

Full Length Research Paper

Analysis of chemical composition of *Chrysanthemum indicum* flowers by GC/MS and HPLC

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***Chrysanthemum indicum* flower is a traditional Chinese medicine with strong aroma and many previous studies focused on its essential oil. GC/MS and HPLC were used to determine its volatiles, flavonoids and flavonoid glycosides. Sixty three volatiles were detected and the abundant volatiles included 2,6,6-trimethyl-bicyclo[3.1.1]hept-2-en-4-ol, 2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4.4]non-3-ene, germacrene D, α -neoclovene, eucalyptol, α -pinene. Ten flavonoids were identified. Quercitrin, myricetin and luteolin-7-glucoside were abundant flavonoids. The bioactivities of the abundant components in *Chrysanthemum indicum* flower were discussed. It is considered that *Chrysanthemum indicum* flower is a good source of natural quercitrin and myricetin, which is significant for the development of potential pharmaceuticals.**

Key words: *Chrysanthemum Morifolium*, volatile, flavonoid, glycosides, GC-MS, HPLC.

INTRODUCTION

Chrysanthemum indicum has been used as a herbal medicine, which is prescribed for anti-inflammatory, analgesic, antipyretic purposes and the treatment of eye disease in Chinese traditional preparations. It showed inhibitory activity against rat lens aldose reductase and against nitric oxide (NO) production in lipopolysaccharide-activated macrophages (Yoshikawa et al., 1999; 2000). It was also proved to be effective to inhibit the agglutination of blood platelet and promote the myocardial blood circulation and white cell phagocytosis, and therefore it was used to treat many diseases such as furuncle (Zhang, 1997). However, the pharmacological activity and bioactive constituents of this natural medicine are left uncharacterized (Yoshikawa et al., 1999).

Flavonoids are proved to be important bioactives in herbal plants (Gohar et al., 2009; Goze et al., 2009; McNulty et al., 2009; Zhou et al., 2009). Several flavonoids and sesquiterpenes have been isolated from the Chinese natural medicine. Volatiles in medicinal plants play a vital role in healthcare systems of the most

traditional and modern medicines (Hassanpouraghdam, 2009). *Chrysanthemum indicum* flower has a strong aroma and many of the previous studies focused on the essential oil of this plant (Shen et al., 2004; Wang et al., 2006; Ye and Deng, 2009). Little information on the flavonoids in *Chrysanthemum indicum* was reported. Chemical composition of this plant was shown to be depended on the soil and climate where it is grown (Shen et al., 2004). Suichang County of Zhejiang Province in China is a major area where wild *Chrysanthemum indicum* flower is harvested. Gas chromatography/ mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) were used to determine the composition of volatiles and flavonoids including flavonoid glycosides in *Chrysanthemum indicum* flower collected from Suichang County of China in present paper.

MATERIALS AND METHODS

Materials and Equipments

Dry flower of *Chrysanthemum indicum*, which was supplied by Agricultural Bureau of Suichang County, Zhejiang Province in

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China, was ground using a EUPA TSK-927S grinder (Cankun Co. Ltd, Shanghai, China) and sifted through 2-mm mesh sifter (Lantian Co. Ltd., Hangzhou, China) before extraction. Reference compounds for HPLC were Sigma products (Sigma-Aldrich, St. Louis, USA). The other chemicals used in the test were HPLC grade reagents.

Equipment for extracting volatiles was a successive distillation extraction (SDE) apparatus described by Liang et al (2005). A gas chromatography/mass spectrometry (GC/MS, Model HP6890/5973, Agilent Technologies Inc., CA, USA) was used to determine volatiles. The column for the GC/MS was a 30 m x 0.32 mm (i.d.) HP-INNO wax fused capillary column with a film thickness of 0.5 μ m (Agilent Technologies Inc., CA, USA). A high performance liquid chromatography (HPLC, Model LC20A, Shimadzu Co., Kyoto, Japan) was used to determine flavonoids and their glycosides, and the HPLC column was a 5- μ m TC-C18 column with 4.6 x 250 mm (Agilent Technologies Inc, CA, USA). There were two HPLC mobile phases used in the test. The HPLC mobile phase A was mixture of acetonitrile /acetic acid /water (3/0.5/96.5,v/v/v) and mobile phase B was a mixture of acetonitrile /acetic acid /water (50/0.5/49.5,v/v/v).

Determination of volatiles

Five grams of the ground sample was used to extract volatiles in a successive distillation extraction (SDE) apparatus as methods described by Liang et al (2005). The weighted samples and 100 μ L internal standard reference ethyl caprate (0.2 μ g μ L⁻¹) were placed in the sample extraction flask containing 250 mL of freshly boiled distilled water. The extraction flask was placed in a boiling water bath. 30 mL of ethyl ether was placed in a volatiles collecting flask which was placed in 50°C water bath. The sample was extracted for 1 hr, during which the volatile compounds were evaporated and absorbed by the ethyl ether in the volatiles collecting flask. When the extraction was finished, the ethyl ether phase was transferred into a 50 mL glass tube and dehydrated with 5 g of Na₂SO₄ for 24 h. The dehydrated ethyl ether phase was then concentrated to about 1.0 mL under reduced pressure at 42°C. The concentrated volatiles sample was used for GC/MS.

The GC/MS was carried out based on a modified method by Kim et al (2007). The injector temperature was held at 250°C and injection volume was 1.0 μ L. The column temperature was programmed at 50°C for 5 min, then from 50°C to 210°C at a rate of 3°C min⁻¹, remained at 210°C for 10 min, and finally increased from 210°C to 230°C at a rate of 3°C min⁻¹. The helium carrier gas flow rate was at 1 mL min⁻¹. The mass spectrometer was worked at ionization voltage 70 eV and ion source temperature at 230°C. The volatile components were qualitatively determined by comparing their Kovats GC retention indices and mass spectra with those of authentic compounds or reported values. Relative concentrations of the identified volatiles were expressed as the ratio of the peak heights of the detected volatiles to that of internal standard reference ethyl caprate.

Determination of soluble solids

The ground flower sample (15 g) was extracted in 300 mL 60% aqueous ethanol in a water bath at 80°C for 200 min. The extracted liquor was filtered through a cotton wool and then cooled on ice. The cold filtrate was then centrifuged at 5000 x g for 15 min. The supernatant was used for determination of soluble solids and flavonoids. Twenty mL of the supernatant was transferred into a weighted dry glass dish (W1) and dried at 80°C overnight and then at 103°C for 3 h. The glass dish with dry soluble solids was transferred to a desiccator to cool for 3 h and then weighted (W2). Soluble solids concentration was calculated according to the weight difference between W2 and W1 and expressed as mg soluble

solids per kg dry flower.

Determination of flavonoids

Ten μ L of the above supernatant was injected into HPLC and the HPLC conditions were: column temperature at 35°C, linear gradient elution from 72.5% A / 27.5% B (v/v) to 65% A / 35% B (v/v) during 0-10 min, from 65% A / 35% B (v/v) to 20% A / 80% B (v/v) during 10-35 min, from 20% A / 80% B (v/v) to 0% A / 100% B (v/v) during 35 - 45 min; mobile phase flow rate 1 mL min⁻¹. The eluant was monitored at 360 nm. The tested flavonoids and flavonoid glycosides were qualitatively and quantitatively determined by comparing their retention times and peak areas with those of authentic reference compounds.

Statistics

The tests in the present paper were carried out in duplicate and the mean values of the duplicate tests were presented. Mean values and standard deviations were calculated on software of the SAS System for Windows version 8.0 (SAS Institute Inc, Cary, NC, USA).

RESULTS AND DISCUSSION

Contents of volatiles

Sixty three volatiles were identified in the sample of *Chrysanthemum indicum* flower by GC/MS. 2,6,6-trimethyl-bicyclo[3.1.1]hept-2-en-4-ol and 2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4.4]non-3-ene were the most abundant volatiles, whose relative concentrations were 89.30 and 88.25 times of that of internal reference ethyl caprate, or amounting for 21.67% and 21.41% of total concentration of the 63 detected volatiles, respectively (Table 1). Germacrene D, α -neoclovene, eucalyptol, α -pinene, 1,4-bis(1-methylethyl)-benzene, β -sesquiphellandrene, longipinane and 7, 11-dimethyl-3-methylene-1,6,10-dodecatriene were the next abundant volatiles, all of which was more than 2% of total volatiles concentration. The concentration of the top ten volatiles was 73.80% of the total detected volatiles, suggesting that they were predominated volatiles in *Chrysanthemum indicum* flower.

Volatiles play a vital role in healthcare systems of medicinal plants (Hassanpouraghdam, 2009). Among the abundant volatiles, there has been no description on the bioactivity of compounds 2, 6, 6-trimethyl-bicyclo [3.1.1] hept-2-en-4-ol and 2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4.4]non-3-ene. Essential oils which were rich in germacrene D exhibited in vitro anti-mycobacterial activity (Juliao et al., 2009). Gas-phase of α -neoclovene could react the NO₃ radical (Canosa-Mas et al., 1999), showing free radical scavenging activity. Caryophyllene oxide was a component of the tested *Chrysanthemum indicum* flower, with 1.25% of total volatiles concentration (Table 1). Plant extracts containing germacrene D, β -sesquiphellandrene, caryophyllene oxide and eucalyptol

Table 1. Relative concentrations of volatiles in *Chrysanthemum indicum* flower.

Volatiles	Relative concentration (%)^a
2,6,6-trimethyl-bicyclo[3.1.1]hept-2-en-4-ol	89.30±3.24 (21.67)
2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4.4]non-3-ene	88.25±2.48 (21.41)
Germacrene D	25.35±0.96 (6.15)
α-Neoclovene	21.02±0.87 (5.10)
Eucalyptol	20.35±1.92 (4.94)
α-Pinene	14.99±0.96 (3.64)
1,4-Bis(1-methylethyl)-benzene	12.49±0.09 (3.03)
β-Sesquiphellandrene	11.95±0.63 (2.90)
Longipinane	11.92±0.47 (2.89)
7, 11-Dimethyl-3-methylene-1,6,10-Dodecatriene	8.93±0.36 (2.17)
β-Myrcene	7.36±0.45 (1.78)
Caryophyllene	7.28±0.63 (1.77)
2,6-Dimethyl-6-(4-methyl-3-pentenyl)-bicyclo[3.1.1]hept-2-ene	7.1±0.08 (1.71)
1,2,3,6-Tetramethyl-bicyclo[2.2.2]octa-2,5-diene	6.74±0.74 (1.64)
4-(1,5-Dimethylhex-4-enyl)cyclohex-2-enone	6.43±1.02 (1.56)
Caryophyllene oxide	5.15±0.43 (1.25)
Isocyclocitral	5.07±0.61 (1.23)
Cadina-1,6,8-triene	4.1±0.12 (0.99)
α,α-4-Trimethyl-3-cyclohexene-1-methanol	3.45±0.11 (0.84)
4-Methylene-1-(1-methylethyl)-bicyclo[3.1.0]hexane	3.04±0.16 (0.74)
3,4-Dihydro-1-Naphthaleneboronic acid diethyl ester	2.92±0.17 (0.71)
Borneol	2.89±0.02 (0.70)
(Z)-3,7-Dimethyl-2,6-octadien-1-ol acetate	2.70±0.07 (0.66)
<i>trans</i> -3-Methyl-6-(1-methylethyl)-2-cyclohexen-1-ol	2.63±0.07 (0.64)
Isobornyl acetate	2.60±0.06 (0.63)
1,3,3-Trimethylcyclohex-1-ene-4-carboxaldehyde	2.46±0.08 (0.60)
Butylated Hydroxytoluene	2.42±0.12 (0.59)
4-Methyl-1-(1-methylethyl)-3-cyclohexen-1-ol	2.36±0.06 (0.57)
(E)-3(10)-Caren-2-ol	2.35±0.03 (0.57)
2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene	2.19±0.14 (0.53)
<i>cis</i> -1-methyl-4-(1-methylethyl)-2-Cyclohexen-1-ol	1.98±0.20 (0.48)
Iridomyrmecin	1.52±0.17 (0.37)
Benzoic acid, 2-(dimethylamino)-methyl ester	1.52±0.03 (0.37)
6,6-Dimethyl-2-methylene-bicyclo[2.2.1]heptan-3-one	1.44±0.12 (0.35)
2-Methyl butanoic acid phenylmethyl ester	1.43±0.10 (0.34)
α-Caryophyllene	1.29±0.08 (0.31)
5-Ethylcyclopent-1-enecarboxaldehyde	1.29±0.09 (0.31)
Camphene	1.25±0.04 (0.30)
1,2,5,5-Tetramethyl-1,3-Cyclopentadiene	1.18±0.02 (0.29)
3,7,11-Trimethyl-2,6,10-Dodecatrien-1-ol	1.18±0.05 (0.29)
Benzyl acetoacetate	1.16±0.11 (0.28)
3,7,11-Trimethyl-1,3,6,10-dodecatetraene	0.87±0.13 (0.21)
6,6-Dimethyl-bicyclo[3.1.1]hept-2-ene-2-methanol	0.86±0.05 (0.21)
4-Dcetyl-3-carene	0.86±0.03 (0.21)
β-Phellandrene	0.78±0.03(0.19)
1-Methyl-8-(1-methylethyl)-tricyclo[4.4.0.02,7]dec-3-ene-3-methanol	0.75±0.02 (0.18)
Copaene	0.68±0.00 (0.17)
1,5,5-Trimethyl-6-methylene-cyclohexene	0.65±0.05 (0.16)
3,4-Dihydro-2,2-dimethyl-2H-1-Benzopyran	0.60±0.12 (0.15)
(S)-2-Methyl-5-(1-methylethenyl)-2-Cyclohexen-1-one	0.57±0.06 (0.14)
4-Methylene-2,8,8-trimethyl-2-vinyl-bicyclo[5.2.0]nonane	0.51±0.02 (0.12)

Table 1. Contd.

2-n-Butyl furan	0.42±0.01 (0.10)
Phytol	0.42±0.03 (0.10)
4,6,6-Trimethyl- bicyclo[3.1.1]hept-3-en-2-ol	0.38±0.03 (0.09)
1,2-Diethyl-3,4-dimethyl-benzene	0.36±0.04 (0.09)
Isoaromadendrene epoxide	0.35±0.01 (0.08)
2,3-Dihydro-2,2,4,6-tetramethyl-benzofuran	0.34±0.02 (0.08)
Alloaromadendrene oxide-(I)	0.33±0.02 (0.08)
4,6-Dimethyl-2-pyrimidone	0.33±0.02 (0.08)
2-Methoxy-4-methyl-4-phenyl-2,5-cyclohexadien-1-one	0.28±0.03 (0.07)
Tetracosane	0.27±0.01 (0.07)
Hexatriacontane	0.25±0.00 (0.06)
3,7-Dimethyl-1,3,6-octatriene	0.24±0.11 (0.06)
Total	412.13±21.74 (100)

^a: 100 μL internal standard reference ethyl caprate ($0.2 \mu\text{g } \mu\text{L}^{-1}$) was extracted with the sample and relative concentration was expressed as the ratio of the peak height of tested volatile to the peak height of ethyl caprate. Data in blanket was percentage of the tested volatile in the total volatile concentration.

showed strong antimicrobial activity and was considered to be potential anti-infective agents (Ibrahim et al., 2009; Oladosu et al., 2009; Ozkan et al., 2010; Runyoro et al., 2010). Activities of free radical scavenging and antioxidation were confirmed in plant extracts containing α -pinene, β -sesquiphellandrene, (Ricci et al., 2005; Sultan et al., 2009). Myrcene, a structural analogue of linalool, and α -pinene and β -caryophyllene had a potent effect on inducing human hepatocellular carcinoma HepG2 cell apoptosis at 100 μM (Usta et al., 2009). The present study showed that *Chrysanthemum indicum* flower is rich source of bioactive phytochemicals and its pharmaceutical effects might be related to these abundant volatiles.

Contents of soluble solids and flavonoids

The extraction by 60% (v/v) aqueous ethanol solution showed that soluble solids concentration of *Chrysanthemum indicum* flower was $39.41 \pm 0.99 \text{ mg g}^{-1}$, which was 30% higher than that of black tea reported by Liang and Xu (2003). It suggests that *Chrysanthemum indicum* flower is a good source of plant material to prepare extract for additive of instant beverage or functional foods.

Fifteen peaks were detected by HPLC in the aqueous ethanol extract of *Chrysanthemum indicum* flower (Figure 1). 10 peaks were qualitatively and quantitatively determined, among which three were glycosides. The other five peaks were not identified owing to lack of authentic references. Total concentration of the detected flavonoids was $137.29 \pm 1.13 \text{ mg g}^{-1}$. Quercitrin was the most abundant flavonoid, and myricetin was the next abundant one (Table 2). The two flavonoids amounted for 65.3% of total concentration of the detected flavonoids.

Vitexin and apigenin were the least abundant flavonoids, being less than 0.1 mg kg^{-1} (Table 2). Quercitrin was reported to have anti-oxidant and anti-carcinogenic activities via its inhibition of neoplastic transformation by blocking activation of the MAPK pathway and stimulation of cellular protection signaling (Hanan et al., 2009; Ding et al., 2010). Quercitrin concentration in the *Chrysanthemum indicum* flower of this study was significantly higher than that detected in *M. edule* shoot extracts reported by Hanen et al (2009).

Myricetin exhibited several pharmacological benefits, and its antioxidant properties were thought to contribute to its cancer-preventive effects. APE₁ (apurinic/aprimidinic endonuclease) performs an essential function in DNA base-excision repair pathway and it has become a target for researchers looking for means to prevent cancer cells from surviving chemotherapy. The knocking down APE₁ could lead to tumor cell sensitivity, thus preventing cancer cells from persisting after chemotherapy (Luo et al., 2008). Myricetin was confirmed to be an inhibitor of APE₁ and it enhanced cellular sensitivity to the alkylating agent methyl methanesulfonate (Simeonov et al., 2009).

Luteolin-7-glucoside, a flavonoid derivative, ranked third abundant flavonoid compound in the tested sample (Table 2). This flavonoid glycoside was reported to have antiasthmatic activity in an ovalbumin - induced lung inflammation via the down-regulation of T helper 2 cytokine transcripts as well as the inhibition of prostaglandin E-2 production (Jin et al., 2009). It also had antioxidant and inflammatory activities (Ha et al., 2006; Silva et al, 2006), hepatoprotective effects (Lima et al., 2006) and inhibitory effect on aortic vascular smooth muscle cell proliferation (Kim et al., 2006). Based on its abundant flavonoids, it is considered that *Chrysanthemum indicum* flower is a good source of natural quercitrin and myricetin. This is of significance for

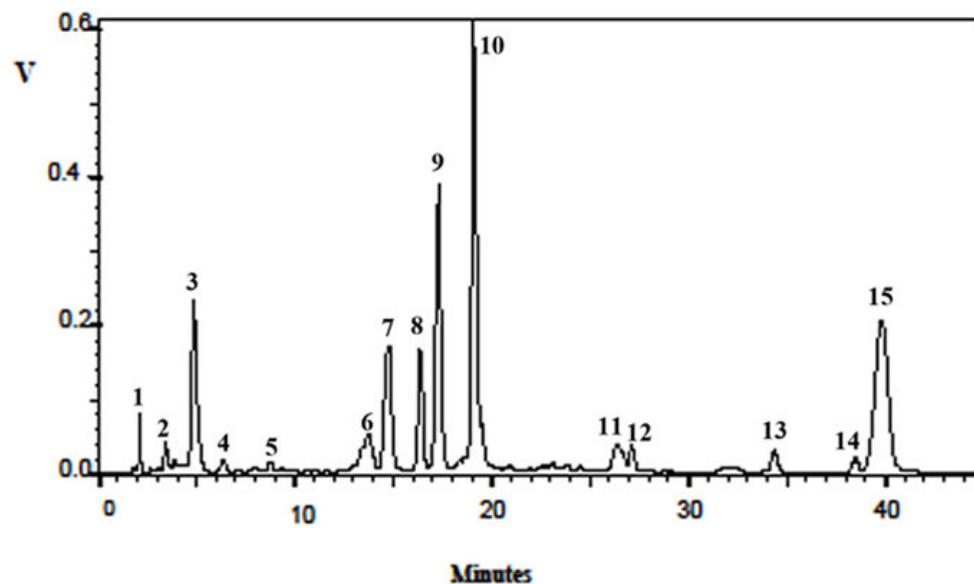


Figure 1. HPLC profile of flavonoids in extract of *Chrysanthemum indicum* flower. Peaks 4: vitexin; 5-rutin; 6: quercetin-3-galactoside; 7: luteolin-7-glucoside; 8: quercetin-3-glucoside; 9: quercitrin; 10-myricetin; 11: luteolin; 12: apigenin; 13: kaempferol. The other peaks (1, 2, 3, 14 and 15) were not identified owing to lack of authentic compounds.

Table 2. Concentrations of flavonoids in *Chrysanthemum indicum* flower (mg g^{-1}).

Vitexin	Rutin	Quercetin-3-galactoside	Luteolin-7-glucoside	Quercetin-3-glucoside	Quercitrin	Myricetin	Luteolin	Apigenin	Kaempferol	Total
0.17±0.01	0.16±0.01	12.55±0.15	17.24±0.41	9.88±0.25	51.88±1.94	37.81±0.76	7.29±0.15	0.09±0.02	0.22±0.01	137.29±1.13

the development of potential pharmaceuticals.

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