

Development of HPLC Method to Evaluate Drug-processing Technique of *Eucommiae Cortex*

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Abstract: **Objective** To establish a RP-HPLC method investigate the processing technique and mechanism of *Eucommiae Cortex*. **Methods** The RP-HPLC method was applied to simultaneously determining six ingredients, geniposidic acid, geniposide, genipin, chlorogenic acid, (+)-pinoresinol-di- β -D-glucopyranoside, and (+)-syringaresinol-di- β -D-glucopyranoside, in the different processed barks of *Eucommia ulmoides*. **Results** The valid method with good accuracy could be well used to study the processing technique of *E. ulmoides*; Besides, target ingredients in *E. ulmoide* were decreased within 6 h when they were processed. **Conclusion** Established RP-HPLC is a reliable method which could be used to research the processing technique of the barks of *E. ulmoides*. Moreover, the result of this study could be provided with significant evidence of processed barks of *E. ulmoides*.

Key words: *Eucommia ulmoides*; genipin; geniposide; geniposidic acid; processing technique; RP-HPLC

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Introduction

In traditional Chinese medicine (TCM), the processed barks of *Eucommia ulmoides* Oliv. have been approved to nourish liver and kidney, increase vigor as well as to stop bleeding and to prevent miscarriage (Pharmacopeia Committee of P. R. China, 2010). The pharmacological functions of *E. ulmoides* were recognized to strengthen the internal organs, bones, and muscles, and prevent senescence in the classic of TCM (Sun and Sun, 2010; Li, 1987). Furthermore, the barks of *E. ulmoides* have been prescribed as a traditional medicine in Japan and Korea (Deyamax, Nishibe, and Nakazawa, 2001). Various active ingredients, such as lignans, phenylpropanoids, and iridoid glycosides, were isolated from the barks of *E. ulmoides* (Deyamax, Nishibe, and Nakazawa, 2001). Considering the bioactivity, lignans such as (+)-pinoresinol-di- β -D-glucopyranoside (PG) and (+)-syringaresinol-di- β -D-glucopyranoside (SG), and phenylpropanoids such as

chlorogenic acid (CGA), were found to be the antioxidant and antibiotic as well as antiviral active ingredients. Iridoid glycosides such as geniposidic acid (GPA), geniposide (GP), and genipin (G), were found to be the active ingredients that possessed anti-hypertensive and antidiabetic properties (Yen and Hsieh, 2000; Lang, 2005; Yen, 2000; Hsieh and Yen, 2000; Zhang *et al.*, 2005; Kwan *et al.*, 2003; 2004). Besides, it possessed the inhibitory effect on adipogenic differentiation against low-density lipoprotein oxidative modification (Lee, Yoon, and Yo, 2004; Yen and Hsieh, 2002). The processed barks of *E. ulmoides* are appreciated because crude barks of *E. ulmoides* have abundant gums and mucilage which are decreased during processing. In addition, the nature of some medicinal herbs can be changed since salt processed medicine can draw the effect of the herb from the upper parts to the lower parts of the human body in TCM (Cai, Hattori, and Namba, 1990; Han *et al.*, 2009).

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Some experiments relevant to processed barks of *E. ulmoides* were performed in many aspects during the past few decades (Di *et al*, 2007). For example, in the changes of microelements degree (Liu *et al*, 1989), the chemical components changed variedly from the crude barks of *E. ulmoides* to the processed barks of *E. ulmoides* confirmed by TLC and HPLC fingerprint (Cao, Jia, and Xu, 2009; Zhang *et al*, 2009; Ping *et al*, 2009). Besides, LC-MS and CE were applied to analyzing the crude barks of *E. ulmoides* and their preparations (Cheung *et al*, 2003; Luo *et al*, 2004). From previous studies, various processing methods were investigated but there were deficiency of objectives and comprehensive evaluations (Zhang, Huang, and Peng, 2009). This experiment was carried out to explore a novel and simple method that could be well utilized to control the quality of the processed barks of *E. ulmoides* as well as its processing technique. The amounts standardization of GPA, GP, PG, SG, G, and CGA was taken into an equivalent standardization and the HPLC method was employed to evaluate and control the processing technique of *E. ulmoides* more objectively than that mentioned above.

Materials and methods

Instruments and chromatographic methods

The HPLC system consisted of a pump (P2695,

USA), an injector (7725i, Rheodyne, USA), and an ultraviolet detector (UVD 2487, Dionex). The output signal was recorded using Empower Data Acquisition and Analysis System. Chromatographic separation was achieved on a Diamonsil C₁₈ column (250 mm × 4.6 mm, 5 μm), the UV detector monitored at 240 and 277 nm, and the mobile phases were composed of CH₃CN (A) and H₂O acidulated with 1% acetic acid (B). The gradient programmer solvent system was fixed at a flow rate of 1.0 mL/min using the following profile: 0–12 min, 8%–13% A; 12–35 min, 13% A; and UV detector wavelengths were 240, 277, and 240 nm during 0–19, 20–26, and 26–35 min, respectively.

Solvents and reagents

CH₃CN (HPLC grade) was obtained from Dikma Chemicals (Poole, UK); Acetic acid (HPLC grade) was purchased from Concord (Tianjin, China); Triple deionized water (Millipore, USA) was used for all preparations. A membrane filter (MF3-13 PTFE, diameter 13 mm, pore size 0.5 μm, Advantech, CA, USA) was used to filter each sample. The standard references of GPA, GP, PG, SG, G, and CGA (purity > 98%) were provided by Prof. SU Yan-fang from Tianjin University, and the chemical structures were demonstrated in Fig. 1.

Plant materials preparation

Decoction pieces of *E. ulmoides* were purchased

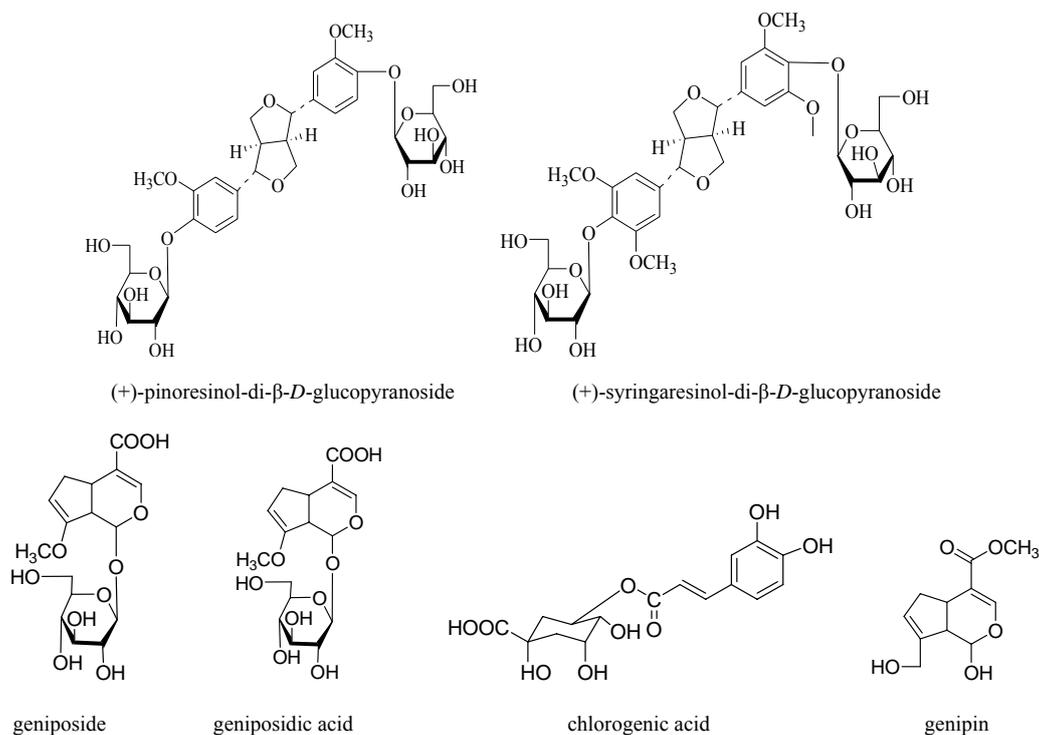


Fig. 1 Chemical structures of six quantitative compounds in *E. ulmoides*

from the Corporation of Medicinal Herbs and Extracted Granule of Qixing (Hebei, China). The batches of *E. ulmoides* were confirmed by qualitative and quantitative analyses in our experiment (Pharmacopeia Committee of P. R. China, 2010). The roasted samples of the processed barks of *E. ulmoides* were prepared at 0, 0.5, 1, 2, 3, 4, 5, and 6 h (S_{0h} , $S_{0.5h}$, S_{1h} , S_{2h} , S_{3h} , S_{4h} , S_{5h} , and S_{6h}). All of them were processed with saline water (1%) before roasting. Each sample was made into powder then passed through 50 sieves, finally weighed precisely before extraction (1.00 g).

Preparation of reference solutions

Stock solutions of G, GP, GPA, PG, SG, and CGA were prepared with 65% MeOH of HPLC grade as solvent at their respective concentrations. Working solutions for calibration were prepared appropriately,

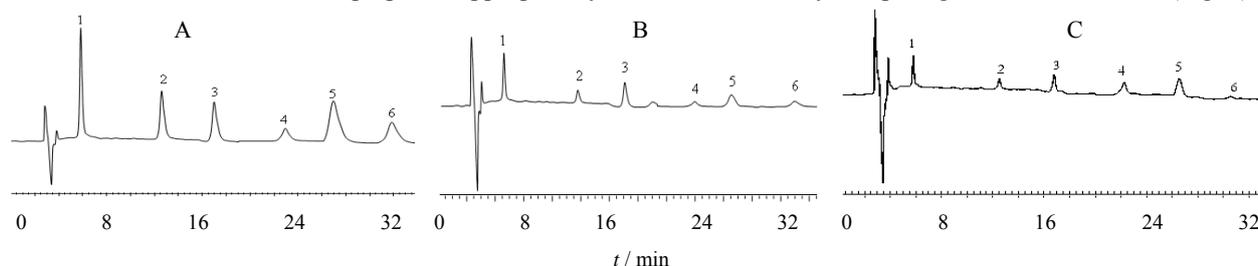


Fig. 2 HPLC chromatograms of stock reference (A), processed barks of *E. ulmoides* (B), and crude barks of *E. ulmoides* (C). Peaks 1–6: GPA, CGA, GP, PG, G, and SG, respectively

Comparison of extraction parameters

Comparison of reflux and ultrasonic extractions was carried out using aqueous 65% MeOH. Three kinds of solvents in the proportions of MeOH, 50%, 65%, and 80% were used to estimate the ability to extract the six reference compounds. The extraction time varied from 0.5 to 2 h. Reflux extraction method was chosen successfully for extracting the six ingredients. The ratio of solvent to powder was 25:1 and the parameter was found to be very nice for reflux extraction at 2 h with 65% MeOH.

Linearity range, LOD, and LQD

Calibration curves were linear in a relatively wide range of concentrations from 3.08 to 332.00 $\mu\text{g/mL}$, stock solution were at concentrations GPA for 230 $\mu\text{g/mL}$, CA 154 $\mu\text{g/mL}$, GP 162 $\mu\text{g/mL}$, PG 260 $\mu\text{g/mL}$, G 217 $\mu\text{g/mL}$, and SG 332 $\mu\text{g/mL}$, and then diluted by sequences of 1:2:1.25:2:2:2.5:2. The six compounds demonstrated good linear regression with high correlation coefficient values between peak area (Y) and amount (X , μg). The LOD and LQD values were determined by means of serial dilution based on a signal to

by successive serial dilution of the stock solution with 65% MeOH.

Results

Selection of HPLC-UV condition

The reverse phase Diamonsil C_{18} column (250 mm \times 4.6 mm, 5 μm) was used. Various mixtures of CH_3CN and water in combination with acetic acid were tested as mobile phase in gradient elution. For the simultaneous detection of these six compounds, the wavelengths must be monitored at 240 and 277 nm, since GPA, GP, SG, G and CGA showed quite well UV absorption at 240 nm and PG at 277 nm. The presence of the six reference compounds in accordance with the extraction of crude and processed barks of *E. ulmoides* was verified by comparing each retention time (Fig. 2).

noise (S/N) ratio of 3:1 and 10:1, respectively. The LOD values of the six standards showed a high sensitivity under this chromatographic condition (Table 1).

Precision

The precision test was carried out by the intra- and inter-day variability of GPA, CGA, GP, PG, G, and SG. The intra-day variability was assayed at same concentrations within one day. Meanwhile, inter-day variability was assayed for successive 3 d. The precisions were 98.91%, 98.73%, 96.97%, 100.70%, 100.01%, and 98.83% for GPA, CGA, GP, PG, SG, and G, respectively. The relative standard deviations (RSD) of intra- and inter-day were less than 3.00% (Table 2). So it was an acceptable precision in this experiment.

Repeatability and stability

Six reduplicative samples were prepared and tested immediately with the valid method. Results showed nice repeatability with the RSD less than 3.00%. The precision was weighed by the amount of samples in comparison to reference at the range of 97.19%–99.87%. The stability test demonstrated that the samples were stable during 12 h.

Table 1 Data of linearity, LOD, and LQD of six ingredients in *E. ulmoides* ($n = 6$)

Compounds	Range / ($\mu\text{g}\cdot\text{mL}^{-1}$)	Regression equations	r^2	LOD / μg	LQD / μg
GPA	3.08–154	$Y_1=14\ 195 X + 7161.9$	0.9998	0.19	0.77
CGA	3.08–154	$Y_5=14\ 884 X + 68.198$	0.9999	0.18	0.75
GP	3.24–162	$Y_2=13\ 387 X + 8808.8$	0.9994	0.20	0.81
PG	5.20–260	$Y_3=3738.4 X - 429.42$	0.9999	0.32	1.30
SG	6.64–332	$Y_4=6799 X + 5608.6$	0.9994	0.41	1.66
G	4.34–217	$Y_6=19\ 310 X + 33\ 815$	0.9999	0.27	1.09

All the analytes showed good linearity ($r^2 > 0.999$) in the concentration ranges. Y refers to the peak area. X is the concentration. r is the correlation coefficient of the equation

Table 2 Analytical results of precision

Compounds	Intra-day ($n = 6$)		Inter-day ($n = 3$)	
	Amount / mg	RSD / %	Amount / mg	RSD / %
GPA	0.11	1.47	0.11	1.56
CGA	0.06	2.24	0.06	2.97
GP	0.08	2.22	0.08	2.08
PG	0.12	1.74	0.12	1.94
SG	0.16	3.00	0.16	2.56
G	0.09	1.84	0.09	1.75

Recovery

The recovery test was carried out by spiking with certain known quantity of the four references to 1.0 g of the pulverized sample of crude barks of *E. ulmoides* and six samples were prepared for the test simultaneously. The recoveries of the quantitative compounds proved to be at the range from 97.61% to 101.63%, and the RSD was less than 2.00% (Table 3). It is an acceptable result for recovery analysis.

Table 3 Recovery of four ingredients for quantitative analysis ($n = 6$)

Compounds	<i>E. ulmoides</i> / μg	Spiked / μg	Found / μg	Recovery / %	RSD / %
GPA	368.86	350.00	722.79	101.63	1.27
CGA	147.51	90.00	237.00	99.91	1.31
GP	107.91	114.00	219.18	97.61	1.33
PG	491.40	514.00	1000.01	98.95	0.83

Sample determination

The valid method was employed to determine the six ingredients in each sample of the three batches, and the contents of the ingredients delineated as shown in Fig. 3. Ordinate axis shows the abundance ($\mu\text{g}/\text{g}$); abscissa axis shows time (h).

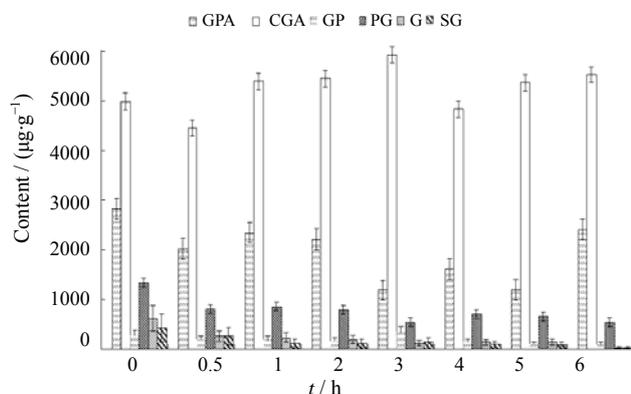


Fig. 3 Contents in processed barks of *E. ulmoides* in accordance with processing time ($\bar{X} \pm s, n = 3$)

Discussion

In TCM, the technique to process the barks of *E. ulmoides* was acknowledged as a result of the vulnerability of the gums and mucilages upon long-term heating, and there were insufficient unified standards to control the processing technique of processed barks of *E. ulmoides*. According to *Chinese Pharmacopoeia 2010*, processed barks of *E. ulmoides* were described as the appearance in burnt black, salty in flavor, and the gums and mucilage were deficiency of resilience when broken. This normality seems to be difficult to control the technique and quality of the processed barks of *E. ulmoides*. We successfully developed a method to control the quality of *E. ulmoides*. This experimental-based method on the multi-components quantification aimed at the various quantity changes of ingredients due to the processing time. Those results indicated that as the processing went on, most of the target ingredients decreased during

the initial two hours, and target ingredients kept stable in the section between two and four hours. In conclusion, four hours was better because it became vulnerable and could be easily broken. This is one of the reasons why barks of *E. ulmoides* should be traditionally processed for clinical application. Furthermore, when the processing time was prolonged, the quantity of GPA decreased. Meanwhile, the amounts of PG, SG, G, and CGA decreased due to the process. These four compounds probably transformed into others because they were unstable. According to some references, GP and G probably transformed into blue pigments and $UV\lambda_{\max}^{\text{MeOH}}$ at about 600 nm (Paik *et al.*, 2001). Meanwhile, caffeic acid might be transformed from CGA or some other compounds, which partly explained why the processed barks of *E. ulmoides* had the properties of stopping bleeding and preventing miscarriage (Zhang, 2002). Further research would be conducted in order to confirm the compounds relevant to the activities of stopping bleeding and preventing miscarriage, and some other factors referring to processing could be discovered to verify those efficacies.

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