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A rapid ultra performance liquid chromatography–tandem mass spectrometric method for the qualitative and quantitative analysis of ten compounds in *Eucommia ulmodies* Oliv.

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ABSTRACT

A rapid method for qualitative and quantitative analysis of constituents in *Eucommia ulmoides* Oliv. was developed by ultra performance liquid chromatography with electrospray ionization–tandem mass spectrometry (UPLC–ESI-MS). Ten compounds including iridoids, phenylpropanoids, and flavonoids were identified and further quantified as marker substances by UPLC-UV using switching UV wavelength within 16 min. The separation was carried out on an Acquity UPLC BEH C_{18} column with 0.1% formic acid aqueous solution and acetonitrile as the mobile phase under gradient conditions. The developed method was applied to the quality assessment of various *E. ulmoides* samples, including different medicinal parts, differently processed bark and bark from different habitats. The results showed that the branches of *E. ulmoides* were not suitable for medicinal use. The process of carbonizing led to significant loss of major components, whereas the process of stir-frying with salt-water enhanced the extraction of chlorogenic acid with negligible loss of the other components. The contents of the investigated compounds varied very remarkably for the bark of *E. ulmoides* collected from different habitats. This study might provide a comprehensive method for the quality assessment of *E. ulmoides*.

1. Introduction

Eucommia ulmoides Oliv., with a Chinese name Duzhong, is a native tree mainly distributed in Guizhou, Sichuan, Hubei, Henan, and Shaanxi provinces of China. According to the Chinese Pharmacopoeia, the medicinal parts of *E. ulmoides* are the bark and leaves, which possess the effects of nourishing the liver and kidney, strengthening the muscles and bones, preventing abortion, and so on [1]. Several types of secondary metabolites including iridoids, phenylpropanoids, and flavonoids have been isolated from *E. ulmoides* [2–4], which possess some specific bioactivities. Modern research shows that various pharmacological properties of *E. ulmoides* are mainly attributed to the lignans and iridoids [3]. Pinoresinol di-*O*-β-D-glucopyranoside and geniposidic acid, the characteristic constituents of *E. ulmoides*, are reported to possess antihypertensive activity [3,5]. Genipin and geniposide show antiinflammatory, antithrombotic, and antitumor effects [6–8]. Chlorogenic acid exhibits antibacterial, antioxidant, and antimutagenic activities [9]. Flavonoids including baicalein, wogonin, and oroxylin A possess antioxidative, anti-inflammatory, and antiviral effects [10,11].

A lot of work has been carried out on the phytochemical constituents of the bark and leaves of *E. ulmoides*. However, to the best of our knowledge, no phytochemical investigations on branches have been reported. It is necessary to compare the differences among the bark, leaves and branches, which would enable us to know whether the branches of *E. ulmoides* could be potentially useful for treating disease and thus reduce the damage of *E. ulmoides* arising from harvesting of bark.

Processing method is adopted based on the intended medicinal use of the *E. ulmoides* bark. Stir-frying with salt-water and carbonizing are two widely used methods nowadays. According to the Traditional Chinese Medicine (TCM) theory, the purpose of carbonizing is to enhance the hemostatic effect and reduce the toxic effect of the crude drug [12], and stir-frying with salt-water

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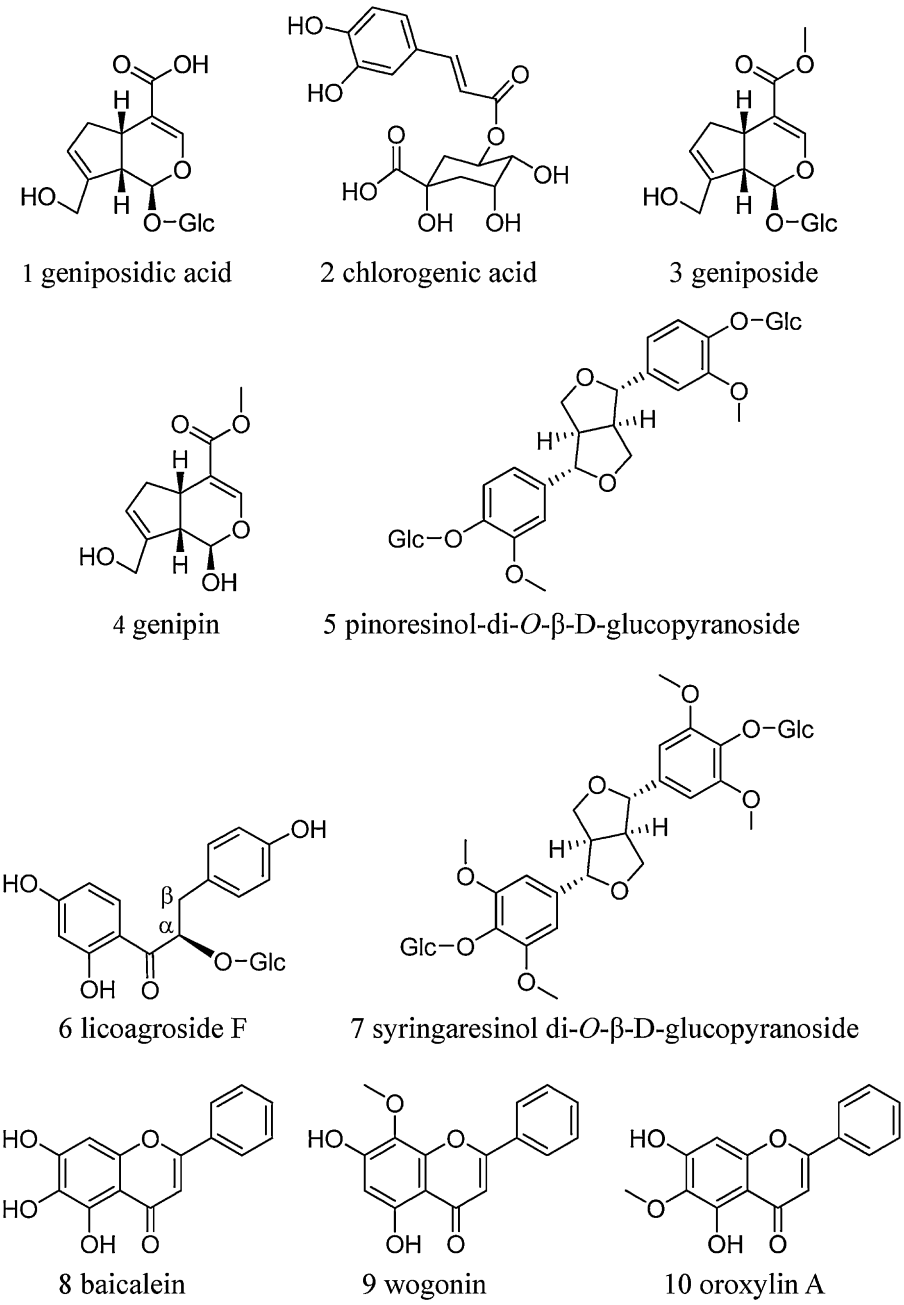


Fig. 1. Chemical structures of the ten compounds isolated from *E. ulmoides*.

enhances the kidney function [13]. For the bark of *E. ulmoides*, the major goal of processing is to break the silk of the gum in the bark, which restricts the extraction of active components. Recent studies were confined to variation of single component, such as pinoresinol di-*O*-β-D-glucopyranoside [14] or chlorogenic acid [15], which could not reflect the overall effects of processing methods to crude drugs. Therefore, it is imperative to elucidate the influence of processing methods on constituents in the *E. ulmoides* bark.

Variations in the medicinal parts, processing methods and habitats may lead to some differences in the content and variety of the constituents. Therefore, development of assessment method of the major compounds in *E. ulmoides* is essential for the effective and safe use of this traditional herb. Several analytical methods including high-performance liquid chromatography (HPLC) [16,17] and capillary electrophoresis (CE) [18] were hitherto employed to quantify some of the compounds for the chemical evaluation or

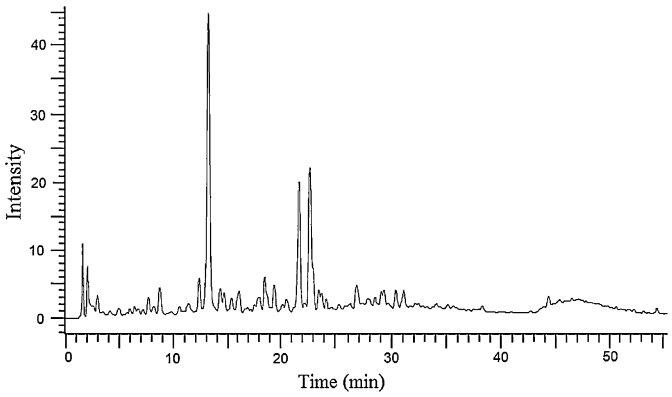


Fig. 2. Chromatogram of HPLC analysis of DZ-1.

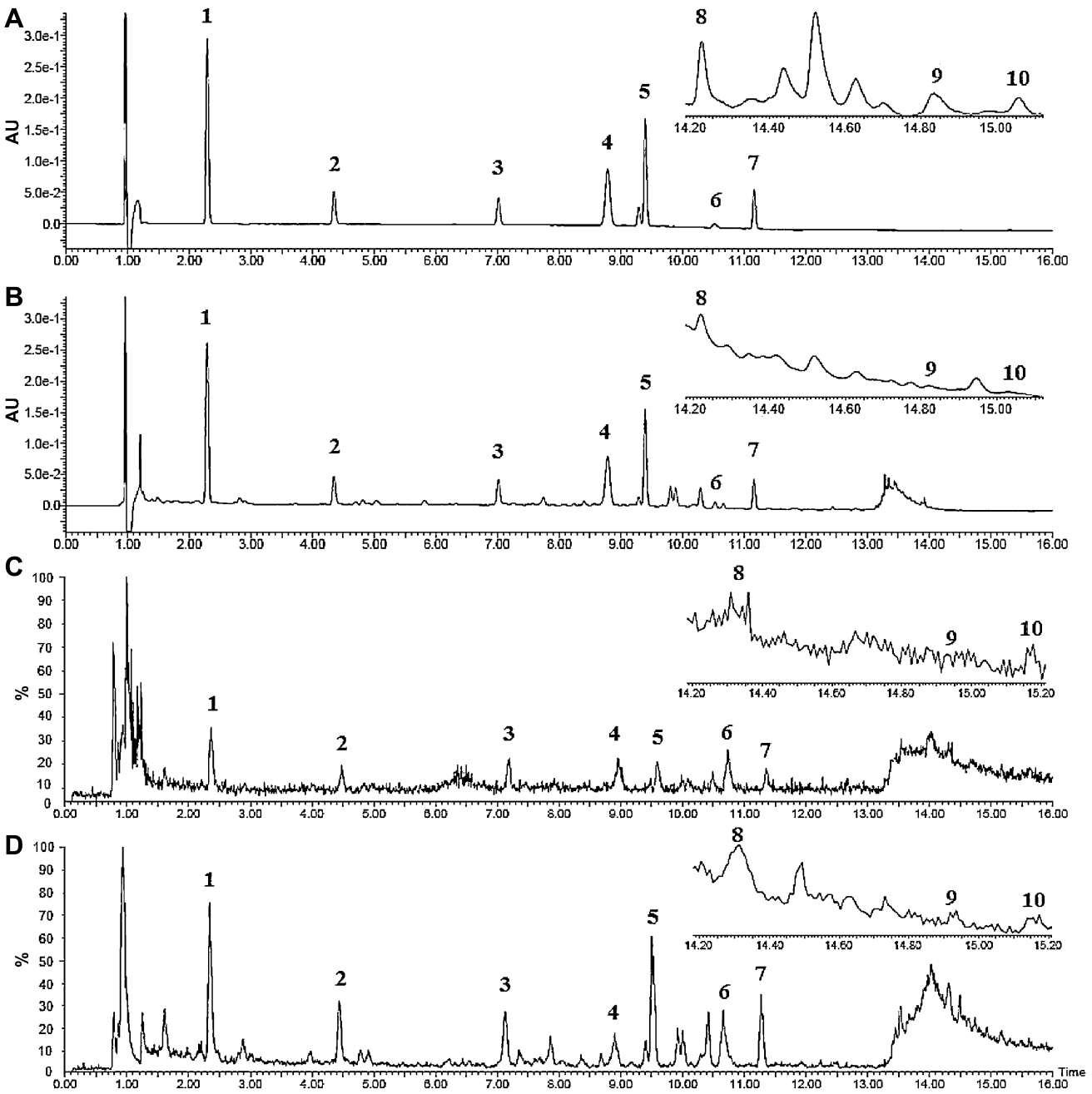


Fig. 3. UPLC-UV chromatograms of standard solution of ten compounds (A) and DZ-1 (B), and UPLC–ESI-MS total ion current chromatograms at the positive (C) and negative (D) ion mode of DZ-1.

standardization of *E. ulmoides*. However, HPLC methods are limited by long analysis time, low resolution and sensitivity. At the same time, it consumes large amounts of organic solvents, which are expensive and potentially harmful. Compared to ultra performance liquid chromatography (UPLC) method, the sensitivity of CE method is unsatisfactory. On the contrary, UPLC offers significant advantages in resolution, speed, reproducibility and sensitivity for analytical determinations with little solvent consumption [19,20]. Recently, UPLC is increasingly recognized as an important analytical separation technique for TCM analysis [21–23].

In this paper, a rapid and reliable UPLC method was developed for the simultaneous quantification of ten compounds within 16 min, namely geniposidic acid, chlorogenic acid, geniposide, genipin, pinoresinol di-*O*-β-D-glucopyranoside, licoagroside F ((α*R*)-α-*O*-β-D-glucopyranosyl-4,2′,4′-trihydroxydihydrochalcone), syringaresinol di-*O*-β-D-glucopyranoside, baicalein, wogonin, and oroxylin A. This developed method was then applied successfully to the quality assessments of various *E. ulmoides* samples.

2. Experimental

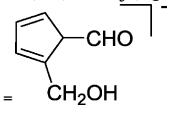
2.1. Chemicals and reagents

Standards of geniposidic acid, chlorogenic acid, geniposide, genipin, pinoresinol di-*O*-β-D-glucopyranoside, licoagroside F, syringaresinol di-*O*-β-D-glucopyranoside, baicalein, wogonin, and

Table 1
Characterization of compounds from *E. ulmoides*.

Peak no.	Retention time (min)	ES$^+$, *m/z*			ES$^-$, *m/z*		Identification
		[M+H]$^+$	[M+Na]$^+$	MS^2	[M−H]$^-$	MS^2	
1	2.29	–	397	235 [M+Na–Glc[a]]$^+$, 217 [M+Na–Glc–H_2O]$^+$, 203 [M+Na–Glc–CH_3OH]$^+$	373	211 [M−H–Glc]$^-$, 167 [M−H–Glc–CO_2]$^-$, 149 [M−H–Glc–CO_2–H_2O]$^-$, 123[b]	Geniposidic acid [33]
2	4.35	–	377	215 [M+Na–Ca[a]]$^+$, 197 [M+Na–Ca–H_2O]$^+$, 185 [M+Na–QA[a]–H_2O]$^+$	353	191 [M−H–Ca]$^-$, 161 [M−H–QA–H_2O]$^-$	Chlorogenic acid
3	7.02	–	411	249 [M+Na–Glc]$^+$, 217 [M+Na–Glc–CH_3OH]$^+$, 203 [M+Na–Glc–HCOOH]$^+$	387	225 [M−H–Glc]$^-$, 207 [M−H–Glc–H_2O]$^-$, 123[b]	Geniposide
4	8.79	–	249	203 [M+Na–HCOOH]$^+$, 171 [M+Na–HCOOH–CH_3OH]$^+$, 111 [M+Na–HCOOH–CH_3OH–$HCOOCH_3$]$^+$	225	207 [M−H–H_2O]$^-$, 123[b]	Genipin
5	9.40	–	705	543 [M+Na–Glc]$^+$, 381 [M+Na–2Glc]$^+$	681	519 [M−H–Glc]$^-$, 357 [M−H–2Glc]$^-$	Pinoresinol di-*O*-Glc
6	10.53	–	459	297 [M+Na–Glc]$^+$, 279 [M+Na–Glc–H_2O]$^+$	435	273 [M−H–Glc]$^-$, 255 [M−H–Glc–H_2O]$^-$	Licoagroside F
7	11.16	–	765	603 [M+Na–Glc]$^+$, 588 [M+Na–Glc–CH_3]$^+$, 441 [M+Na–2Glc]$^+$	741	579 [M−H–Glc]$^-$, 417 [M−H–2Glc]$^-$	Syringaresinol di-*O*-Glc
8	14.22	271	–	169 [M+H–EB[a]]$^+$, 123 [M+H–EB–CO–H_2O]$^+$, 103 [EB+H]$^+$, 95 [M+H–EB–2CO–H_2O]$^+$	269	241 [M−H–CO]$^-$, 223 [M−H–CO–H_2O]$^-$, 195 [M−H–2CO–H_2O]$^-$, 167 [M−H–EB]$^-$	Baicalein
9	14.82	285	–	270 [M+H–CH_3]$^+$, 168 [M+H–CH_3–EB]$^+$	283	268 [M−H–CH_3]$^-$	Wogonin
10	15.04	285	–	270 [M+H–CH_3]$^+$, 168 [M+H–CH_3–EB]$^+$	283	268 [M−H–CH_3]$^-$	Oroxylin A

[a] Glc, glucose; Ca, caffeoyl; QA, quinic acid; EB, ethynylbenzene.

[b] *m/z* 123 = 

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oroxylin A were isolated from *E. ulmoides* which were identified by direct comparison of their spectra data (MS, ^{1}H NMR and ^{13}C NMR) with those reported in the literatures [24–31]. Their structures are displayed in Fig. 1. The purities were above 98% using LC analysis.

LC-grade water was produced by Milli-Q water purification system (Millipore, Bedford, MA, USA). Formic acid, methanol, and acetonitrile of LC grade were purchased from Fisher Scientific (Pittsburg, PA, USA).

2.2. Plant materials

The leaves and branches of *E. ulmoides* were collected by ourselves from Xinyang city, Henan province of China, in July 2008,

Table 2
Quantitative results of ten compounds in DZ-1 extracted by different methods.

Methods	Content (μg/g crude drug)									
	Geniposidic acid	Chlorogenic acid	Geniposide	Genipin	Pinoresinol di-*O*-Glc	Licoagroside F	Syringaresinol di-*O*-Glc	Baicalein	Wogonin	Oroxylin A
25%[a], 30 min[b], 50 mL[c]	4100.32	820.97	791.20	1339.50	2700.31	91.14	774.14	0.382	0.0779	0.179
50%, 30 min, 50 mL	4480.31	932.47	978.04	1408.70	2939.77	93.71	776.07	0.523	0.0864	0.214
75%, 30 min, 50 mL	4482.04	924.31	836.63	1423.96	2843.73	67.38	773.52	0.528	0.0862	0.219
100%, 30 min, 50 mL	2962.40	308.71	839.24	1289.46	862.46	32.87	536.39	0.528	0.0863	0.211
50%, 10 min, 50 mL	4414.35	882.94	897.89	1297.65	2811.12	78.01	707.02	0.419	0.0691	0.197
50%, 20 min, 50 mL	4446.18	903.07	914.98	1321.06	2844.23	80.85	718.49	0.505	0.0739	0.201
50%, 30 min, 50 mL	4480.31	932.47	978.04	1408.70	2939.77	93.71	776.07	0.523	0.0864	0.214
50%, 40 min, 50 mL	4466.20	938.37	980.53	1403.55	2950.56	93.45	775.95	0.524	0.0863	0.223
50%, 30 min, 25 mL	4402.41	924.88	872.37	1379.19	2936.01	92.46	771.83	0.516	0.0809	0.214
50%, 30 min, 50 mL	4480.31	932.47	978.04	1408.70	2939.77	93.71	776.07	0.523	0.0864	0.214
50%, 30 min, 75 mL	4475.76	937.66	976.69	1407.17	2930.92	93.70	777.77	0.526	0.0853	0.216

[a] Extracting solvent: 25%, 50%, 75% methanol aqueous and 100% methanol.
[b] Ultrasonic time: 10 min, 20 min, 30 min and 40 min.
[c] Sample–solvent ratio: 25 mL, 50 mL and 75 mL per gram of sample.

Table 3
Regressive equations, linear ranges, LOD and LOQ of ten investigated compounds.

Analyte	Regressive equation[a]	R^2	Linear range (μg/mL)	LOD[b] (ng/mL)	LOQ[c] (ng/mL)
Geniposidic acid	$y = 139.327x + 5.714$	0.9998	12.225 – 319.200	8.1	27
Chlorogenic acid	$y = 128.894x - 3.044$	0.9998	2.726 – 87.217	12	36
Geniposide	$y = 116.164x - 5.216$	0.9997	2.672 – 85.517	19	53
Genipin	$y = 249.046x + 0.741$	0.9998	4.022 – 128.700	25	87
Pinoresinol di-*O*-Glc	$y = 129.054x + 32.680$	0.9998	8.013 – 256.400	11	32
Licoagroside F	$y = 237.534x + 41.478$	0.9991	0.263 – 8.425	4.2	10
Syringaresinol di-*O*-Glc	$y = 148.338x - 11.050$	0.9996	2.631 – 84.183	7.7	23
Baicalein	$y = 430.170x + 0.302$	0.9991	0.00394 – 0.126	0.24	0.79
Wogonin	$y = 1537.930x + 0.002$	0.9997	0.000497 – 0.0159	0.029	0.11
Oroxylin A	$y = 519.671x + 0.092$	0.9995	0.000998 – 0.0319	0.048	0.17

[a] The regressive equations were presented as $y = ax + b$. y and x were defined as peak area and concentration of compound, respectively.
[b] LOD, limit of detection, $S/N = 3$.
[c] LOQ, limit of quantification, $S/N = 10$.

Table 4
Stability, intra-day and inter-day precision of the proposed method (μg/mL).

Analyte	Stability ($n = 7$)		Intraday ($n = 6$)[a]		Interday ($n = 3$)[b]	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Geniposidic acid	4731.71 ± 16.07	0.34	4733.11 ± 9.67	0.20	4619.29 ± 200.16	4.33
Chlorogenic acid	1014.53 ± 5.50	0.54	1012.38 ± 4.11	0.41	1004.44 ± 15.97	1.59
Geniposide	938.51 ± 10.13	1.08	938.46 ± 5.28	0.56	918.77 ± 33.31	3.63
Genipin	1422.97 ± 6.64	0.47	1425.81 ± 2.88	0.20	1396.86 ± 33.31	2.38
Pinoresinol di-*O*-Glc	3004.53 ± 13.11	0.44	3008.58 ± 7.32	0.24	2982.48 ± 55.30	1.85
Licoagroside F	94.32 ± 0.72	0.76	93.76 ± 1.79	1.91	92.95 ± 0.82	0.89
Syringaresinol di-*O*-Glc	819.94 ± 2.30	0.28	820.92 ± 3.44	0.42	803.72 ± 20.83	2.59
Baicalein	0.52 ± 0.008	1.59	0.52 ± 0.010	1.97	0.52 ± 0.003	0.55
Wogonin	0.082 ± 0.0003	0.36	0.082 ± 0.0009	1.04	0.082 ± 0.0003	0.33
Oroxylin A	0.22 ± 0.002	1.08	0.22 ± 0.002	1.09	0.22 ± 0.0005	0.22

[a] Intraday precision on 1 day for tested six times.
[b] Interday precision on 3 successive days.

and marked as DZL and DZB, respectively. The five batches of the bark of *E. ulmoides* were collected in different locations and labelled as DZ-1 (Henan, China), DZ-2 and DZ-3 (Sichuan, China), DZ-4 (Hubei, China), and DZ-5 (Guangxi, China). The plant materials were authenticated by Prof. Tianxiang Li, Tianjin University of Traditional Chinese Medicine, and the voucher specimens were deposited in the herbarium of pharmacognosy, Tianjin University of Traditional Chinese Medicine (Tianjin, China). The bark of *E. ulmoides* from Henan province were parallel processed at twice using two different methods, namely stir-frying with salt-water and carbonizing as depicted in the Chinese Pharmacopoeia [1,32]. The four groups of roasted bark were labelled as DZS-1, DZS-2, DZC-1, and DZC-2.

2.3. Sample preparation

The bark, leaves and branches of *E. ulmoides* were pulverized into fine powder. The powdered sample (0.5 g) was extracted separately with 50% methanol aqueous solution (25 mL) in an ultrasonic water bath at room temperature for 30 min. The solution was centrifuged at 14,000 rpm for 10 min. Aliquot (3 μL) of the supernatant solution was injected into UPLC for analysis.

2.4. Standard solutions

Accurately weighed ten compounds were dissolved in DMSO to prepare stock solutions. A certain amount of each stock solution was placed in a 5 mL volumetric flask and diluted to volume with 50% methanol aqueous solution at the concentration of 391.2 μg/mL geniposidic acid, 87.22 μg/mL chlorogenic acid, 85.52 μg/mL geniposide, 128.7 μg/mL genipin, 256.4 μg/mL pinoresinol di-*O*-β-D-glucopyranoside, 8.425 μg/mL licoagroside F, 84.18 μg/mL syringaresinol di-*O*-β-D-glucopyranoside, 0.126 μg/mL baicalein, 0.0159 μg/mL wogonin, and 0.0319 μg/mL oroxylin A. The combined solution was then diluted stepwise with 50% methanol aqueous solution to give six different concentrations for construction of calibration curves.

2.5. LC analysis

The Hitachi LaChrom Elite LC system (Hitachi, Japan) equipped with Agilent Eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 μm) was used for the analysis. The mobile phase was a mixture of acetonitrile (A) and water containing 0.1% formic acid (B) at flow rate of

Table 5
Recovery of the proposed method ($n = 6$).

Analyte	Original (μg)	Spiked (μg)	Detected (μg)	Average recovery (%)	RSD (%)
Geniposidic acid	1148.63	1129.70	2247.10	97.24	2.04
Chlorogenic acid	260.12	261.65	516.86	98.12	2.55
Geniposide	239.24	256.55	501.20	102.11	3.78
Genipin	362.77	343.20	702.30	98.93	2.14
Pinoresinol di-*O*-Glc	765.05	769.05	1519.77	98.14	2.97
Licoagroside F	23.44	25.28	47.74	96.13	2.05
Syringaresinol di-*O*-Glc	198.46	210.46	408.10	99.61	3.45
Baicalein	0.1288	0.1262	0.2551	100.07	3.08
Wogonin	0.0204	0.0199	0.0402	99.50	1.45
Oroxylin A	0.0545	0.0639	0.1176	98.79	2.98

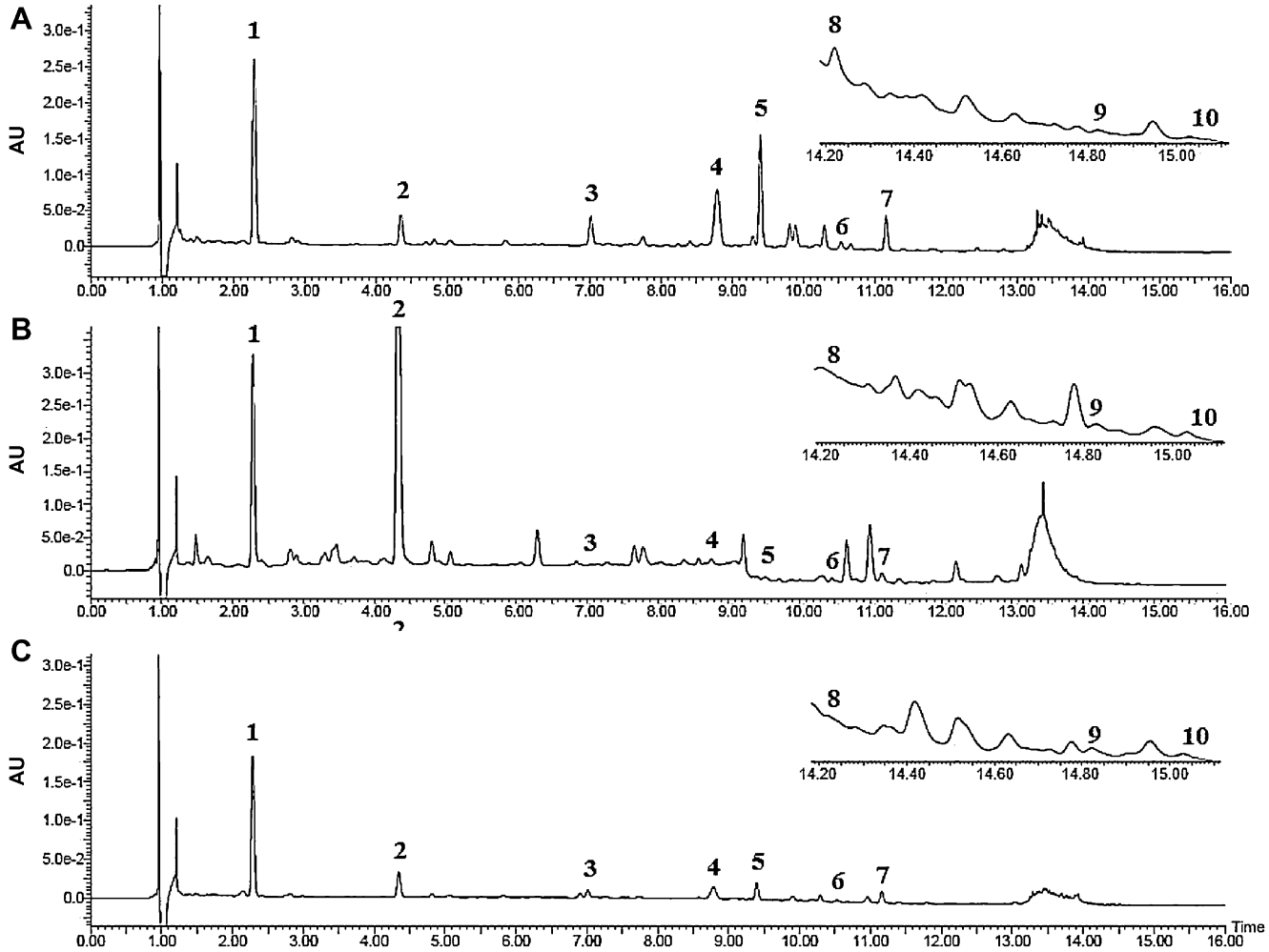

Fig. 4. UPLC-UV chromatograms of the bark (A), leaves (B), and branches (C) from the same *E. ulmoides* tree.

1 mL/min. A gradient elution program was used as follows: 5–25% A at 0–40 min, 25–50% A at 40–50 min, then linearly increased to 60% A at 55 min, and decreased from 60% to 5% A at 55–56 min. The composition was held at 5% A for a further 10 min for reequilibration. The detection wavelength was set at 277 nm, and analysis was performed at ambient temperature.

2.6. UPLC analysis

UPLC analyses were carried out using a Waters Acquity UPLC system (Waters, Milford, MA, USA), composed of a column heater, a sample manager, a binary solvent manager and a TUV detector. The chromatographic separation was performed on an Acquity UPLC™ BEH C_{18} column (100 mm × 3.0 mm, 1.7 μm particle size; Waters) by fixing the column heater at 50 °C. The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (B) with the flow rate at 0.5 mL/min. A gradient elution program was employed as follows: 5–16% A at 0–12 min, 16–50% A at 12–13 min, 50–58% A at 13–16 min, and decreasing from 58% to 5% A at 16–16.1 min. The composition was held at 5% A for a further 3 min for reequilibration. The detection wavelength was set at 237 nm in 0–3 min, 277 nm in 3–6 min, 237 nm in 6–9.2 min, 227 nm in 9.2–12 min, and 275 nm in 12–16 min.

2.7. Mass spectrometry

For UPLC–MS analysis, a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) was connected to the Waters Acquity UPLC system via an ESI interface. The LC effluent was directly introduced into the ESI source without split. Nitrogen was used as desolvation gas at flow rates of 600 L/h for ESI(+) and 700 L/h for ESI(−); the cone gas was set at 50 L/h. The desolvation temperature was 350 °C; the source temperature was 110 °C. Capillary voltage was 3200 V for ESI(+) and 2800 V for ESI(−); cone voltage was 30 V. Argon was employed as collision gas at flow rate of 0.20 mL/min; collision energy was 35 V. The spectra were recorded in the range of *m*/*z* 150–800 for full scan MS analysis and *m*/*z* 50–800 for MS/MS analysis.

3. Results and discussion

3.1. Comparison study of chromatographic performance

In our preliminary study, an LC method was tested to determine DZ-1 in 55 min (Fig. 2). However, as far as the routine analysis of *E. ulmoides* is concerned, the LC method was not feasible. Compared to LC analysis, UPLC could complete the chromatographic analysis in 16 min (Fig. 3), which allowed shortening the analysis time up to threefold with better resolution. Therefore, UPLC was a powerful tool for the analysis of complex system such as TCM.

3.2. Optimization of UPLC conditions

Allowing for the presence of phenolic compounds in *E. ulmoides*, 0.1% formic acid was added into the mobile phase

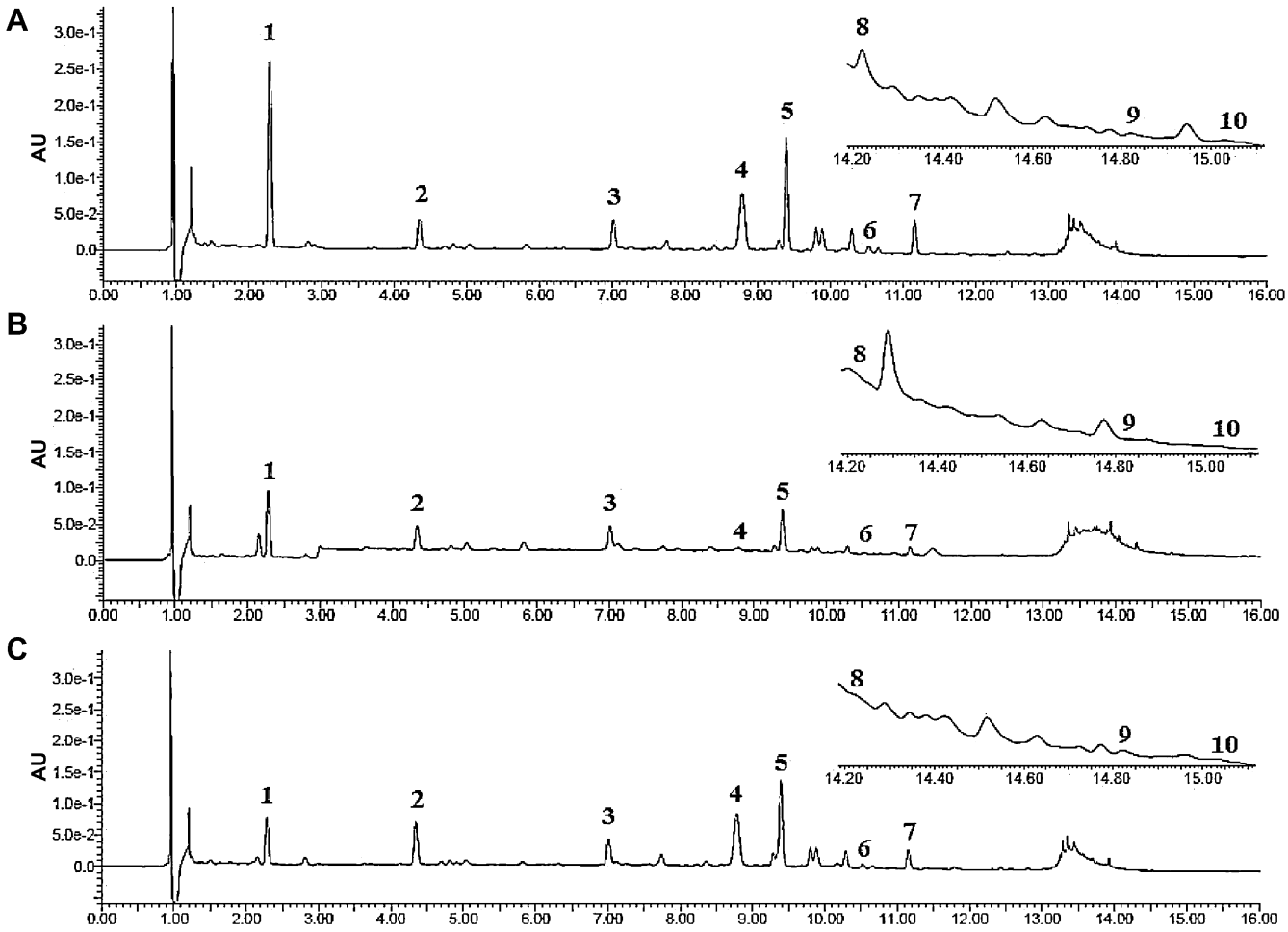

Fig. 5. UPLC-UV chromatograms of unroasted bark (A), carbonized bark (B), and bark stir-fried with salt-water (C).

to prevent dissociation. Higher column temperature was used at the flow rate of 0.5 mL/min, which resulted in improved separation and peak shape. In order to attain the satisfactory sensitivity, resolution and lower noise, three wavelengths, 227 nm, 237 nm and 277 nm, were selected as the detective wavelength for determining the different compounds in *E. ulmoides*. According to absorption curve of the tested analytes, the iridoids had good sensitivity at 237 nm, chlorogenic acid and the flavones exhibited maximum absorption at 277 nm, pinoresinol di-*O*-β-D-glucopyranoside, licoagroside F, and syringaresinol di-*O*-β-D-glucopyranoside showed good sensitivity at 227 nm. Thus, a switching UV wavelength method was established by using a variable-wavelength spectrophotometric detector. Under the optimized UPLC-UV conditions, the investigated analytes were well separated and detected in 16 min (Table 1).

3.3. Optimization of extraction methods

To achieve an efficient extraction of active components in *E. ulmoides*, such key factors as extracting solvent (25%, 50%, 75% methanol aqueous solution and pure methanol), ultrasonic time (10 min, 20 min, 30 min and 40 min) and sample-solvent ratio (25 mL, 50 mL and 75 mL per gram of sample) were investigated independently. Consequently, as shown in Table 2, 50% methanol aqueous solution, 30 min of ultrasonic time and 50 mL per gram of sample emerged as the conditions at which efficient extraction was attained.

3.4. UPLC method validation

Method validation of quantitative analysis was performed under the UPLC analytical conditions described above. The linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, accuracy, and stability for the investigated compounds were validated. Each calibration curve was performed with six different concentrations in triplicate. All calibration curves were of good linearity with high correlation coefficient ($R^2 \geq 0.9991$) over the tested range, which are summarized in Table 3. The LOD and LOQ listed in Table 3 were 0.029–25 ng/mL and 0.11–87 ng/mL for the ten compounds quantified in this study, respectively. They were determined by serial dilution of standard solution using the described UPLC conditions. As shown in Table 4, ten analytes proved to be stable in sample solution within 12 h at room temperature with their RSD values below 1.59%. The analysis of intra- and inter-day precisions was conducted by six repetitive injections on the same day and on the consecutive three days. Both RSD values of intra- and inter-day precisions did not exceed 4.33% (Table 4). Recovery tests were performed by spiking the authentic standards to the samples. Six samples of DZ-1 were spiked with the standard solution of each compound, then treated as sample preparation procedure. The overall recoveries of the ten compounds were in the range of 96.13–102.11% with RSD values not more than 3.78%, as displayed in Table 5.

In general, the assay results proved to be satisfactory in sensitivity, reproducibility and accuracy. Accordingly, ten analytes in various *E. ulmoides* samples were determined by the validated method.

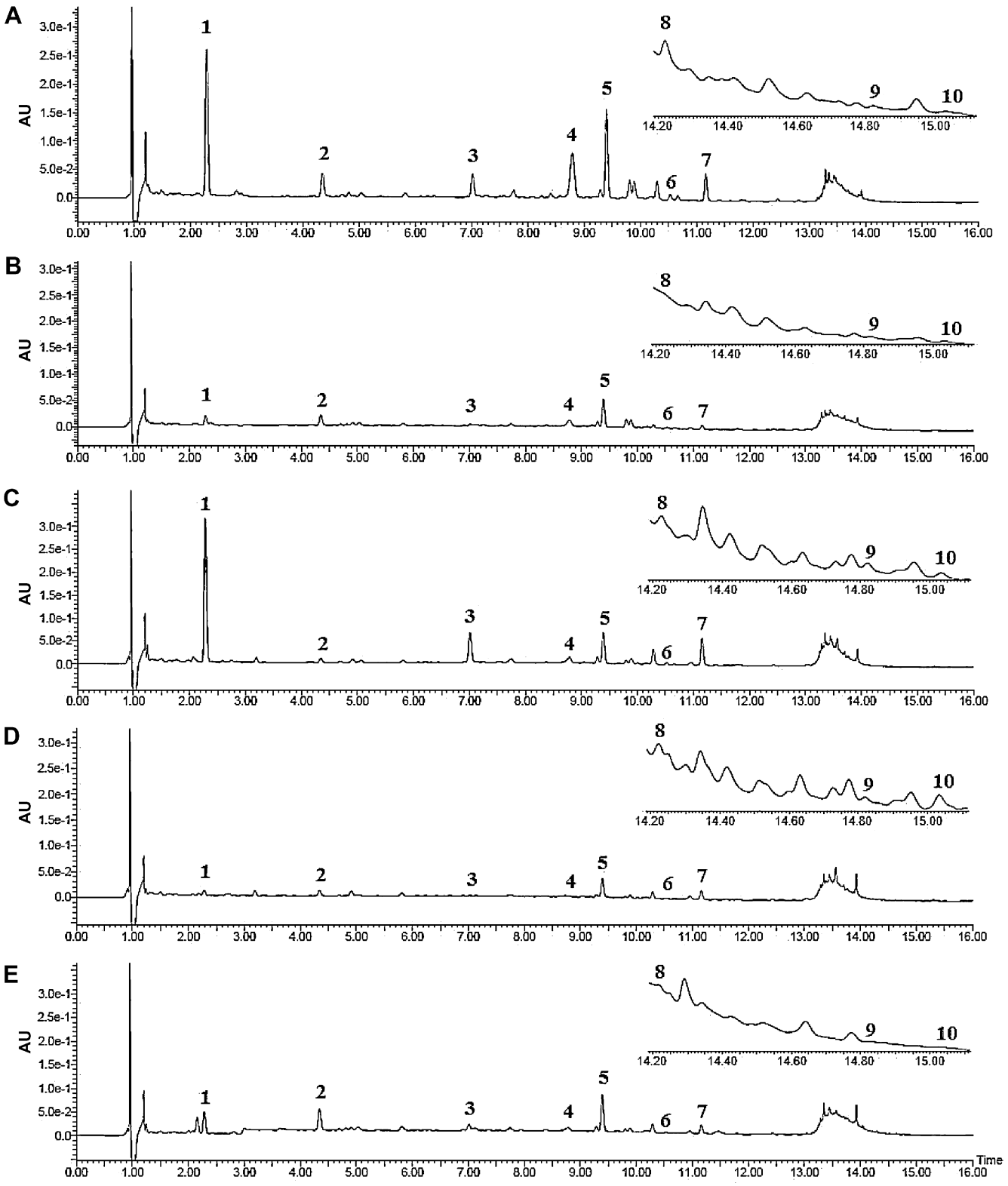

Fig. 6. UPLC-UV chromatograms of *E. ulmoides* bark from different regions: DZ-1 (A), DZ-2 (B), DZ-3 (C), DZ-4 (D), and DZ-5 (E).

3.5. Qualitative identification and quantitation of ten compounds in E. ulmoides by UPLC–MS

Identification of the compounds in *E. ulmoides* was accomplished by UPLC–ESI-MS technique by comparison of their retention times and MS spectra with reference standards. The ten investigated analytes in *E. ulmoides* covered three chemical types, namely iridoids (compounds **1**, **3** and **4**), phenylpropanoids (compounds **2**, **5** and **7**), and flavonoids (compounds **6**, **8**, **9** and **10**). Under the validated UPLC method, various *E. ulmoides* samples were subjected to UPLC-UV analysis. The typical UPLC-UV chromatograms of standard compounds and DZ-1, and total ion current

chromatograms acquired with two ES modes of DZ-1 are shown in Fig. 3. The UPLC-UV chromatograms of various *E. ulmoides* samples, including different medicinal parts, differently processed bark and bark from different habitats are shown in Figs. 4–6. The mass spectra of the identified compounds are summarized in Table 1.

3.6. Quantitative analysis of different medicinal parts

Three medicinal parts, namely bark, leaves and branches, were collected from the same tree by ourselves. As shown in Table 6 and Fig. 4, the contents of geniposidic acid and pinoresinol di-*O*-β-D-glucopyranoside were very high in the bark from Henan province (DZ-1), the contents of chlorogenic acid, geniposide, genipin, and syringaresinol di-*O*-β-D-glucopyranoside were relatively high, and the contents of four flavonoids were found to be relatively low. The leaves from Henan province (DZL) contained great amount of chlorogenic acid, about eighteen folds relative to the bark. The content of geniposidic acid was relatively high, a little more than the content in the bark, and the contents of the other compounds were significantly low. So chlorogenic acid and geniposidic acid might be the effective components of the leaves. The content of each analyte was significantly reduced in the branches (DZB) compared with the bark. The total content of the ten investigated compounds in the branches was less than half of the total content in the bark, which could explain why branches are not suitable for medicinal use.

3.7. Quantitative analysis of different processed bark

As shown in Table 6 and Fig. 5, the content of each analyte in carbonized bark (DZC) declined in varying degrees from 20% to 95% compared with the unroasted bark, among which the content of genipin declined most significantly. The total content of ten analytes was only one third of that in the unroasted bark. In addition, the contents of the investigated analytes varied significantly between the two groups of carbonized bark, which may be caused by inconsistent judgments about processing end-point. For the bark stir-fried with salt-water (DZS), the data of the two groups were basically consistent. The content of chlorogenic acid slightly increased compared with the unroasted bark, whereas the content of geniposidic acid was one quarter of that in the unroasted bark, and the contents of the other analytes did not markedly reduce.

Based on these results, the process of carbonizing was hard to control and may cause serious loss of major components. By comparison, the process of stir-frying with salt-water enhanced the extraction of chlorogenic acid with little loss of the other components. In summary, different processing methods led to variation in the content and proportion of the components in the bark of *E. ulmoides*, which might cause the different pharmacological effects and clinical application.

3.8. Quantitative analysis of bark from different habitats

Based on analytical results of the five samples of *E. ulmoides* gathered from different places (Table 6 and Fig. 6), the total contents of the investigated analytes varied remarkably, which showed eightfold difference between the maximum and minimum. As for the three chemical types, the total contents of iridoids varied most remarkably with RSD value upon 110%, followed by flavonoids and phenylpropanoids with RSD values of 76% and 55%, respectively. Among the investigated analytes, the content of genipin varied most significantly with RSD value of 149%, and the contents of pinoresinol di-*O*-β-D-glucopyranoside, baicalein, and oroxylin A were relatively stable with RSD values below 60%. Many factors could be responsible for the variations, such as climate, regions of growth, growing year, seasons of harvest among others.

Table 6
Quantitative results of ten compounds in various *E. ulmoides* samples ($n = 3$).

Samples	Content (mean ± SD) (μg/g crude drug)										
	Geniposidic acid	Chlorogenic acid	Geniposide	Genipin	Pinoresinol di-*O*-Glc	Licoagroside F	Syringaresinol di-*O*-Glc	Baicalein	Wogonin	Oroxylin A	Total[b]
DZL	5797.87 ± 36.69	18,816.59 ± 89.05	27.40 ± 1.00	111.69 ± 2.33	23.91 ± 0.67	8.97 ± 0.41	268.30 ± 5.74	0.28 ± 0.010	0.20 ± 0.0027	0.09 ± 0.0040	25,055.30
DZB	3274.23 ± 39.78	671.20 ± 4.40	211.46 ± 1.43	250.55 ± 0.71	384.15 ± 19.67	28.78 ± 0.81	275.53 ± 0.99	0.23 ± 0.002	0.19 ± 0.0030	0.04 ± 0.0004	5096.36
DZC-1	1643.07 ± 29.88	740.63 ± 16.59	663.09 ± 6.12	77.40 ± 2.98	1140.16 ± 20.06	13.38 ± 0.03	204.14 ± 2.37	0.41 ± 0.005	ND[a]	ND	4482.28
DZC-2	638.63 ± 8.27	907.19 ± 6.35	408.39 ± 3.33	14.07 ± 0.28	1079.00 ± 11.87	9.12 ± 0.12	145.57 ± 0.35	0.38 ± 0.001	ND	ND	3202.35
DZS-1	1364.21 ± 36.47	1502.25 ± 43.86	947.25 ± 0.26	1490.05 ± 8.92	2895.51 ± 12.07	62.84 ± 1.61	513.82 ± 12.51	0.52 ± 0.009	0.13 ± 0.0035	0.03 ± 0.0012	8776.61
DZS-2	1366.62 ± 53.87	1490.91 ± 28.77	672.37 ± 23.89	1014.05 ± 1.82	2923.49 ± 26.54	68.78 ± 1.58	553.32 ± 24.81	0.51 ± 0.002	0.10 ± 0.0003	0.01 ± 0.0003	8090.16
DZ-1	4592.36 ± 172.48	1040.01 ± 26.09	956.52 ± 44.01	1450.42 ± 26.34	3058.79 ± 83.64	93.71 ± 1.22	793.48 ± 21.90	0.52 ± 0.005	0.08 ± 0.0003	0.22 ± 0.002	11,986.11
DZ-2	284.09 ± 6.52	426.53 ± 5.90	47.18 ± 1.08	182.22 ± 5.13	923.92 ± 33.05	20.31 ± 0.69	156.39 ± 1.05	1.01 ± 0.007	0.13 ± 0.0049	0.15 ± 0.0058	2041.93
DZ-3	5768.16 ± 103.72	186.47 ± 0.03	1609.42 ± 26.11	211.22 ± 4.72	1348.80 ± 36.17	44.56 ± 0.63	1063.27 ± 16.67	2.25 ± 0.014	0.48 ± 0.0092	0.34 ± 0.0032	10,234.97
DZ-4	156.71 ± 6.09	223.64 ± 2.22	22.82 ± 0.77	20.49 ± 0.51	756.80 ± 26.07	20.43 ± 0.51	305.10 ± 1.43	1.45 ± 0.020	0.32 ± 0.0011	0.59 ± 0.0136	1508.35
DZ-5	822.06 ± 4.95	1003.71 ± 10.27	248.09 ± 0.86	122.46 ± 1.54	1502.68 ± 10.28	20.63 ± 0.28	327.64 ± 9.22	0.79 ± 0.006	ND	0.22 ± 0.0037	4048.28

[a] ND, not detected.
[b] Total content of ten investigated compounds.

In general, the contents of pinoresinol di-*O*-β-D-glucopyranoside were relatively high and stable, but the contents of the iridoids varied very remarkably. In the Chinese Pharmacopoeia 2010 edition, pinoresinol di-*O*-β-D-glucopyranoside alone was assigned as the marker species for the quality evaluation of the bark of *E. ulmoides* [1]. However, iridoids possess many specific bioactivities, such as antiinflammatory, antithrombotic, and antitumor effects. We believe that a more effective quality control should include assay of a set of major iridoids.

4. Conclusion

A rapid, sensitive and reliable method was established to assess the quality of *E. ulmoides*. With the lower flow rate and much shorter analysis time, the current UPLC–ESI-MS method helped to identify and quantify ten active compounds in various *E. ulmoides* samples. Our results indicated that the branches of *E. ulmoides* may not be suitable for medicinal use. Different processing methods led to variation in the content and proportion of the components in the bark of *E. ulmoides*. The contents of the investigated compounds varied very remarkably in the bark of *E. ulmoides* collected from different regions.

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