.

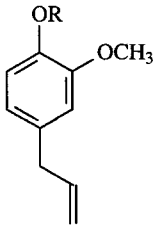

Fig. 1. Structure of eugenol (R=H) and methyleugenol (R=CH_3).

The animals were sacrificed by exsanguination from the abdominal aorta under slightly anesthesia with CO_2 gas. The liver was exhaustively perfused with ice-cold normal saline through the portal vein until uniformly pale and weighed. The thiobarbituric acid (TBA) reactive substance in the liver was maesured as a marker of lipid peroxidation by the method of Ohkawa *et al.* (1979). An aliquot (0.4 ml) of 10% liver homogenate in 0.9% NaCl was added to 1.5 ml of 8.1% SDS, 1.5ml of 20% acetate buffer (pH 3.5) and 1.5 ml of 0.8% TBA solution. The mixture was heated at 95°C for 1 h. After cooling, 5.0 ml of n-butanol: pyridine (15:1) was added for extraction, and the absorbance of the n-butanol:pyridine layer at 532 nm was measured for the determination of TBA reactive substance.

Results and Discussion

Many peaks on gas chromatogram of the essential oil of *Chrysanthemum sibiricum* were shown. Mass spectra of relatively abundant eleven peaks were taken and compared with the data of mass spectral library. Based on our GC-MS data and the literatures, 8 compounds were identified as 2-methoxythioanisol (**1**), (+)-camphor (**2**), geraniol (**3**), citral (**4**), thymol (**5**), eugenol (**6**), β-caryophyllene oxide (**7**), β-caryophyllene (**8**), β-eudesmol (**10**), juniper camphor (**11**) together with an unknown substance (**9**) The compounds 2-methoxythioanisol (**1**) and eugenol (**6**) with molecular ions at the base peak has aromatic substances whereas thymol (**5**) exhibited the base peak at m/z 135.1 [M-CH_3].

It has been reported that many volatile substances have cytotoxic activities. Generally, the volatile oil contains phenylpropenes in addition to terpenoids, though the former is less common that the latter. We assummed that the volatile

Table 1. Composition of the essential oil obtained from the herbs of *Chrysanthemum sibiricum*.

Compound (Peak No)	Rt. time	MS {m/z (%)}	Total ion peak Area (mm^2)	Identif.
2-Mehtoxythianisol (1)	3′ 44″	154.2 (100), 108.1 (82), 81.1. (64)	13.5	MS
(+)-Camphor (2)	5′ 03″	152.2 (60), 95.1 (100), 81.2 (63)	82.5	MS, GC
Geraniol (3)	5′ 20″	154.2 (4), 110.2 (58), 95.0 (100)	97.6	MS
Citral (4)	5′ 34″	152.2 (8), 91.1 (56), 79.1 (100)	16.5	MS, GC
Thymol (5)	6′ 23″	150.1 (34), 135.1 (100), 95.1 (43)	19.9	MS, GC
Eugenol (6)	7″ 5″	164.1 (100), 149.1 (37), 103.1 (23)	65.7	MS, GC
Caryophyllene oxide (7)	8′ 57″	220.3 (22), 205.2 (100), 119.1 (78)	18.3	MS, GC
β-Caryophyllene (8)	9 00″	204.3 (18), 161.1 (63), 79.1 (100)	19.6	MS
Unknown (9)	9′ 17″	220.3 (22), 159.1 (61), 119.1 (100)	20.3	–
β-Eudesmol (10)	9′ 24″	222.3 (8), 161.2 (100), 95.1 (60)	22.4	–
Juniper camphor (11)	9′ 32″	222.2 (35), 204.3 (100), 135.1 (79)	22.6	MS

oil has cytotoxic and antioxidant properties. The cytotoxicities of terpenoids and phenylpropenes may be mainly dependent on oxidative stress based on the characteristic structure, e.g., including the partial structure of exomethylene, allyl or α,β-unsaturated carbonyls. Since eugenol that containing both phenolic and allylic structure, the cytotoxicities and free radical scavenging effect of eugenol and methyleugenol together with the essential oil (CS-oil) of *C. sibiricum* were tested.

By the MTT test, the IC_{50} of CS-oil was calculated. (12.5 μg/ml against HL-60; 102.4 μg/ml against HepG2). Eugenol showed the lower IC_{50} values (39.2 μg/ml against HL-60; 183.6 μg/ml against HepG2 whereas methyleugenol the lowest values (76.3 μg/ml against HL-60; 222.2 μg/ml against HepG-2), suggesting that eugenol do not reach the potency of CS-oil. It is also notable that methylation of eugenol decrease its cytotoxicity. It should be also mentioned that the ring-activation of *p*-positioned methoxy to allyl group is weaker than that of hydroxy. We presumed that *p*-substitution to allyl may contribute to the electrophilicity potency of exomethylene and the effect of *p*-OCH_3 is weaker than that of *p*-OH.

On the other hand, eugenol showed potent free radical-scavenging effect (IC_{50} 7.7 μg/ml) than other two substances methyleugenol (IC_{50} 92.4 μg/ml) and CS-oil (IC_{50} 97.2 μg/ml) in DPPH assay. The ionizable phenolic OH may critically contribute to the stability of eugenol radical. CS-oil containing eugenol exhibited weak antioxidant ability.

Bromobenzene can be metabolized in the animal to bromobenzene 3,4-oxide by hepatic microsomal enzymes accompanied by the generation of superoxide anion radical (·O2-) which results in the formation of malondialdehyde. Bromobenzene treatment increased the malondialdehyde from 18.9±0.72 nmole/g tissue of the normal rat to 49.3± 1.98 nmole/g tissue. In the oil-preteated group, the MDA value was shown by 38.7±2.42 nmole/g tissue (35% inhibition).

Generally, the flavor components that can be classified into essential oil are perfumed from the periderm of plant organ. Several of the components function as oxidant or antioxidant abilities based on the characteristic structures. Even a single compound could include the partial structures that induce oxidative and antioxidative functions. Furthermore, CS-oil would be beneficial for a cytotoxic substance as well as for an antioxidant one. The principles may be attributed to the volatile terpenoids, e.g., maybe β-caryophyllene, β-caryophyllene oxide and others. These results may contribute to the availability of this oil.

Acknowledgements

This research was supported by the grant for 2001 Good Health R & D project (01-PJ2-PG3-21604-0008) by the Ministry of Health and Welfare, Korea.

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(Accepted September 20, 2002)