

# High performance liquid chromatography time of flight electrospray ionization mass spectrometry for quantification of sesquiterpenes in *Chrysanthemi indicis* Flos active extract

Ling Fu, Pan Wang, Yiqun Sun, Yangyang Wang, Jing Zhao, Yuting Ye, Yanbin Zhang, Yuefeng Bi

Department of Pharmacognosy, Zhengzhou University School of Pharmaceutical Science, Ke Xue Da Dao 100, Zhengzhou, 450001, P. R. China

Submitted: 09-12-2014

Revised: 07-01-2015

Published: 16-09-2015

## ABSTRACT

**Background:** *Chrysanthemi indicis* Flos, a traditional herbal medicine is used to clearing heat-toxicity, removing the liver fire, and improving eyesight. In our preliminary work, an active extract of CTC in *C. indicis* Flos with anti-hepatitis B virus and liver protective activity was found by HepG2.2.1.5 test and experiment of protein synthesis in mice's injured liver. In this work, we aimed to study the active fraction CTC further by qualitative and quantitative analysis method. **Materials and Methods:** High performance liquid chromatography time of flight electrospray ionization mass spectrometry (HPLC TOF ESI-MS) analysis method of the CTC was established. Cumambrin A and angeloylcumambrin B in CTC were analyzed by high performance liquid chromatography-ultraviolet-evaporative light scattering detector (HPLC-UV-ELSD) analysis methods. A binary gradient elution program was conducted for chromatographic separation with acetonitrile (A) and ultrapure water (B) as follows: 0–10 min, 42–46% A; 10–20 min, 46–55% A; 20–25 min, 55–60% A; and 25–35 min, 60–75% A. The column temperature and UV wavelength were set at 30°C and 205 nm. **Result:** Ten constituents including (3R, 5R, 6S, 7S, 10R)-7-(2-hydroxy-2-propyl)-10-methyl-4-methyleneperry, dronaphthal-ene-3, 5, 6-triol acetone solvate; (+)-edusmance-4, (14)-ene-11, 13-diol; linarin; luteolin; apigenin; triclin; 5, 3',4'-trimethyl-6,7-dimethoxy flavones; cumambrin A; acacetin; and angeloylcumambrin B in CTC were identified by HPLC TOF ESI-MS. The contents of sesquiterpenes in CTC were decreased by storing years. **Conclusions:** The results showed that both UV and ELSD methods were feasible, accurate, and the determination results were in good consistency. The contents of two sesquiterpenes decreased with storing years. Two sesquiterpenes could be used as quality control for *C. indicis* flos CTC.

**Key words:** Angeloylcumambrin B, *Chrysanthemi indicis* Flos, cumambrin A extract high performance liquid chromatography time of flight electrospray ionization mass spectrometry, high performance liquid chromatography-ultraviolet-evaporative light scattering detector

## INTRODUCTION

*Chrysanthemi indicis* Flos (*Chrysanthemum indicum* L.), a traditional herbal medicine for clearing heat-toxicity, removing the liver fire and improving eyesight have a long application history, and wide distribution in China.<sup>[1]</sup> *C. indicis* Flos is also well-known and usually used as Chrysanthemum tea, Chrysanthemum pillow, food additives, medicated bath in the folk.<sup>[2-4]</sup>

In our preliminary work, an active extract of (CTC) in *C. indicis* Flos with anti-hepatitis B virus and liver protective activity was found by HepG2.2.1.5 test and experiment of protein synthesis in mice's injured liver. The results of HepG2.2.1.5 test show that the inhibition ratio to expression of hepatitis B surface antigen was 92.2% (40 µg/mL), and the inhibition ratio to expression of hepatitis B e antigen was 94.2% (40 µg/mL).<sup>[5]</sup> Then, the influence of CTC on liver protein synthesis action injured by D-amino galactose was analyzed by the levels of total protein (TP), albumin (ALB), and alanine transaminase (ALT) in serum, liver, and spleen indexes.<sup>[6]</sup> It made us excited that the different dose groups of CTC could reduce the liver, spleen indexes, and ALT level but enhance TP and ALB

### Address for correspondence:

Dr. Yuefeng Bi, Zhengzhou University School of Pharmaceutical Science, Ke Xue Da Dao 100, Zhengzhou, 450001, P.R. China.  
E-mail: zzubyf@126.com

### Access this article online

#### Website:

www.phcog.com

#### DOI:

10.4103/0973-1296.165574

#### Quick Response Code:



levels, especially the high dose group of CTC [Figure 1]. Given the promising bioactivities of *C. indicii* extract CTC investigations on the chemical constituents and quality control methods were very meaningful. Systematical analysis on the constituents of the active extract indicated that CTC mainly contained sesquiterpenes (about 41.7%) and flavonoids (38.8%).<sup>[7]</sup>

Up till now, there are some reports on analysis methods of the active constituents in *C. indicii* Flos. However, most of them mainly focused on the contents of flavonoids and volatile oils such as luteolin, linarin, camphor, and borneol.<sup>[8-11]</sup> There is no paper reported on the contents of active sesquiterpenes<sup>[12-16]</sup> in *C. indicii* Flos. As we have studies, the content of flavonoids such as luteolin and linarin. Hence, in this paper, the high performance liquid chromatography-ultraviolet-evaporative light scattering detector (HPLC-UV-ELSD) analysis methods were established to determinate the contents of two significant active sesquiterpenes, cumambrin A, and angeloylcumambrin B in CTC. The results showed that the developed methods were convenient and accurate.

## MATERIALS AND METHODS

### Plant Material

*C. indicii* Flos were obtained from Xinyang Henan province and identified by professor Yuefeng Bi. Voucher specimens (No. 201110) were deposited at the herbarium in Zhengzhou University School of Pharmaceutical Science (Zhengzhou, China). The dried *C. indicii* Flos were powdered and passed through a 40-mesh sieve before extraction.

### A standard procedure for active extract in *C. indicii* Flos preparation

Air-dried flowers (3.9 kg) were extracted with 70% EtOH (3 × 6 l) by a tissue-smashing extractor, and the combined extracts were concentrated (yield 1.07 kg). The

residue was suspended in H<sub>2</sub>O (5 l) and then partitioned successively with petroleum ether, petroleum ether (60–90°)/AcOEt 1:1, and AcOEt (5 × 4 l). The petroleum ether/AcOEt 1:1 fraction was separately concentrated which afforded 46.6 g of extract. This extract is CTC. The CTC (42.1 mg) was dissolved in methanol (25 mL) and filtered through a nylon membrane filter (0.22 μm micro-pore, phenomenex, USA) for HPLC analysis.

### Chemical reagents

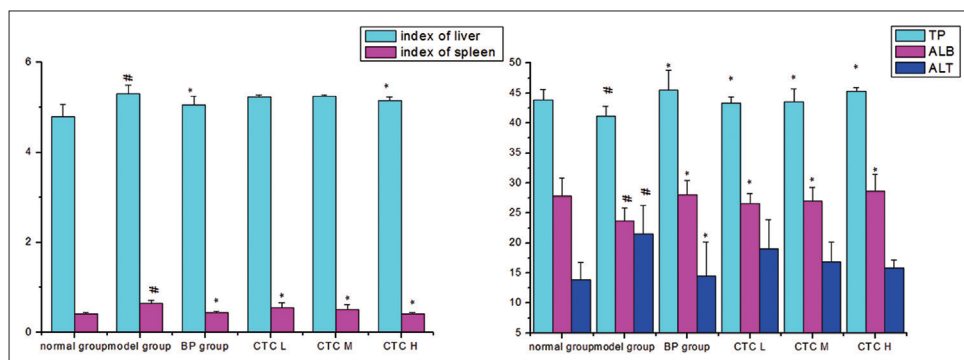
HPLC grade acetonitrile (MeCN) and MeOH were purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared by using a Millipore Milli-Q-Plus system (Bedford, MA, USA). (3R, 5R, 6S, 7S, 10R)-7-(2-hydroxy-2-propyl)-10-methyl-4-methyleneperry, dronaphthal-ene-3, 5, 6-triol acetone solvate; (+)-edusmance-4, (14)-ene-11, 13-diol; linarin; luteolin; apigenin; tricin; 5, 3',4'-trimethyl-6, 7-dimethoxy flavones; cumambrin A; acacetin; angeloylcumambrin B were prepared and purified by our lab. The purity was > 98%. The chemical structures of all reference substances are shown in Figure 2.

### Standard solutions

A mixed standard stock solution containing 10 reference substances was prepared in MeOH for HPLC analysis. Another mixed stock solution of cumambrin A and angeloylcumambrin B reference substances was prepared in MeOH and further diluted to six data points, and each standard dilution was injected in triplicate for the calibration curve. All solutions were passed through a 0.22 μm micropore membrane before injection and stored at 4°C.

### Qualitative analysis of the active extract in *Chrysanthemi indicii* Flos by high performance liquid chromatography time of flight electrospray ionization mass spectrometry

High performance liquid chromatography time of flight electrospray ionization mass spectrometry (HPLC TOF



**Figure 1:** Bifedate Pills group, active extract in *Chrysanthemi indicii* Flos low dosage group, active extract in *Chrysanthemi indicii* Flos middle dosage group, active extract in *Chrysanthemi indicii* Flos high dosage group. Left: Effects of active extract in *Chrysanthemi indicii* Flos on the index of liver and spleen in mice injured by D-gal. Right: Effects of active extract in *Chrysanthemi indicii* Flos on the total protein, albumin, and alanine transaminase in serum of mice injured by D-gal

ESI-MS) analysis of CTC was performed on Waters Xevo G2 Q-TOF (Milford, MA, USA). A YMC column (250 mm \*4.6 mm i.d., 5  $\mu$ m particles) was used to separate the CTC. A gradient elution was used for chromatographic separation with 0.5% methanoic acid (A, pH 3, 0.1 M) and MeCN (B) as follow: 0–30 min, 25–35% B; 30–35 min, 35–43% B; 35–50 min, 43–45% B; 50–65 min, 45–57% B; and 65–80 min, 57–70% B. The flow rate and the injection volume were 0.8 mL/min and 10  $\mu$ L, respectively. The column temperature and wavelength were set at 35°C and 205 nm. For MS detection, a positive ion mode ESI was used. Nitrogen was used as the desolation and cone gas with the flow rate of 50 L/h. The optimal MS parameters were as follows: Capillary, 3.0 kV; extraction and sampling cone were fixed to four and 35, respectively; source temperature, 120°C; desolation temperature, 400°C [Table 1].

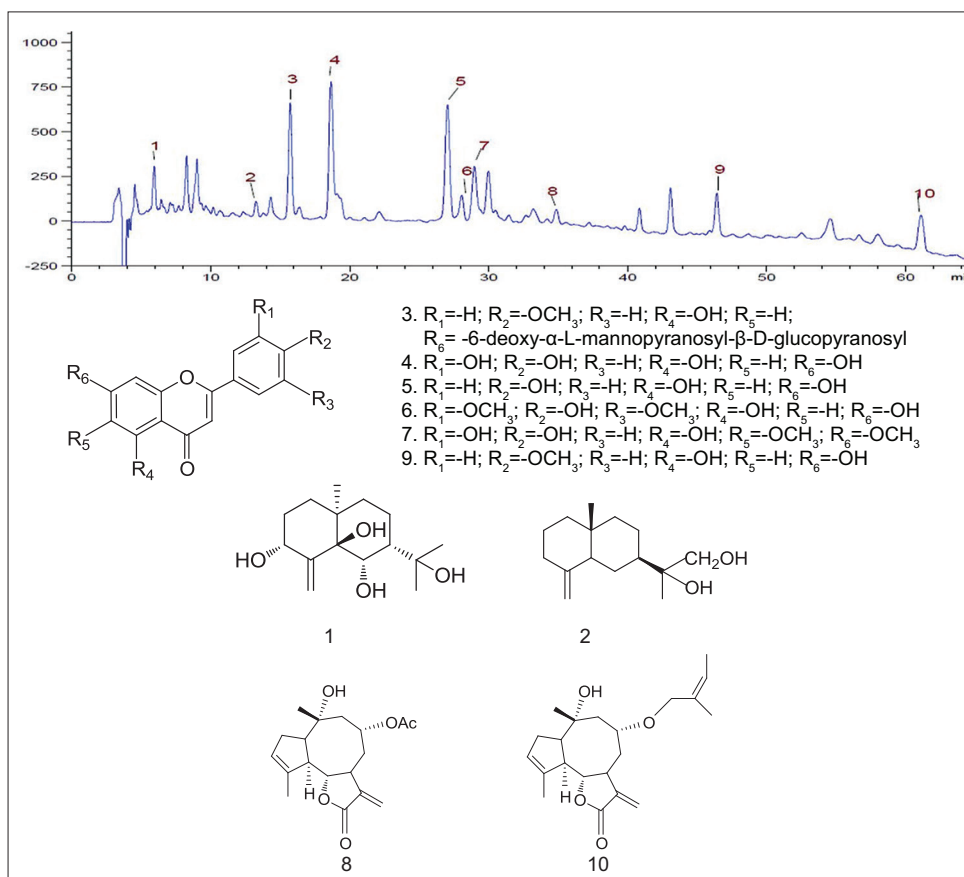
#### Quantitative analysis of sesquiterpenes by high performance liquid chromatography-ultraviolet-evaporative light scattering detector

HPLC analysis was performed on Waters system (Milford, MA, USA) equipped with separation module unit and 2487 detector, an Agilent ZORBAX SB-C<sub>18</sub> column (4.6 mm

\*250 mm i.d., 5  $\mu$ m particles). ELSD analysis was performed with an All tech 2000 ES evaporative light scattering detector. The flow rate of carrier gas (N<sub>2</sub>) and temperature of drift tube were 3.0 mL/min and 90°C, respectively. The injection volume was 80  $\mu$ L. A binary gradient elution program was conducted for chromatographic separation with MeCN (A) and ultrapure water (B) as follows: 0–10 min, 42–46% A; 10–20 min, 46–55% A; 20–25 min, 55–60% A; and 25–35 min, 60–75% A. The flow rate and the injection volume were 1.0 mL/min and 10  $\mu$ L. The column temperature and UV wavelength were set at 30°C and 205 nm.

#### Ultraviolet and evaporative light scattering detector methodology investigation

Peak areas for the samples and reference substances were integrated by UV detector and ELSD detector, respectively. Limits of detection (LOD) and limits of quantitation (LOQ) were determinates on the basis of their signal to noise (S/N) ratios of 3:1 and 10:1. Precision, the intra-day and inter-day stability, recovery of the two sesquiterpenes were analyzed by UV and ELSD detectors, respectively [Table 2].



**Figure 2:** The high performance liquid chromatography chromatograms of active extract in *Chrysanthemi indicii* Flos and structures formulas of Peak 1, (3R, 5R, 6S, 7S, 10R)-7-(2-hydroxy-2-propyl)-10-methyl-4-methylenepiperhy, dronaphthal-ene-3, 5, 6-triol acetone solvate; Peak 2, (+)-edusmance-4, (14)-ene-11, 13-diol; Peak 3, linarin; Peak 4, Luteolin; Peak 5, apigenin; Peak 6, triclin; Peak 7, 5, 3', 4'-trimethyl-6,7-dimethoxy flavones; Peak 8, cumambrin A; Peak 9, acacetin; Peak 10, angeloylcumambrin B

### Statistical analysis

Experimental results were expressed as mean  $\pm$  standard deviations (SD). Statistical analysis was performed by one-way analysis of variance. Statistical significance was determined as  $P < 0.05$ . Each calibration curve was constructed by running standard solutions of six different concentrations. All samples and chromatographic analysis were performed in triplicate.

## RESULTS AND DISCUSSION

### Result of high performance liquid chromatography time of flight electrospray ionization mass spectrometry on the active extract in *Chrysanthemi indicis* Flos

To develop the quality control, here, the fingerprint of the CTC was established, and 10 constituents including (3R, 5R, 6S, 7S, 10R)-7-(2-hydroxy-2-propyl)-10-methyl-4-methylenepery, dronaphthal-ene-3, 5, 6-triol acetone solvate; (+)-edusmance-4, (14)-ene-11, 13-diol; linarin; luteolin; apigenin; triclin; 5, 3', 4'-trimethyl-6,7-dimethoxy

flavones; cumambrin A; acacetin; and angeloylcumambrin B were identified by HPLC TOF ESI-MS [Figure 2] and [Table 1]. Among these identified constituents in the CTC, there are six flavones and four sesquiterpenes. The two class constituents have wide distribution in the Composite family such as *Taraxacum*,<sup>[17,18]</sup> *Ligularia*,<sup>[19,20]</sup> Sesquiterpene 1 (3R, 5R, 6S, 7S, 10R)-7-(2-hydroxy-2-propyl)-10-methyl-4-methylenepery, dronaphthal-ene-3, 5, 6-triol acetone solvate, and sesquiterpene 2 (+)-edusmance-4, (14)-ene-11, 13-diol are eudesmane, sesquiterpene 8 cumambrin A, and sesquiterpene 10 angeloylcumambrin B are guaiane. As terpenoids, cumambrin A, and angeloylcumambrin B have better resolution and contents for quality control [Figure 2].

### Quantification the contents of two sesquiterpenes in the active extract in *Chrysanthemi indicis* Flos

The results of the contents of the two sesquiterpenes in CTC analyzed by HPLC-UV-ELSD show that methods were feasible, accurate, and worthy of spreading. UV

**Table 1: MS structural information of 10 constituents in positive ion mode**

Compound	[M+H] <sup>+</sup>	[M+Na] <sup>+</sup>	Formula
(3R, 5R, 6S, 7S, 10R)-7-(2-hydroxy-2-propyl)-10-methyl-4-methylenepery, dronaphthal-ene-3, 5, 6-triol acetone solvate	-	293.1722	C <sub>15</sub> H <sub>26</sub> O <sub>4</sub>
(+)-edusmance-4, (14)-ene-11, 13-diol	-	261.2011	C <sub>15</sub> H <sub>26</sub> O <sub>2</sub>
Linarin	593.1876	-	C <sub>28</sub> H <sub>32</sub> O <sub>14</sub>
Luteolin	287.0566	-	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>
Apigenin	271.0611	-	C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>
Tricin	331.0823	-	C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>
5, 3', 4'-trimethyl-6,7-dimethoxy flavones	331.0813	-	C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>
Cumambrin A	-	329.1373	C <sub>17</sub> H <sub>22</sub> O <sub>5</sub>
Acacetin	285.0764	-	C <sub>16</sub> H <sub>12</sub> O <sub>5</sub>
Angeloylcumambrin B	-	369.1685	C <sub>20</sub> H <sub>26</sub> O <sub>5</sub>

MS: Mass spectrometry

**Table 2: The Calibration curves, linear range, LOD, LOQ, R<sup>2</sup>, recovery, and RSD of two sesquiterpenes**

Analytes	Method	Calibration curve	Linear range (µg.mL <sup>-1</sup> )	LOD (µg.mL <sup>-1</sup> )	LOQ (µg.Lm <sup>-1</sup> )	R <sup>2</sup>
Cumambrin A	UV	Y=23840X-4839.5	2.87-57.40	0.23	0.69	0.9999
	ELSD	Y=14239X+1900.8	2.87-57.40	0.57	1.62	0.9999
Angeloylcumambrin B	UV	Y=10674X-10695	2.60-52.00	0.52	1.62	0.9999
	ELSD	Y=6069.5X-1107.6	2.60-52.00	1.04	3.12	0.9999

LOD: Limits of detection; LOQ: Limits of quantitation; RSD: Relative standard deviation

**Table 3: The contents of cumambrin A and angeloylcumambrin B in CTC by UV and ELSD (% , n=3)**

Sample no	Year	Cumambrin A content		Angeloylcumambrin B content	
		UV	ELSD	UV	ELSD
1	2008	0.0678±0.0002	0.0655±0.0001	0.0857±0.0012	0.0805±0.0009
2	2009	0.2071±0.0012	0.1929±0.0013	0.1641±0.0011	0.1349±0.0026
3	2010	0.2007±0.0019	0.1871±0.0020	0.1437±0.0007	0.1154±0.0020
4	2011	0.2621±0.0008	0.2538±0.0018	0.2005±0.0013	0.1796±0.0009

The UV was monitored at 205nm (MeCN-water gradient elution). The temperature of drift tube of ELSD was set at 90°C, and the flow rate of carrier gas was set at 3.0 mL/min. UV: Ultraviolet; ELSD: Evaporative light scattering detector; CTC: *Chrysanthemi indicis* active extract

detector have better sensitivity for LOD and LOQ but baseline drift for edge absorption of 205 nm, while ELSD have good recovery and widely application for eliminating the baseline drift [Table 2]. The contents of two sesquiterpenes decreased with storing years. The contents in 2010 years were lower than that in 2009 years for the terrible harvest quality [Table 3]. Our study offered two different analysis methods for the main sesquiterpenes in CTC. The study also demonstrates that CTC is an active CTC measured by pharmacological action and HPLC TOF ESI-MS.

## CONCLUSIONS

From the results mentioned above, we could see the two main groups including flavones and sesquiterpenes in CTC. It was allowed to propose that the enrichment of flavones and sesquiterpenes in the CTC could influence on the pharmacological action of *C. indic* extract. However, how does CTC work? And how the simple constituent plays a part in CTC? We did not know, either. Hence, it is necessary to research the connection between the simple constituent and CTC based on the activity of anti-hepatitis B virus and liver protective activity further.

## ACKNOWLEDGMENTS

The work was supported by the National Natural Science Foundation of China (30973872 and 81273392) and Henan provincial key scientific and technological project (122102310085).

## REFERENCES

- Chinese Pharmacopoeia Commission. Chinese Pharmacopoeia. Beijing: Chemical Industry Press; 2010. p. 295.
- Yue C, Huang Z, Chen C. Research of *Chrysanthemum* soft beverage. Food Sci Technol 2003;11:68-70.
- Wang T, Qiu Y, Li B, Xiao S, Wan F. Extracting technology of yellow pigment from *Chrysanthemum indicum* flos. Food Machinery 2002;8:726-7.
- Sun T, Meng L, Wang S. Comparison the effect of bathing with different concentrations of *Chrysanthemum indicum* flos soup for new born erythema venenatum. J Nurs Sci 2011;15:39-40.
- Chen YY. The Immunomodulatory and Liver Protect Effects of Two Antagonistic Compounds of Sesquiterpene and Flavonoid Purified from *Chrysanthemum indicum* L.[D]. Zhengzhou University; 2012. p. 6.
- Li GD, Chen YY, Wang P, Guan RF, Ye YT, Zhao J, et al. Protection of terpenes and flavonoids from *Chrysanthemum indicum* Flos on immunological liver injury. Chin Tradit Herb Drugs 2013;44:3510-4.
- Bi YF, Lu J, Shi SP, Sun XL, Chen YY, Zhang YB. New sesquiterpenes from the flowers of *Chrysanthemum indicum* L. Helv Chim Acta 2010;93:1953-9.
- Shen S, Sha Y, Deng C, Zhang X, Fu D, Chen J. Quality assessment of Flos *Chrysanthemi indici* from different growing areas in China by solid-phase microextraction-gas chromatography-mass spectrometry. J Chromatogr A 2004;1047:281-7.
- Zhang Q, Li J, Wang C, Sun W, Zhang Z, Cheng W. A gradient HPLC method for the quality control of chlorogenic acid, linarin and luteolin in Flos *Chrysanthemi indici* suppository. J Pharm Biomed Anal 2007;43:753-7.
- Deng C, Mao Y, Yao N, Zhang X. Development of microwave-assisted extraction followed by headspace solid-phase microextraction and gas chromatography-mass spectrometry for quantification of camphor and borneol in Flos *Chrysanthemi indici*. Anal Chim Acta 2006;575:120-5.
- Tan XJ, Jia Y, Qi DD, Chen XH, Bi KS. Establishment of chromatographic fingerprint and quality assessment of Flos *Chrysanthemi indici* by RP-HPLC. Chin J Pharm Anal 2007;27:29-32.
- Kim Y, Sung J, Sung M, Choi Y, Jeong HS, Lee J. Involvement of heme oxygenase-1 in the anti-inflammatory activity of *Chrysanthemum boreale* Makino extracts on the expression of inducible nitric oxide synthase in RAW264.7 macrophages. J Ethnopharmacol 2010;131:550-4.
- Rüngeler P, Castro V, Mora G, Gören N, Vichnewski W, Pahl HL, et al. Inhibition of transcription factor NF-kappaB by sesquiterpene lactones: A proposed molecular mechanism of action. Bioorg Med Chem 1999;7:2343-52.
- Haruna M, Kato M, Ito K, Nikai T, Sugihara H, Murat H. Angeloylcumambrin-B, an anti-microbial sesquiterpene lactone from *Chrysanthemum ornatum* var. Spontanum. Phytochemistry 1981;20:2583-4.
- Akihisa T, Yasukawa K, Tokuda H. Potentially cancer chemopreventive and anti-inflammatory terpenoids from natural sources. Stud Nat Prod Chem 2003;29(Part J):73-126.
- Muftuoglu Y, Mustata G. Pharmacophore modeling strategies for the development of novel nonsteroidal inhibitors of human aromatase (CYP19). Bioorg Med Chem Lett 2010;20:3050-64.
- Kisiel W, Barszcz B. Further sesquiterpenoids and phenolics from *Taraxacum officinale*. Fitoterapia 2000;71:269-73.
- Williams CA, Goldstone F, Greenham J. Flavonoids, cinnamic acids and coumarins from the different tissues and medicinal preparations of *Taraxacum officinale*. Phytochemistry 1996;42:121-7.
- Liu JQ, Zhang M, Zhang CF, Qi HY, Bashall A, Bligh SW, et al. Cytotoxic sesquiterpenes from *Ligularia platyglossa*. Phytochemistry 2008;69:2231-6.
- Li S, Huang H, Liu J, Song T, Wu K, Han Y, et al. Two new sesquiterpenes from *Ligularia dictyoneura*. Phytochem Lett 2013;6:315-7.

**Cite this article as:** Fu L, Wang P, Sun Y, Wang Y, Zhao J, Ye Y, et al. High performance liquid chromatography time of flight electrospray ionization mass spectrometry for quantification of sesquiterpenes in *Chrysanthemi indici* Flos active extract. Phcog Mag 2015;11:740-4.

**Source of Support:** The work was supported by the National Natural Science Foundation of China (30973872 and 81273392) and Henan provincial key scientific and technological project (122102310085),

**Conflict of Interest:** None declared.