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Full Length Research Paper

Distribution of seven polyphenols in several medicinal plants of Boraginaceae in China

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Polyphenols are one important type of compounds investigated in the family Boraginaceae, a simple and accurate high performance liquid chromatography with diode-array detector (HPLC-DAD) method was established for simultaneous determination of seven polyphenols including five flavonoid glucosides and two phenolic acids in *Ehretia thyrsiflora* and several medicinal plants of the family Boraginaceae in China. This is the first report which obtained from the distribution of these polyphenols in these medicinal plants in China or the world and to provide a fast and effective method to evaluate the polyphenols quantification of these medicinal plants. The results showed that rosmarinic acid distributed in all of the tested species of the family Boraginaceae. *E. thyrsiflora* of the subfamily Ehretioideae contained relatively high amount of total flavonoid glycosides but low amount of phenolic acids compared with all the other species from the subfamily Boraginoideae.

Key words: *Ehretia thyrsiflora*, Boraginaceae, LC-DAD, flavonoid glucosides, phenolic acids.

INTRODUCTION

Family Boraginaceae including about 100 genera 2000 species in all over the world is divided into four subfamilies: Boraginoideae, Heliotropioideae, Cordioideae and Ehretioideae (Gottschling et al., 2001). In China, the family Boraginaceae is represented by more than 200 species belonging to 49 genera (Kong and Wang, 1989), and about 30 species belonging to 17 genera have medicinal therapeutics (Wu, 1990). The main medicinal plants distribute in genera *Cynoglossum*, *Lappula*, *Lithospermum*, and *Onosma* of the subfamily Boraginoideae (Sun et al., 2009). Alkaloids, naphthoquinones, polyphenols, phytosterols, terpenoids and fatty acids are the main secondary metabolites of the family Boraginaceae (Zhou and Duan, 2005; Iqbal et al., 2005). Polyphenols including phenolic acids and flavonoids distributed in the family Boraginaceae have diverse pharmaceutical activities such as antioxidant, anti-inflam-matory, anti-viral, anti-bacterial and hepato-protecting activity (Wu, 1990; Zeng and Zeng, 1994; Iqbal et al., 2005). More over, plentiful polyphenols are though to be the main pharmaceutical contributors in tea, red-wine, beverages, plant foods and in some other traditional therapies (Balasundram et al., 2006).

In order to directly access the active polyphenols in several medicinal plants of Boraginaceae in China, an accurate and rapid LC-DAD method is established for quantitation of the major active polyphenols quercetin 3-*O*-arabinosylgalactoside Balasundram et al. (2006), isoquercetin (China Pharmacopoeia Committee, 2005), quercetin-3-*O*-*α*-D-arabinoside Gottschling et al. (2001), astragalin Hu et al. (2006), kaempferol-3-*O*-*α*-D-arabinoside Iqbal et al. (2005), caffeic acid Kong and Wang (1989), and rosmarinic acid Li et al. (2009a) in the medicinal plants of *Ehretia thyrsiflora*, *Cynoglossum*

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Table 1. The folk therapeutic uses and the distribution in China of the tested Boraginaceous medicinal plants.

Botanical name	Distribution in China	Parts used	Ailments treated and therapeutic effects	References
E. thyrsiflora	South of China	Leaves; Heart of wood; Branches	Antipyretic, Used for cold and migraine; Wounds, Sore Pain, Furuncle, Alcer, Fractures; Convergence, Used for enteritis and diarrhea	Wu, 1990
C. amabile	Southwest of China; Gansu province	All herbs	Blood depurative; Relieving swelling and cough; Used for malaria, hepatitis, dysentery, dysurea, leucorrhea, and pulmonary tuberculosis	Zeng and Zeng, 1994
C. amabile via pauciglochidiatum	Southwest of Si chuan province; Northwest of Yunnan province	All herbs	Blood depurative; Diuresis and relieving swell	Wu, 1990
C. lanceolatum	Shanxi and Gansu province; Southwest of China	All herbs	Blood depurative; Diuresis; Relieving swell and facilitating the flow of blood; Treatment for the acute nephritis, periodontitis, periodontal abscess, and acute lymphadenitis of mandibular	Wu, 1990; Zeng and Zeng, 1994
O. paniculatum	Southwest of China	Roots; Root barks	Blood depurative; Facilitating the flow of blood; Prevention and treatment for various ulcer disease, measles, and pox poison	Zeng and Zeng, 1994; Wu, 1990
L. heteracantha	Northeast and central areas of China	Fruits	Appetizer; Insecticide	Wu, 1990
B. paridiformis	Northeast of China; Heilongjiang, Jilin, and Liaoning province	Fruits	Blood depurative; Antipyretic; Appetizer;	Zeng and Zeng, 1994

amabile, *Cynoglossum amabile via pauciglochidiatum*, *Cynoglossum lanceolatum*, *Onosma paniculatum*, *Lappula heteracantha* and *Brachybotrys paridiformis*. This is the first time to provide the evidences of polyphenol distribution of these medicinal plants.

MATERIALS AND METHODS

Plant materials, reagents and apparatus

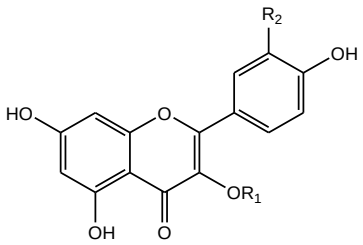
All investigated species have been used traditionally in China. The folk therapeutic uses and the distribution of the tested Boraginaceous medicinal plants in China are summarized in Table 1. HPLC grade acetonitrile was procured from Merck (Darmstadt, Germany); deionized water from Wahaha (Hangzhou, China); analytical grade solvents for extraction and chromatography from Beijing Beihua Fine Chemicals Co. (Beijing, China). Seven reference compounds quercetin 3-*O*-arabinosylgalactoside (Balasundram et al., 2006), isoquercetin (China Pharmacopoeia Committee, 2005), quercetin-3-*O*-*α*-D-arabinoside (Gottschling et al., 2001), astragalin (Hu et al., 2006), kaempferol-3-*O*-*α*-D-arabinoside (Iqbal et al., 2005), caffeic acid (Kong and Wang, 1989) and rosmarinic acid (Li et al., 2009a) were purified from *E. thyrsiflora* by the author's laboratory and their structures were elucidated by various spectrophotometric methods (Viz. UV-Vis spectroscopy, MS, ^{1}H NMR and ^{13}C NMR) and were in comparison with the reported literatures (Li et al., 2009a). The purity of each compound was all above 98% and was estimated by HPLC analysis. The structures are illustrated in Table 4.

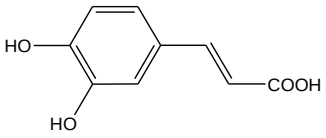
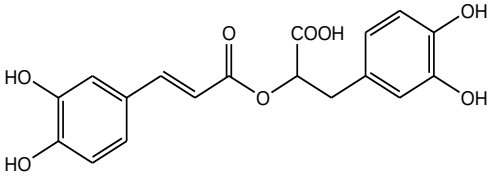
Chromatographic separation was carried out using an Agilent 1200LC series system (Agilent Technologies, PaloAlto, CA, USA) equipped with online vacuum degasser, quaternary pump, auto sampler, thermostated column compartment, and a diode array detector (DAD). Agilent technologies Chemstation software for LC (B.02.01) was used. The HPLC separation was performed on Agilent Eclipse XDB-C_{18} reserved-phase column (5 µm, 150 × 4.6 mm).

Quantitative analyses of polyphenols in several medicinal plants of Boraginaceae

Stock standard solutions of compounds quercetin 3-*O*-arabinosylgalactoside (1, 4.08 mg), isoquercetin (2, 2.51 mg), quercetin-3-*O*-*α*-D-arabinoside (3, 3.62 mg), astragalin (4, 4.20 mg), kaempferol-3-*O*-*α*-D-arabinoside (5, 3.80 mg), caffeic acid (6, 8.03 mg) and rosmarinic acid (7, 4.15 mg) were dissolved with 10 ml methanol. Standard solutions were prepared by diluting the mixed standard solution to appropriate concentration range for the establishment of calibration curves. The stock standard solutions were all stored in dark brown calibrated flasks at 4°C. The powder of all drying samples in shade (~1 g, 40 meshes) was weighed accurately, suspended in 50 ml ethanol (75%, v/v) and extracted by ultrasonication for 30 min at 25°C. The extract solution was then cooled down to the room temperature and diluted to 50 ml. The solutions were filtered through a 0.45 µm filter and 5 µl of the filtrate

Table 4. Chemical structure of the 7 standards (**1 - 7**) for HPLC-DAD analysis.



		R_1	R_2
1	Quercetin 3-*O*-arabinosylgalactoside	Ara-(1→6)-Gal	OH
2	Isoquercetrin	Glc	OH
3	Quercetin 3-*O*-*α*-D-arabinoside	Ara	OH
4	Astragalin	Glc	H
5	Kaempferol 3-*O*-*α*-D-arabinoside	Ara	H
6	Caffeic acid		
7	Rosmarinic acid		

was subjected to HPLC analysis.

The optimum wavelength was set at 270 nm. The mobile phase was composed of aqueous phosphonic acid (0.1%, v/v) (A) and acetonitrile (B) using a gradient elution of 5 - 12% B at 0 - 12 min, 12 - 16% B at 12 - 14 min, 16% B maintained at 14 - 21 min, 16 - 20% B at 21 - 27 min, 20 - 25% B at 27 - 37 min and the re-equilibration time of gradient elution was 15 min. The flow rate was 1.0 ml/min.

The column temperature was maintained at 30˚C. The seven calibration curves were obtained with known concentrations of the standard working solutions by plotting the peak area versus the concentration of each analyte. The content of each analyte was calculated from the corresponding calibration curve.

RESULTS AND DISCUSSION

Optimization of chromatographic and sample extraction conditions

Acetonitrile–water and methanol–water in different ratios as the mobile phase were investigated. The results indicated that 0.1% (v/v) aqueous phosphonic acid and acetonitrile could make all seven standard compounds eluted with baseline separation in 35 min. On the basis of the absorption maxima of the seven compounds in UV spectra acquired by diode-array detection (DAD), the monitoring wavelength was set at 270 nm. The column temperature was controlled at 30˚C thermostatically. Typical chromatograms of the authentic standards and the samples are presented in Figure 1. 75% (v/v) methanol, 75% (v/v) ethanol, 50% (v/v) methanol, 50% (v/v) ethanol and water as the extraction solvent were employed. 75% (v/v) ethanol was finally selected because it could make the peak areas of the target compounds reach the highest values and the affection of impurities reach the least.

Three duplicate samples were extracted with 75% (v/v) ethanol in an ultrasonic bath for 20, 30, and 40 min, respectively. At last, the optimal extraction time was selected as 30 min because of exhaustive extraction of all analyzed compounds in 30 min.

Validation of the quality evaluation method

The linearity, precision (intra-day and inter-day assay), stability and recovery were evaluated according to guidelines of Chinese Pharmacopoeia (2005); Ma et al. (2009) (Table 2). The limit of detection (LOD) and quantification (LOQ) were calculated as the concentrations of injected sample showing S/N ratios of 3 and 10, respectively. Therefore, this verified RP-HPLC method was precise, accurate and reliable for the quantitative evaluation of the main polyphenols in the medicinal plants of family Boraginaceae.

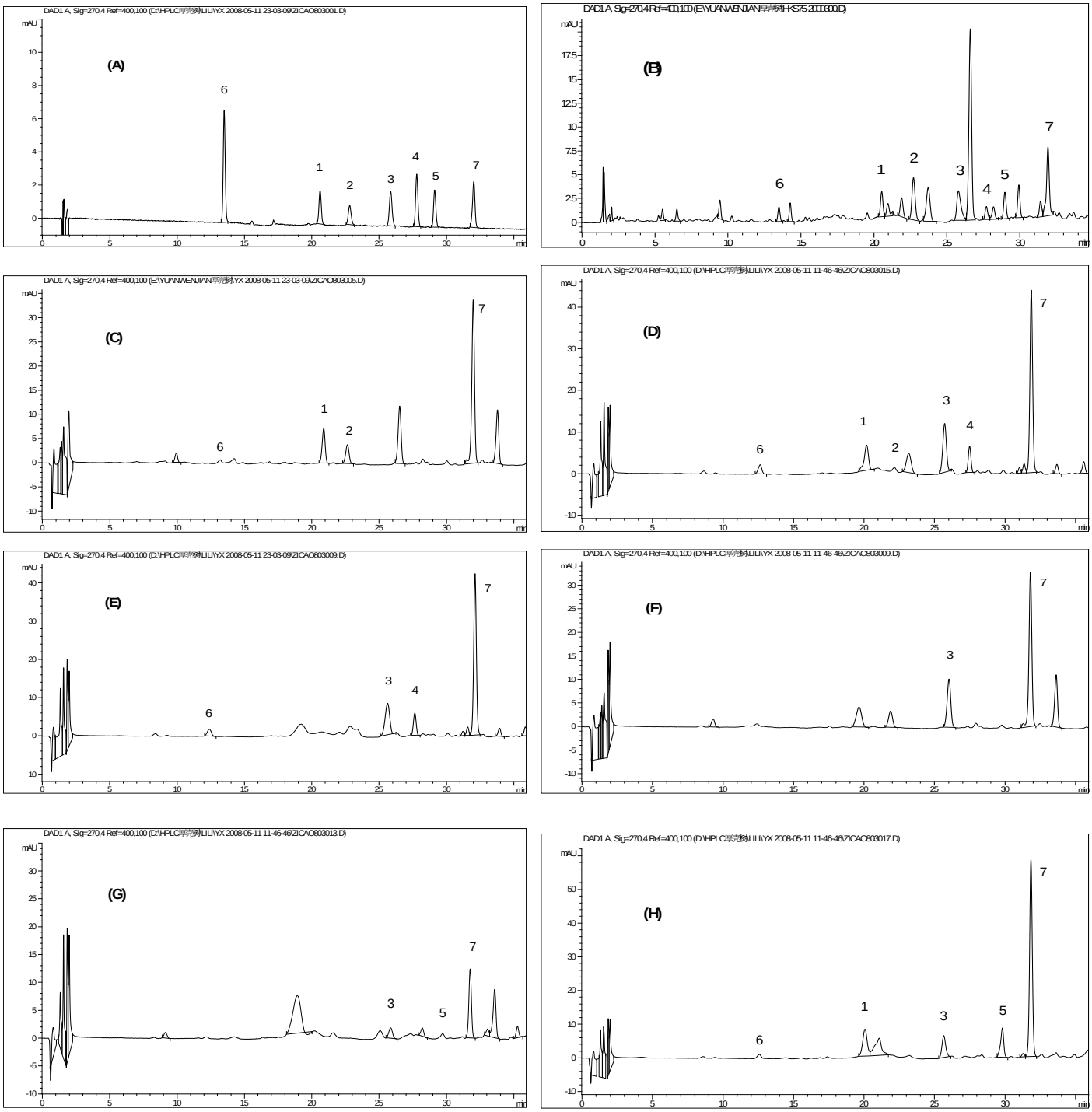


Figure 1. Representative HPLC-DAD (270 nm) chromatograms of the Standard mixture (A) and the samples (B-H).
(A) Standard mixture; (B) *E. thyrsiflora*; (C) *C. amabile*; (D) *C. amabile via pauciglochidiatum*; (E) *C. lanceolatum*; (F) O. paniculatum; (G) *L. heteracantha*; (H) *B. paridiformis*

The distribution of polyphenols in Boraginaceae species

In our previous work, large number of polyphenols has been separated from *E. thyrsiflora* and the potent antioxidant activities of these compounds have been evaluated (Li et al., 2008; Li et al., 2009a; b). In the pre-sent study, the tested species *E. thyrsiflora* is originated from the subfamily Ehretioideae. All of the other tested species are originated from another subfamily Boraginoideae.

Table 2. Results of regression analysis on calibration curves and detection limits.

Compounds	Regression equation	Linear range (μg/ml)	r^2	LOD (μg/ml)	LOQ (μg/ml)
1	$Y = 6.8487\ X + 1.0925$	1.80-21.16	0.9999	0.04	0.14
2	$Y = 4.7198\ X + 3.0757$	1.68-40.32	0.9997	0.07	0.23
3	$Y = 7.4486\ X + 2.7497$	1.86-22.32	0.9992	0.02	0.07
4	$Y = 8.4416\ X + 3.2337$	2.10-25.20	0.9997	0.08	0.28
5	$Y = 6.2273\ X + 2.0362$	2.01-24.00	0.9999	0.05	0.16
6	$Y = 16.072\ X + 0.1850$	0.60-28.80	0.9994	0.04	0.15
7	$Y = 17.148\ X + 10.599$	0.84-33.60	0.9995	0.05	0.20

Y peak area, *X* concentration of the compounds (μg/ml), r^2 correlation coefficient of the equation, LOD limit of detection, LOQ limit of quantification

Table 3. The contents of the 7 polyphenols in the tested Boraginaceous medicinal plants (mg/g).

Plants\Compound	1	2	3	4	5	6	7
E. thyrsiflora	0.48 ± 0.005	1.45 ± 0.007	0.80±0.007	0.39±0.006	0.43±0.007	0.11±0.009	0.53±0.004
C. amabile	1.71 ± 0.008	1.19 ± 0.003	Nd	Nd	Nd	0.08±0.001	2.40±0.012
C. amabile via pauciglochidiatum	1.81 ± 0.005	0.18 ± 0.001	1.72±0.011	0.91±0.006	Nd	0.12±0.002	3.31±0.013
C. lanceolatum	Nd	Nd	1.61±0.008	0.82±0.005	Nd	0.09±0.001	2.83±0.002
O. paniculatum	Nd	Nd	1.69±0.008	Nd	Nd	Nd	2.72±0.011
L. heteracantha	Nd	Nd	0.45±0.005	Nd	0.11±0.004	Nd	0.93±0.006
B. paridiformis	1.85±0.014	Nd	1.03±0.008	Nd	0.64±0.002	0.07±0.001	4.01±0.015

Results are mean ± SD (n = 3); Nd: not detected.

The results have shown that rosmarinic acid Li et al. (2009a) distributes in all of the tested species of the family Boraginaceae. The contents of rosmarinic acid Li et al. (2009a) in species *C. amabile* (2.40 ± 0.012 mg/g), *O. paniculatum* (2.72 ± 0.011 mg/g) and *B. paridiformis* (4.01 ± 0.015 mg/g) are relatively high. But the other phenolic acid Kong and Wang (1989) is in trace amount or not detected in these species. Rosmarinic acid has been shown to possess anti-inflammatory, antioxidative, antiviral as well as antibacterial activity in various *in vitro* assays (Petersen and Simmonds, 2003) and *in vivo* studies (Youn et al., 2003). *O. paniculatum* which has been shown to have large amount of rosmarinic acid also contains the most naphthoquinones and it is already included in the Yunnan Standard of Chinese Material Medical (Hu et al., 2006).

The distribution of the flavonoid glycosides investigated is relatively minor compared with rosmarinic acid Li et al. (2009a) except *E. thyrsiflora*. Quercetin-3-*O*-*α*-D-arabinoside Gottschling et al. (2001) is the most commonly flavonoid glycoside (0.45 - 1.72 mg/g) found in each species of family Boraginaceae except *C. amabile*. The contents of quercetin 3-*O*-arabinosylgalactoside Balasundram et al. (2006) are relatively high in species *E. thyrsiflora* (0.48 ± 0.005 mg/g), *B. paridiformis* (1.85 ± 0.014 mg/g), *C. amabile via pauciglochidiatum* (1.81 ± 0.005 mg/g), and *C. amabile* (1.71 ± 0.008 mg/g). A small amount of the other flavonoid glycosides distributes in species of the subfamily Boraginoideae. The results also indicate that *E. thyrsiflora* contains relatively high amount of total flavonoid glycosides but low amount of phenolic acids compared with all the other species from the subfamily Boraginoideae (Table 3 and Figure 1).

Conclusion

A simple, specific, rapid and reproducible RP-HPLC method has been established for quantification of seven major polyphenols (quercetin 3-O-arabinosylgalactoside, isoquercetrin, quercetin-3-O-*α*-D-arabinoside, astragalin, kaempferol-3-O-*α*-D-arabinoside, caffeic acid and rosmarinic acid) in *E. thyrsiflora* and related Boraginaceous species. The results indicated that this LC-DAD method could be considered as a convenient method for the quality evaluation of these medicinal plants. Moreover, the different distribution of these polyphenols in several medicinal plants of the family Boraginaceae in China was also discussed in this paper. The applicable prospects of these medicinal plants with abundance polyphenols

would be extensive and diverse in further studies.

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