Identification of Major Alkaloids in Rat Urine by HPLC/DAD/ESI-MS/MS Method Following Oral Administration of Cortex *Phellodendri* Decoction

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A rapid, sensitive, and specific high-performance liquid chromatography (HPLC), diode-array detection, and mass-spectrometry techniques were developed for an identification of the constituents of Cortex *Phellodendri* and their metabolites in rat urine. The dose of 10 ml/kg of Cortex *Phellodendri* decoction was used for rats' oral administration. 0–24-h Urine was purified using a *C18* solid-phase extraction cartridge, and then analyzed by an on-line MS detector. A total of 13 characteristic HPLC peaks were detected in the urine samples. Nine of them, including five alkaloids and four of their metabolites, were tentatively elucidated as magnoflorine (**1**), the glucuronide conjugate of demethyleneberberine (**2**), menisperine (**3**), jatrorrhizine 3-*O*-glucuronide (**4**), berberubine 9-*O*-glucuronide (**5**), jatrorrhizine (**6**), the monomethyl and monohydroxy catabolite of berberubine (**7**), palmatine (**8**), and berberine (**9**). Identification and structural elucidation of the metabolites were performed by comparing their MS^n spectra data with those reported.

Introduction. – Traditional Chinese medicines (TCMs) have been widely used for disease treatment and made essential contributions to the growth and prosperity in China for centuries. At present, TCMs are facing difficulties because their curative effects are largely based on the prescribing doctor's prior knowledge and experiences, and their effective constituents and mechanisms of action are unclear, which seriously restricts their development for release into the international market. Unlike western medicines, TCMs contain hundreds of chemically different constituents. It is impossible to obtain their effective constituents by traditional methods like fractionation to obtain pure compounds for structure elucidation, followed by pharmacological testing of individual compounds. Therefore, methods to reveal the effective substances are in great demand [1].

In fact, for most of orally administrated TCMs, only the constituents successfully assimilated into blood and kept in a considerable concentration level in target organs have the possibility to be responsible for the curative effects. If these constituents can be identified, the number of pharmacologically active constituents will be considerably reduced. Together with a subsequent preparation of these identified compounds from plants or by synthesis and following pharmacological tests, the effective constituents can be found and action mechanisms of TCMs will conceivably be elucidated. Nevertheless, if TCMs are directly administrated in form of decoction, the much lower

concentrations of constituents in serum would make it very hard to detect without any pooling preparations. Furthermore, these preparations are often complex, time-consuming, and usually relate to lots of big animals, even pigs [2]. Such difficulty seriously limits the ongoing research on TCMs and their pharmacologically active constituents. On the other hand, due to renal tubular reabsorption, the concentrations of the potentially effective constituents in urine are much higher than those in serum. By rather using urine as biological matrix instead of blood, the presence of the potentially active compounds of TCMs would be easier to detect.

Cortex *Phellodendri* (*Huangbai* in Chinese), the dried bark of *Phellodendron chinensis* or *Phellodendron amurense* (Fam. Rutaceae), is a well-known traditional Chinese medicinal herb and officially listed in the Chinese Pharmacopoeia. It has been widely used in China to remove *damp-heat*, quench *fire*, counteract toxicity, and relieve consumptive fever. In TCMs' clinical applications, this herb has proved effective in treating dysentery, jaundice and morbid leucorrhea caused by *damp-heat*, urinary infections, weakness and oedema of legs, consumptive fever and night sweating, seminal emission, sores and skin infection with local redness and swelling, eczema with itching [3]. The constituents of Cortex *Phellodendri* are numerous and diverse, including aporphine alkaloids, protoberberine alkaloids, and flavonoids. Even though some active compounds have been isolated by traditional phytochemical methods from this medicine in previous research, their assimilation and metabolites research *in vivo*, which is very important for them showing their pharmacological effectiveness, has not been reported when the decoction was directly administrated.

Recently, HPLC/MS^n had been proved to be a modern powerful tool for the identification of substances in biofluids or in TCM extracts [2][4–12]. This approach takes the advantage in sensitivity and specificity. In addition, rich structural information of analytes of interest can be acquired by MS^n techniques. In this research, a more efficient and convenient method utilizing SPE and HPLC/DAD/MS/MS techniques for potentially active compounds detection in urine following oral administration of Cortex *Phellodendri* decoction was developed. The identification of nine compounds including five alkaloids and four of their metabolites in rat urine would pave the way for any action mechanism research to follow.

Results and Discussion. – MS total ion current (*TIC*) chromatograms of the blank rat urine (*a*), the rat urine sample collected from 0 to 24 h after oral administration of Cortex *Phellodendri* decoction (*b*) and Cortex *Phellodendri* decoction (*c*) are shown in *Fig. 1*. Based on direct comparison with chromatograms of the blank rat urine and Cortex *Phellodendri* decoction, nine compounds (**1**–**9** in *Fig. 1*,*b*), including five alkaloids and four of their metabolites, were tentatively elucidated as magnoflorine (**1**), the glucuronide conjugate of demethyleneberberine (**2**), menisperine (**3**), jatrorrhizine 3-*O*-glucuronide (**4**), berberubine 9-*O*-glucuronide (**5**), jatrorrhizine (**6**), the monomethyl and monohydroxy product of berberubine (**7**), palmatine (**8**), and berberine (**9**). Compounds **10**–**13** could not be identified by the present UV and LC/MS^n data.

The UV spectra of compounds **1**–**9** can be classified into two different profiles, which are typical for two kinds of alkaloids which exist in Cortex *Phellodendri*, the aporphine alkaloid structure of compounds **1** and **3** (*Fig. 2*,*a* and *c*), and the protoberberine alkaloid structure of compounds **2** and **4**–**9** (*Fig. 2*,*b* and *d*–*i*).

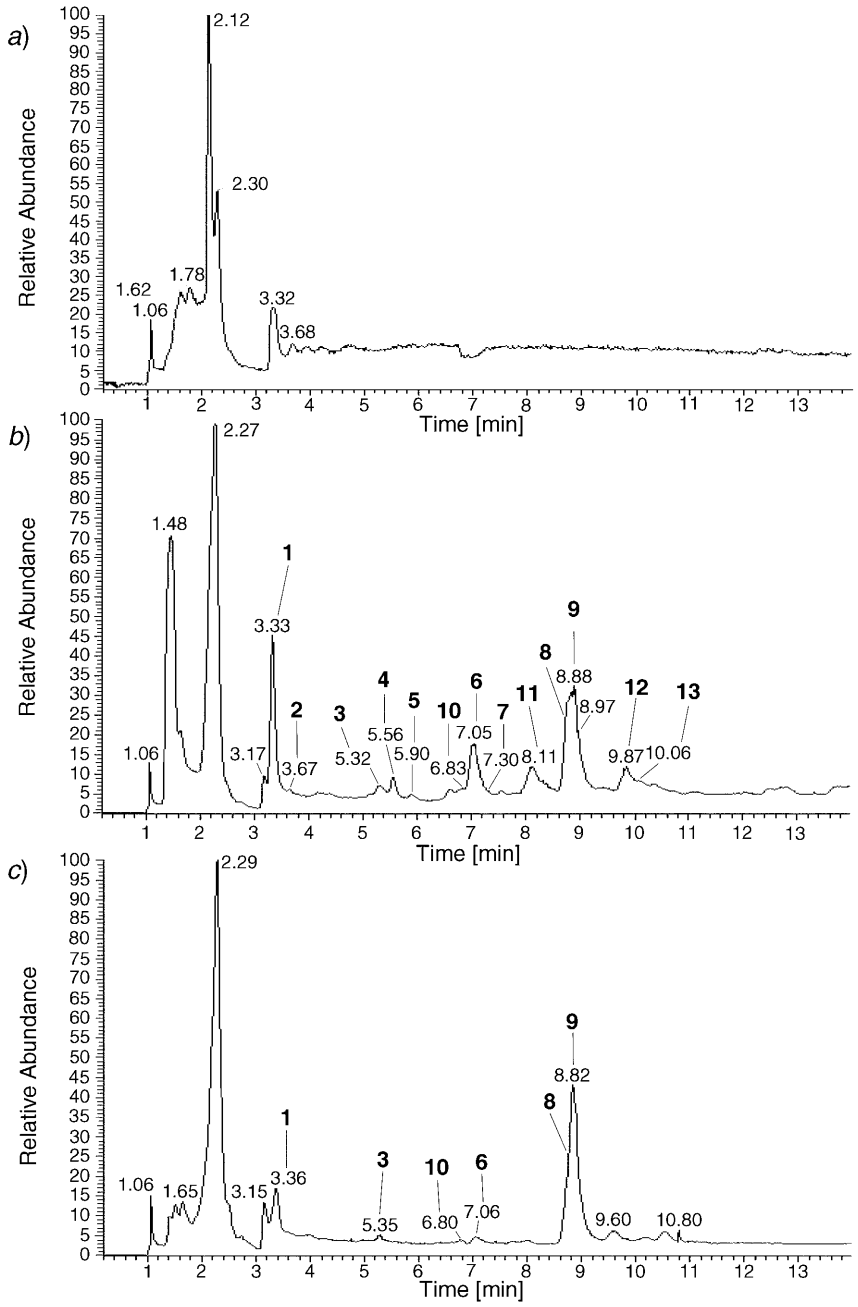


Fig. 1. *MS Total ion current chromatograms of* a) *blank rat urine,* b) *the urine sample collected from 0 to 24 h after oral administration of Cortex* Phellodendri *decoction, and* c) *Cortex* Phellodendri *decoction*. Labeled chromatographic peaks were identified by mass spectrometry as follows: magnoflorine (**1**), the glucuronide conjugate of demethyleneberberine (**2**), menisperine (**3**), jatrorrhizine 3-*O*-glucuronide (**4**), berberubine 9-*O*-glucuronide (**5**), jatrorrhizine (**6**), the monomethyl and monohydroxy product of berberubine (**7**), palmatine (**8**), and berberine (**9**).

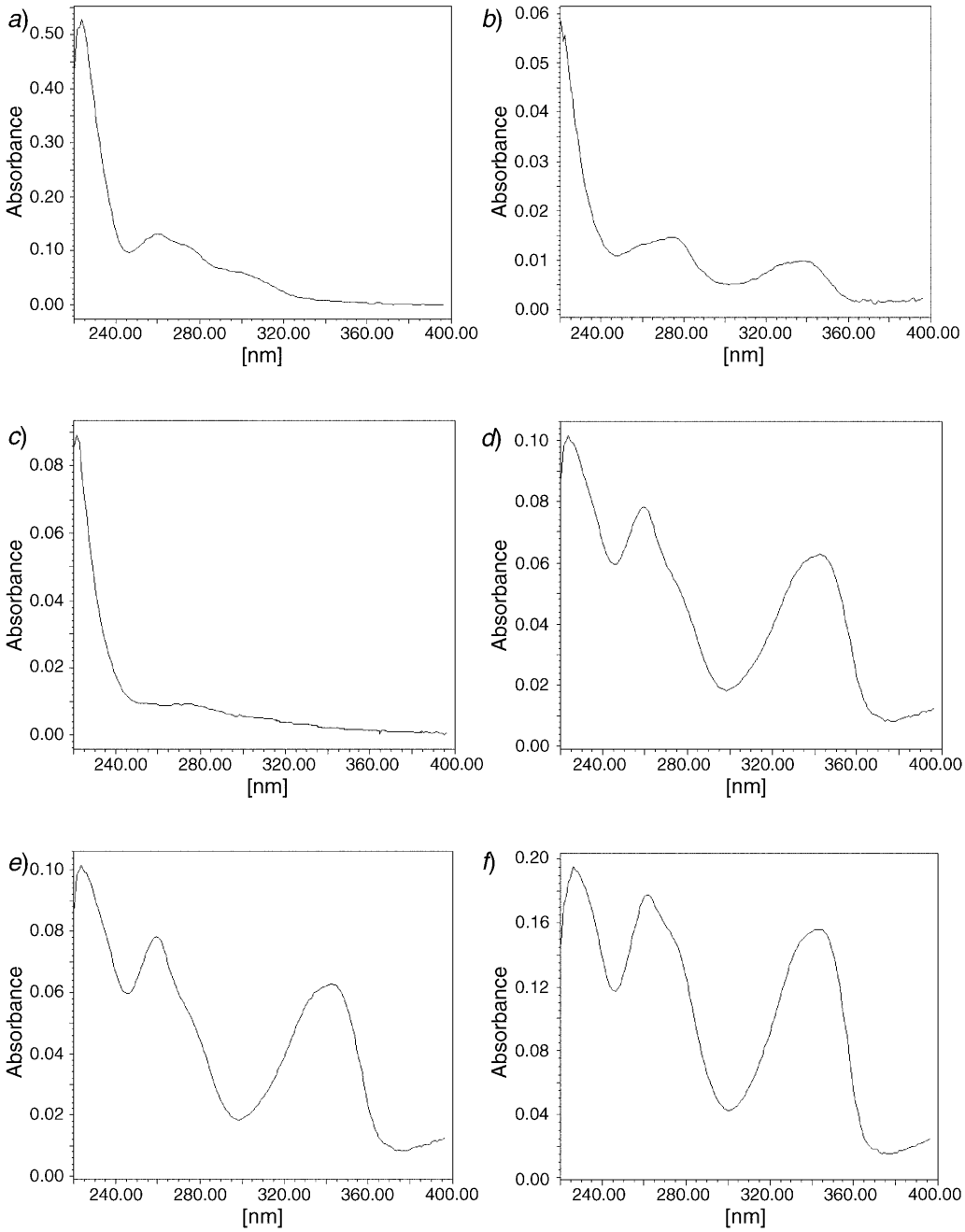


Fig. 2. *UV Spectra of nine HPLC peaks* (**1**–**9**) *detected in the urine of rats orally administrated Cortex* Phellodendri *decoction. a*) Magnoflorine (**1**), *b*) the glucuronide conjugate of demethyleneberberine (**2**), *c*) menisperine (**3**), *d*) jatrorrhizine 3-*O*-glucuronide (**4**), *e*) berberubine 9-*O*-glucuronide (**5**), *f*) jatrorrhizine (**6**), *g*) the monomethyl and monohydroxy product of berberubine (**7**), *h*) palmatine (**8**), and *i*) berberine (**9**).

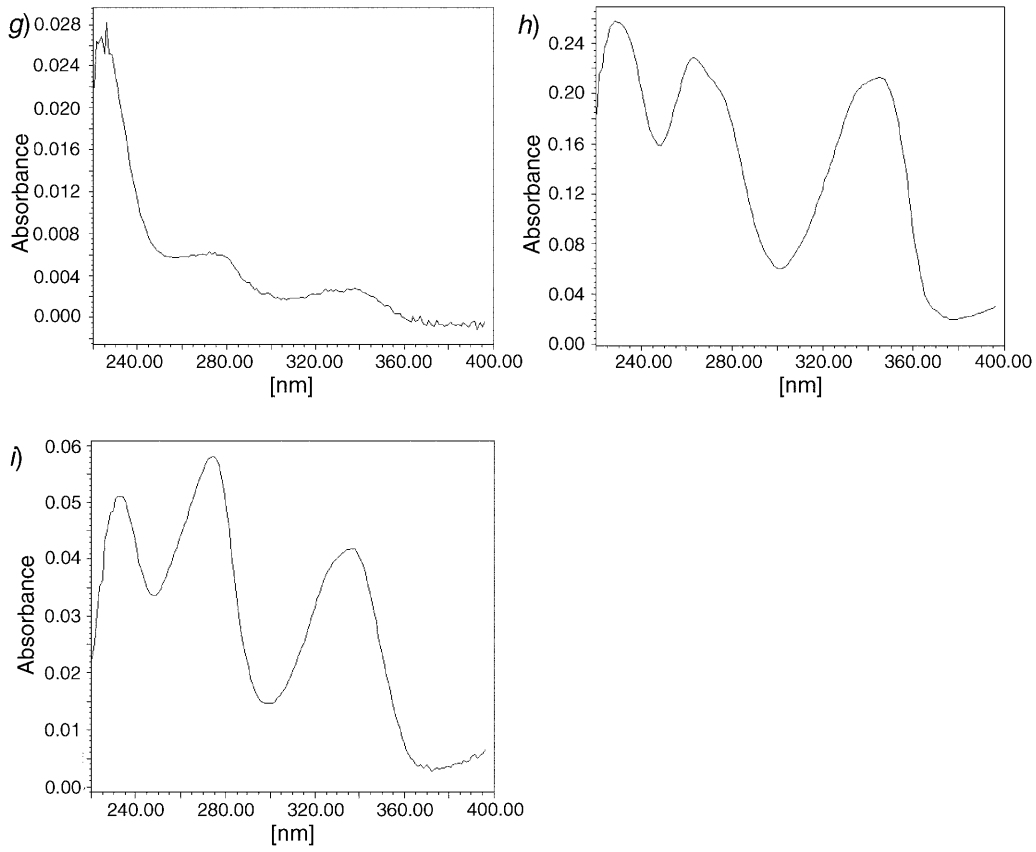


Fig. 2 (cont.)

Compound **1** (t_R 3.33 min) had UV absorption maxima at 225, 262 and 305 nm (*Fig. 2,a*). A singly charged ion at *m/z* 341.8 (M^+) was observed in the ESI mass spectrum (positive ion mode) of **1**. Additionally, when the cone voltage was set at 35 V, nine product ions at *m/z* 311.0 ($[M-MeO]^+$), 296.9 ($[M-C_2H_7N]^+$), 281.8 ($[M-C_2H_7N-Me]^+$), 264.8 ($[M-C_2H_7N-MeOH]^+$), 236.8 ($[M-C_2H_7N-MeOH-CO]^+$), 221.8 ($[M-C_2H_7N-MeOH-CO-Me]^+$), 208.8 ($[M-C_2H_7N-MeOH-CO-C_2H_4]^+$), 191.5 ($[M-C_2H_7N-MeOH-CO-C_2H_4-OH]^+$), and 176.7 ($[M-C_2H_7N-MeOH-CO-C_2H_4-OH-Me]^+$) were detected in the collision induced dissociation (CID) spectrum of the ion peak at *m/z* 341.8 (*Fig. 3*). These results are consistent with those of magnoflorine ($C_{20}H_{24}NO_4$, molecular weight 342) [13], which is a major constituent of Cortex *Phellodendri* [14]. The mechanistic pathway for fragments formed may be explained according to *Scheme 1*.

Compound **2** (t_R 3.67 min) had UV absorption maxima at 277 and 340 nm (*Fig. 2,b*). A singly charged ion at *m/z* 499.8 (M^+) was observed in the ESI mass

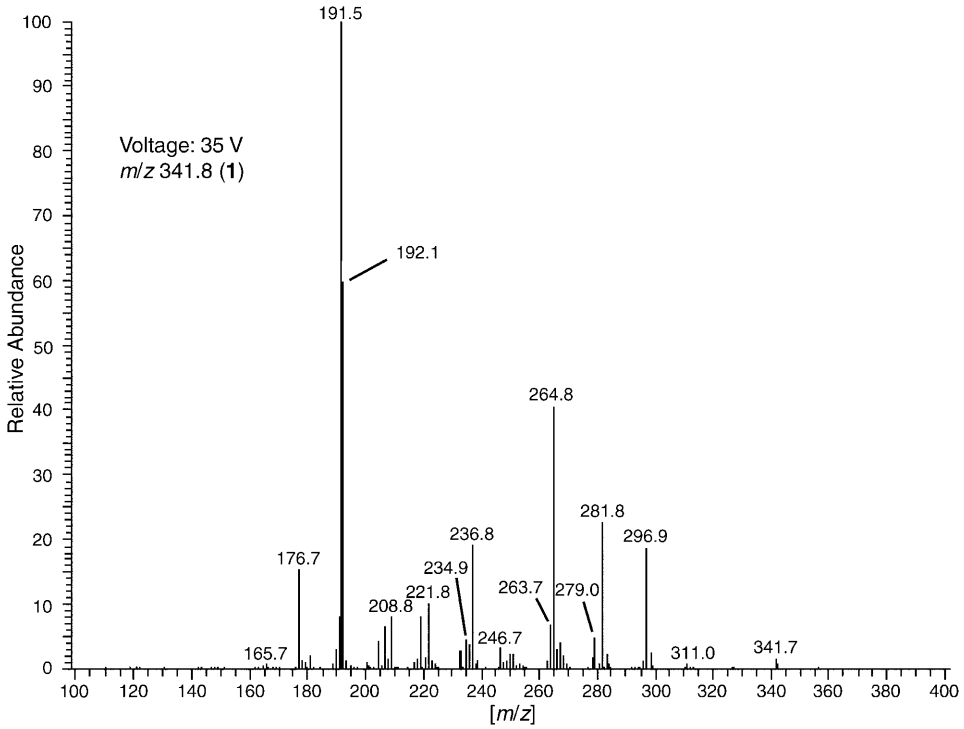
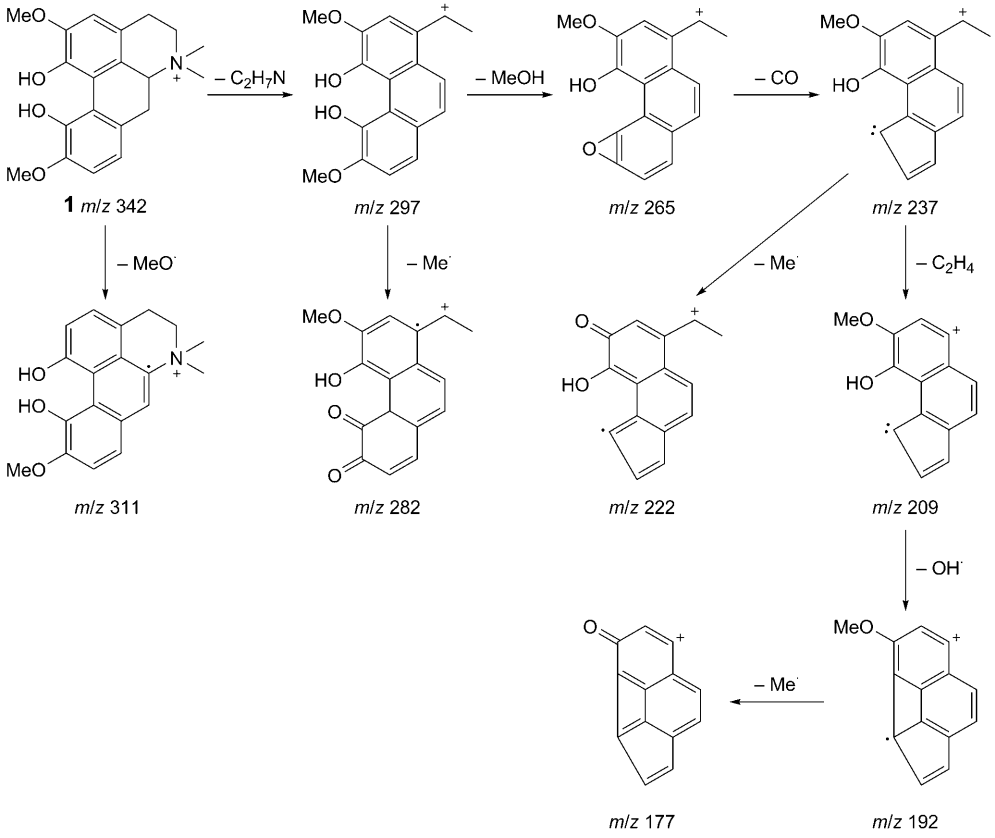


Fig. 3. *CID Spectrum of the ion at* m/z *341.8 of* **1**

spectrum (positive ion mode) of **2**. The molecular weight of **2** was 176 u higher than that of the ion at *m*/*z* 324.1. Additionally, when the cone voltage was set at 45 V, six product ions at *m*/*z* 324.1 ($[M-C_6H_8O_6]^+$), 309.0 ($[M-C_6H_8O_6-Me]^+$), 307.9 ($[M-C_6H_8O_6-Me-H]^+$), 293.9 ($[M-C_6H_8O_6-2\,Me]^+$), 279.9 ($M-C_6H_8O_6-Me-H-CO]^+$), and 265.9 ($[M-C_6H_8O_6-2\,Me-CO]^+$) were detected in the CID spectrum of the ion at *m*/*z* 499.8 (*Fig. 4*), suggesting a demethyleneberberine moiety which is one of the metabolites of berberine *in vivo* [13][15]. Moreover, berberine is an abundant alkaloid in Cortex *Phellodendri* [16]. Based on these data, compound **2** was identified as the glucuronide conjugate of demethyleneberberine ($C_{25}H_{26}NO_{10}$, molecular weight 500, phase II metabolite) which might have originated from the metabolite of berberine or related same skeleton structures *in vivo*. The mechanistic pathway for fragments formed may be explained according to *Scheme 2*.

Compound **3** (t_R 5.32 min) had UV absorption maxima at 223 and 274 nm (*Fig. 2*,*c*). A singly charged ion at *m*/*z* 355.8 (M^+) was observed in the ESI mass spectrum (positive ion mode) of **3**. Additionally, when the cone voltage was set at 45 V, five product ions at *m*/*z* 325.1 ($[M-MeO]^+$), 279.0 ($[M-C_2H_7N-MeOH]^+$), 250.9 ($[M-C_2H_7N-MeOH-CO]^+$), 191.8 ($[M-C_2H_7N-MeOH-CO-MeO]^+$), and 176.8 ($[M-C_2H_7N-MeOH-CO-MeO-Me]^+$) were detected in the CID spectrum of the ion peak at *m*/*z* 355.8 (*Fig. 5*). These results are consistent with those of menisperine ($C_{21}H_{26}NO_4$, molecular weight 356) [13] which is present in Cortex

Scheme 1. *Proposed Mechanistic Pathway for Fragments Formed in MS² of* **1**



Phellodendri [17]. A proposed mechanistic pathway for fragments formed in MS^2 is shown in *Scheme 3*.

Compound **4** (t_R 5.56 min) had UV absorption maxima at 260 and 344 nm (*Fig. 2,d*). A singly charged ion at *m/z* 513.9 (M^+) was observed in the ESI mass spectrum (positive ion mode) of **4**. When the cone voltage was set at 40 V, four product ions at *m/z* 337.8 ($[M-C_6H_8O_6]^+$), 322.8 ($[M-C_6H_8O_6-Me]^+$), 307.9 ($[M-C_6H_8O_6-2\,Me]^+$), and 293.6 ($[M-C_6H_8O_6-Me-CO-H]^+$) were detected in the CID spectrum of the ion peak at *m/z* 513.9 (*Fig. 6*) and suggested a jatrorrhizine moiety [13]. Additionally, the 176 u higher molecular weight of **4** compared to that of jatrorrhizine, which was detected in Cortex *Phellodendri* before [18], suggested that compound **4** was jatrorrhizine 3-*O*-glucuronide ($C_{26}H_{28}NO_{10}$, molecular weight 514, phase II metabolite). This compound might have originated from the metabolite of jatrorrhizine or related same skeleton structures *in vivo*. The mechanistic pathway for fragments formed may be explained according to *Scheme 4*.

Compound **5** (t_R 5.90 min) had UV absorption maxima at 260 and 344 nm (*Fig. 2,e*). A singly charged ion at *m/z* 497.8 (M^+) was observed in the ESI mass spectrum

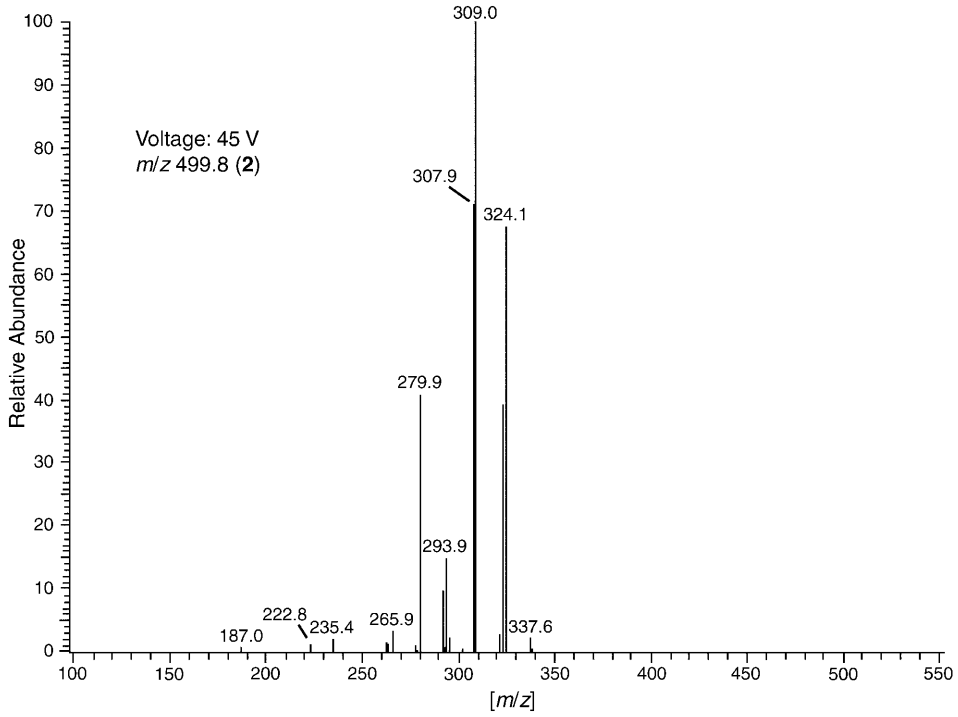
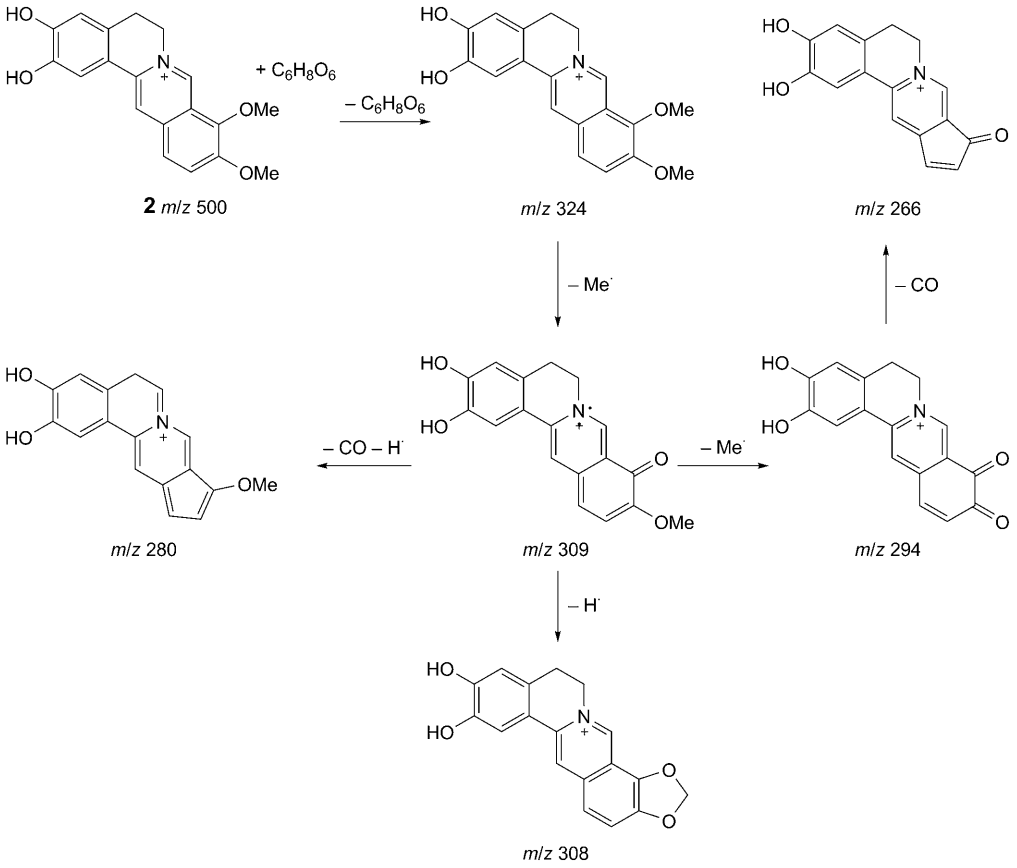


Fig. 4. *CID Spectrum of the ion at* m/z *499.8 of* **2**

(positive ion mode) of **5**. When the cone voltage was set at 40 V, three product ions at *m*/*z* 321.9 ($[M-C_6H_8O_6]^+$), 306.7 ($[M-C_6H_8O_6-Me]^+$), and 278.6 ($[M-C_6H_8O_6-Me-CO]^+$) were detected in the CID spectrum of the ion at *m*/*z* 497.8 (*Fig. 7*). These results are in agreement with the formula and structure of a berberubine moiety in the Cortex *Phellodendri* extract [19]. Additionally, the 176 u higher molecular weight of **5** compared to that of berberubine, which was detected in Cortex *Phellodendri* before [20], suggested that compound **5** was berberubine 9-*O*-glucuronide ($C_{25}H_{24}NO_{10}$, molecular weight 498, phase II metabolite). This compound might have originated from the metabolite of berberubine or related same skeleton structures *in vivo*. The mechanistic pathway for the observed fragments formed from berberubine may be explained according to *Scheme 5*.

Compound **6** (t_R 7.05 min) had UV absorption maxima at 260 and 344 nm (*Fig. 2*,*f*). A singly charged ion at *m*/*z* 337.7 (M^+) was observed in the ESI mass spectrum (positive ion mode) of **6**. When the cone voltage was set at 35 V, three product ions at *m*/*z* 321.8 ($[M-Me-H]^+$), 306.9 ($[M-2\,Me-H]^+$), and 278.8 ($[M-2\,Me-H-CO]^+$) were detected in CID spectrum of the ion at *m*/*z* 337.7 (*Fig. 8*). These results are consistent with those of jatrorrhizine ($C_{20}H_{20}NO_4$, molecular weight 338) [13] which is present in Cortex *Phellodendri* [18]. The mechanistic pathway for the fragments formed may be explained according to *Scheme 6*.

Scheme 2. *Proposed Mechanistic Pathway for Fragments Formed in MS² of* **2**



Compound **7** (t_R 7.30 min). This compound had UV absorption maxima at 278 and 340 nm (*Fig. 2,h*). In the ESI mass spectrum (positive ion mode) of **7**, a singly charged ion at *m/z* 353.8 (M^+) was observed. The 30 u higher molecular weight compared to that of berberubine suggested that this compound might be a further metabolite of berberubine. When the cone voltage was set at 45 V, five product ions at *m/z* 337.5 ($[M-Me-H]^+$), 335.8 ($[M-Me-3\,H]^+$), 322.0 ($[M-Me-OH]^+$), 309.5 ($[M-Me-H-CO]^+$), and 293.9 ($[M-Me-OH-CO]^+$) were detected in the CID spectrum of the ion at *m/z* 353.8 (*Fig. 9*), which suggested that **7** was the monomethyl and monohydroxy product of berberubine ($C_{20}H_{18}NO_5$, molecular weight 354, phase II metabolite). The mechanistic pathway for the fragments formed may be explained according to *Scheme 7*.

Compound **8** (t_R 8.63 min) had UV absorption maxima at 230, 265, and 348 nm (*Fig. 2,h*). A singly charged ion at *m/z* 351.8 (M^+) was observed in the ESI mass spectrum (positive ion mode) of **8**. When the cone voltage was set at 45 V, five product ions at *m/z* 337.1 ($[M-Me]^+$), 335.8 ($[M-Me-H]^+$), 321.8 ($[M-2\,Me]^+$), 307.9

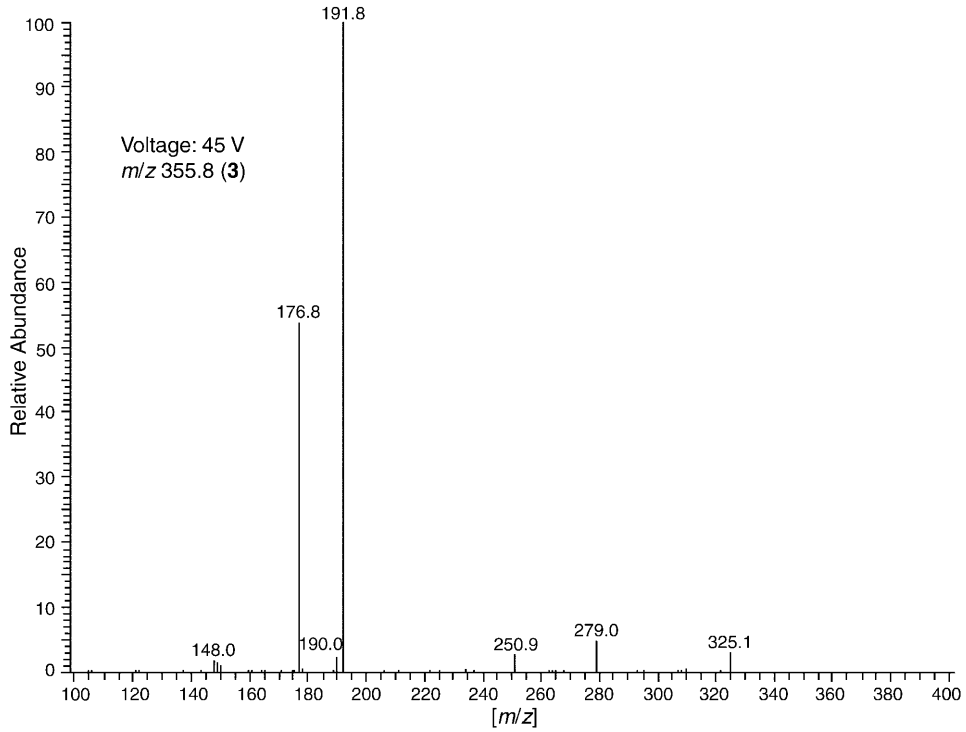
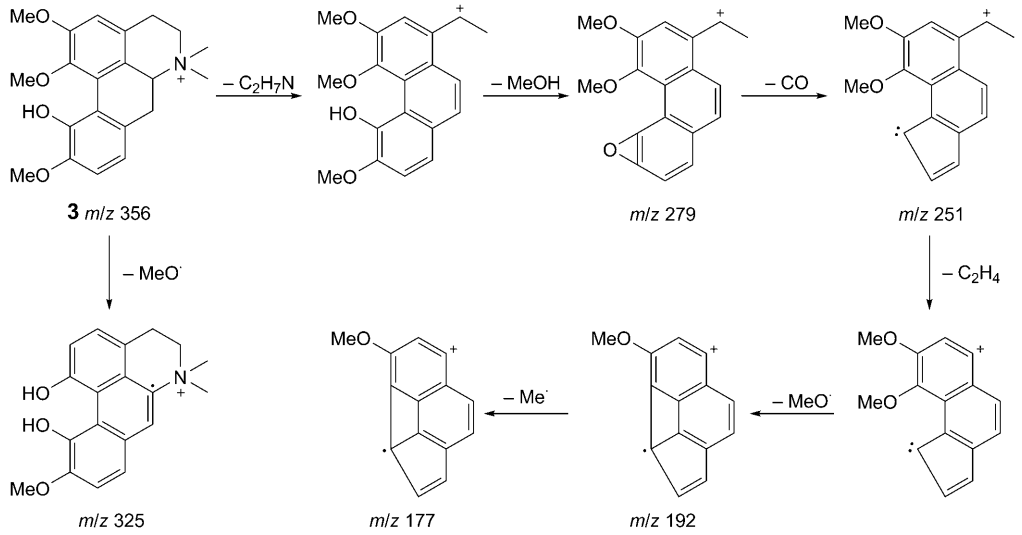


Fig. 5. *CID Spectrum of the ion at* m/z *355.8 of* **3**

Scheme 3. *Proposed Mechanistic Pathway for Fragments Formed in* MS^2 *of* **3**



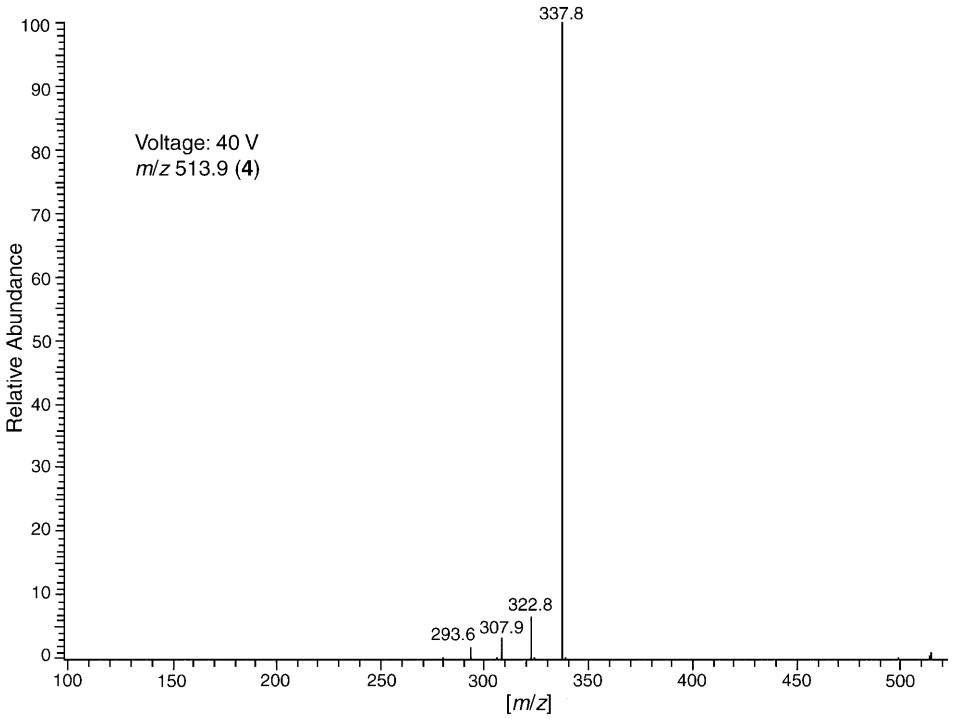


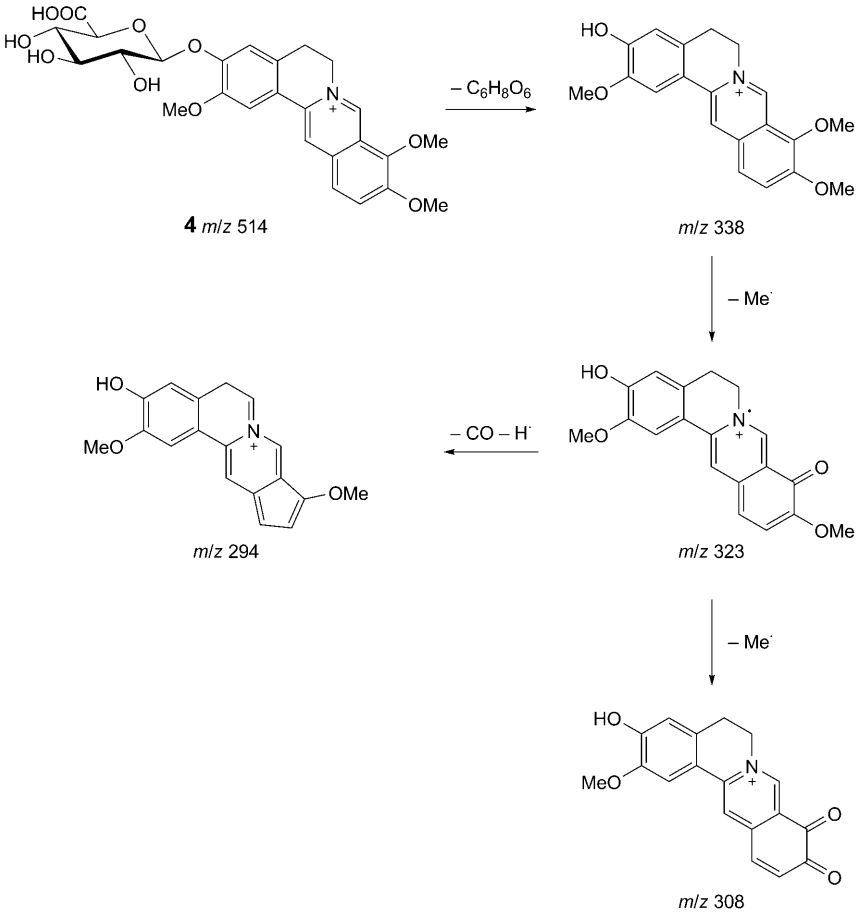
Fig. 6. *CID Spectrum of the ion at* m/z *513.9 of* **4**

($[M-\mathrm{Me}-\mathrm{H}-\mathrm{CO}]^+$), and 293.9 ($[M-2\,\mathrm{Me}-\mathrm{CO}]^+$) were detected in the CID spectrum of the ion at *m*/*z* 351.8 (*Fig. 10*). These results are consistent with those of palmatine ($C_{21}H_{22}NO_4$, molecular weight 352) [13][21] and indicated its presence in Cortex *Phellodendri* [16]. The mechanistic pathway for the fragments formed may be explained according to *Scheme 8*.

Compound **9** (t_R 8.88 min) had UV absorption maxima at 232, 275, and 340 nm (*Fig. 2*,*i*). A singly charged ion at *m*/*z* 335.7 (M^+) was observed in the ESI mass spectrum (positive ion mode) of **9**. When the cone voltage was set at 30 V, five product ions at *m*/*z* 321.1 ($[M-\mathrm{Me}]^+$), 319.9 ($[M-\mathrm{Me}-\mathrm{H}]^+$), 306.0 ($[M-2\,\mathrm{Me}]^+$), 292.1 ($[M-\mathrm{Me}-\mathrm{H}-\mathrm{CO}]^+$), and 277.9 ($[M-2\,\mathrm{Me}-\mathrm{CO}]^+$) were detected in the CID spectrum of the ion at *m*/*z* 335.7 (*Fig. 11*). These results are consistent with those of berberine ($C_{20}H_{18}NO_4$, molecular weight 336) [22][23] and indicated its presence in Cortex *Phellodendri* [16]. The mechanistic pathway for the fragments formed may be explained according to *Scheme 9*.

Conclusions. – By combined HPLC/APCI-MS/MS techniques, a total of 13 characteristic HPLC peaks were detected from rats' urine after oral administration of a Cortex *Phellodendri* decoction. Nine compounds including five alkaloids (**1**, **3**, **6**, **8**, and **9**) and four of their metabolites (**2**, **4**, **5**, and **7**) were identified, which indicated that the alkaloids in Cortex *Phellodendri* were easily assimilated *in vivo*. Among them, **2**, **4**, **5**,

Scheme 4. *Proposed Mechanistic Pathway for Fragments Formed in MS²* of **4**



and **7** might have originated from the phase II metabolites of berberine, jatrorrhizine, and berberubine or other similar compounds. This is also the first reported detection of these compounds in body fluids. Actually, in order to assign the substitution positions of MeO, OH, glucuronide, and other groups in these compounds correctly, additional information (for example, nuclear magnetic resonance spectra) will be required. Therefore, the chemical structures of **1**–**9** proposed in this paper are only tentative.

Modern pharmacological studies showed that these natural isoquinoline alkaloids in Cortex *Phellodendri* possess a wide range of biochemical and pharmacological activities. Magnoflorine (**1**) and jatrorrhizine (**6**) can act as potent scavengers of peroxy radicals [24]. Furthermore, magnoflorine (**1**) is the antifeedant against *Spodoptera frugiperda* [25]. Palmatine (**8**) possesses antibiotic activity against bacteria, fungi, and viruses [26][27], and exerts vasodilatory [28], sedative [29], and hepatoprotective effects [30]. Berberine (**9**) shows antidiarrheal, anti-arrhythmic, antitumor [31–33],

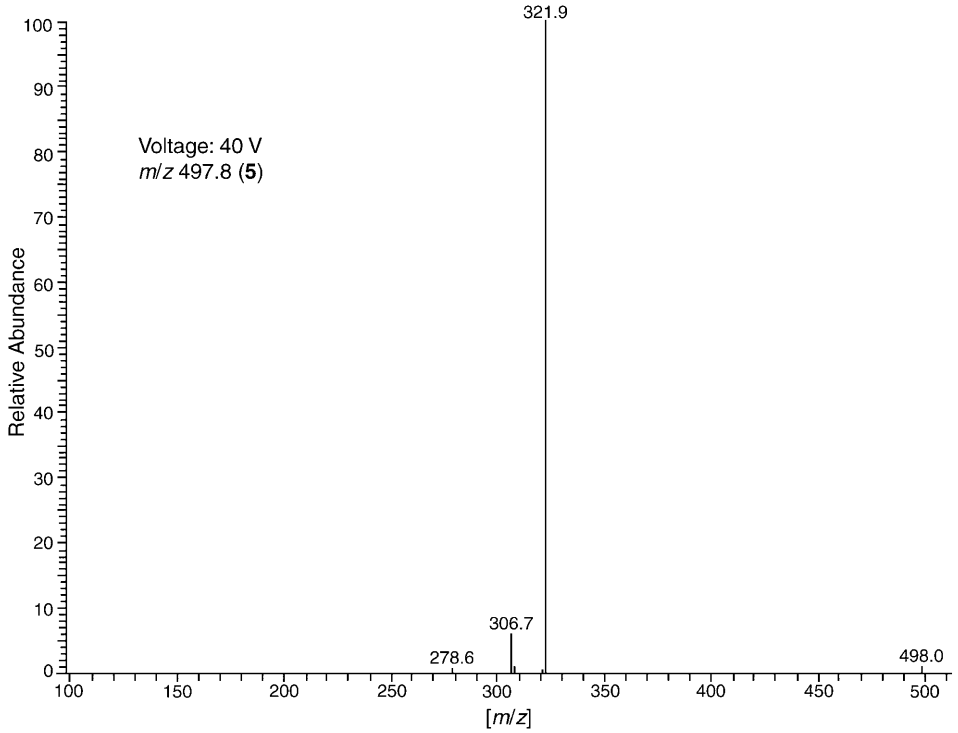
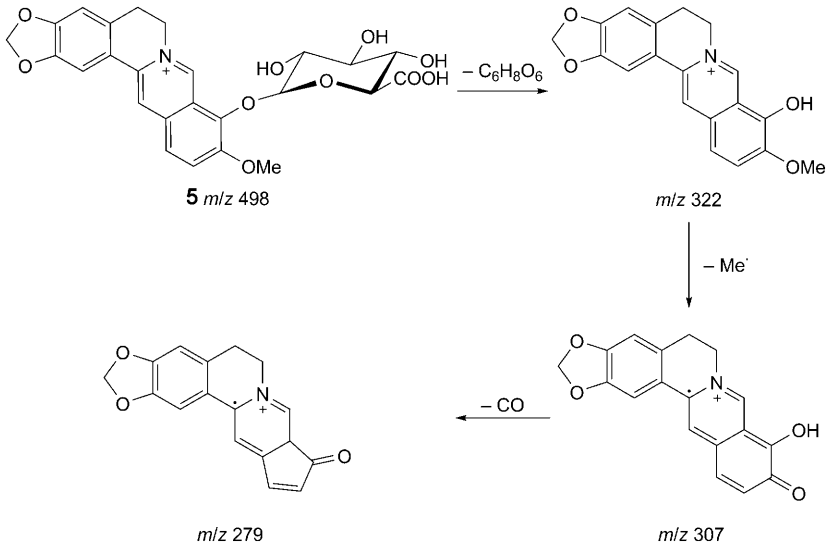


Fig. 7. *CID Spectrum of the ion at* m/z *497.8 of* **5**

Scheme 5. *Proposed Mechanistic Pathway for Fragments Formed in MS² of* **5**



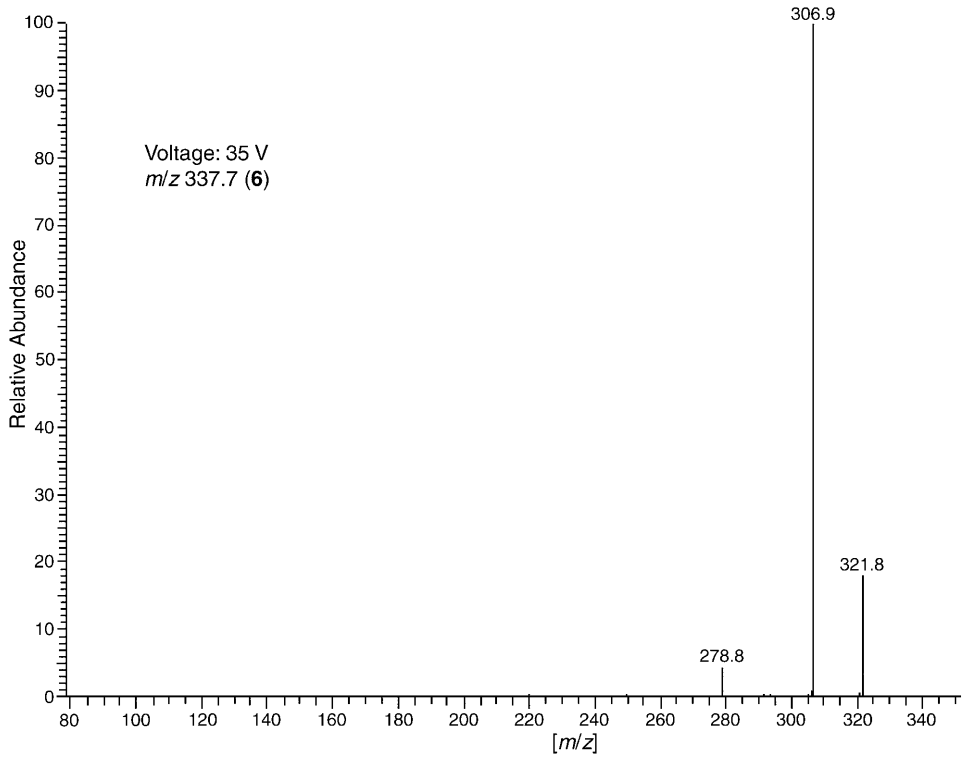
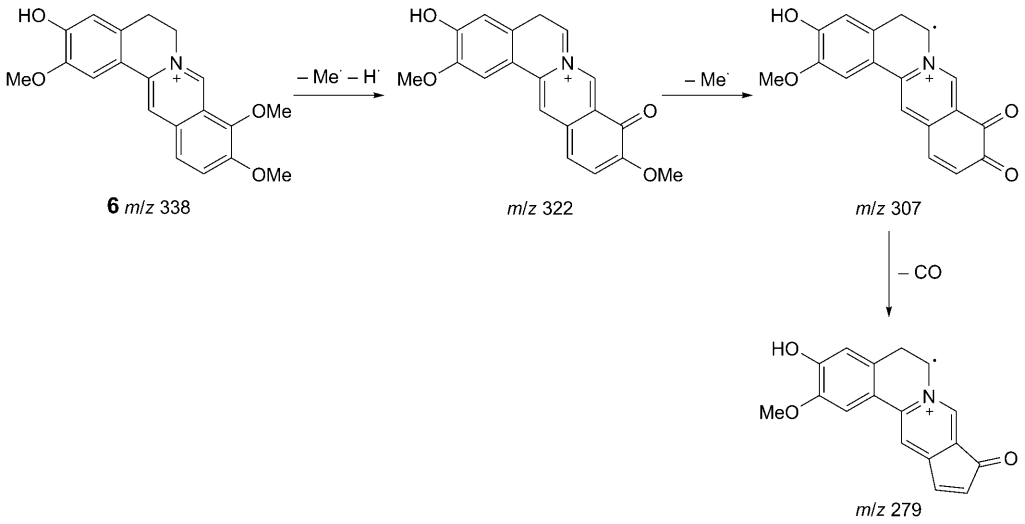


Fig. 8. *CID Spectrum of the ion at* m/z *337.7 of* **6**

Scheme 6. *Proposed Mechanistic Pathway for Fragments Formed in* MS^2 *of* **6**



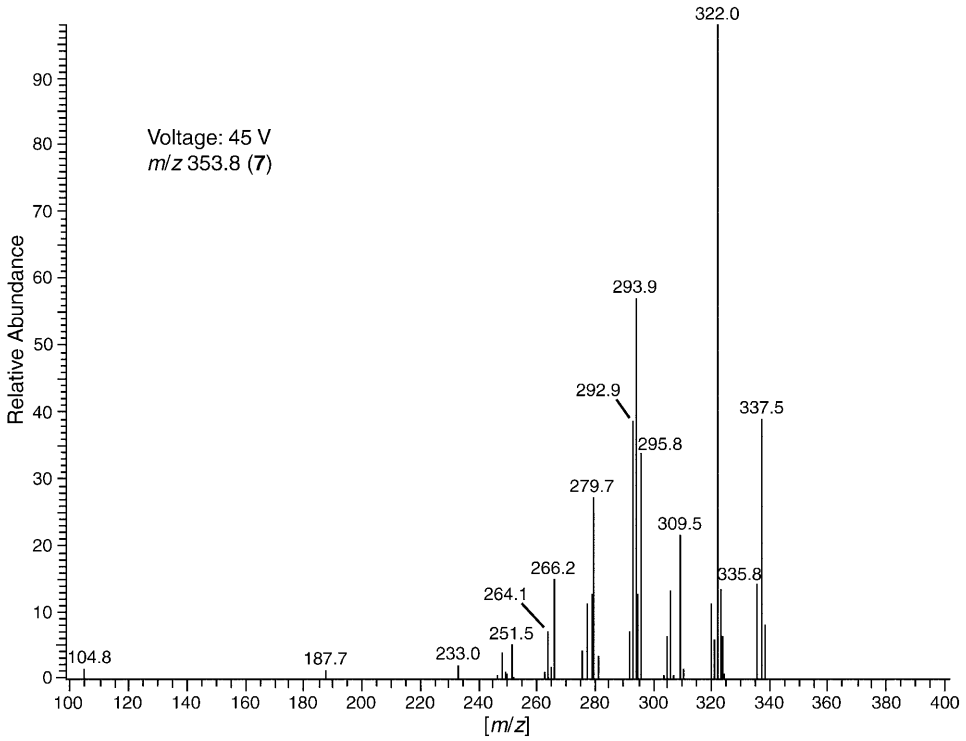


Fig. 9. *CID Spectrum of the ion at* m/z *353.8 of* **7**

antidepressant-like [34], antiviral [35], and hypoglycemic effects [36], and it also can inhibit the cell growth and induct the apoptosis in several human cancer cell lines [37–39].

In fact, oral administration is the most popular drug-delivering method for TCMs when used in a clinic setup. Only the constituents successfully absorbed into blood and kept in a considerable concentration level in target organs might possess the possibility to cure diseases. The results of the present research significantly narrowed the range of effective constituents to be found and would be very helpful for the follow-up action mechanism research on Cortex *Phellodendri* decoction in treating various diseases.

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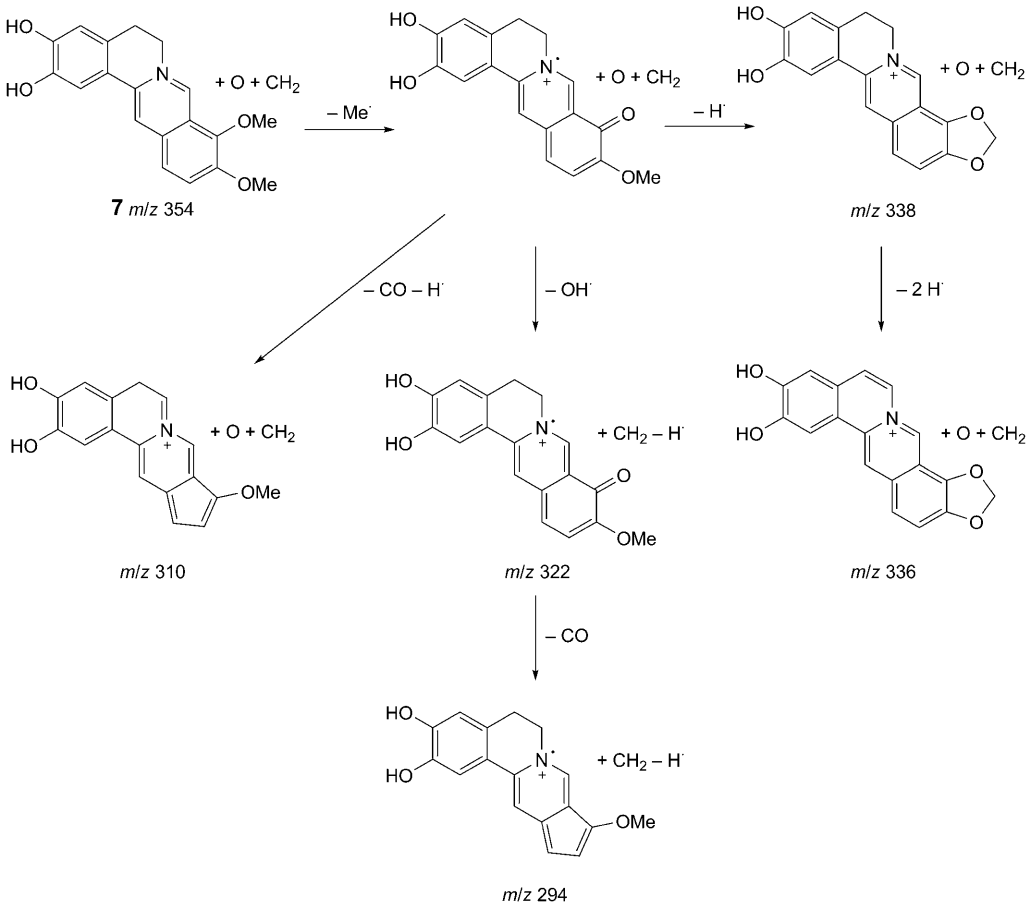
Experimental Part

Reagents and Materials. HPLC Grade MeCN (*Tedia*, Fairfield, OH) was used for HPLC analysis. H_2O for the HPLC mobile phase was purified with a *Milli-Q* system (*Millipore*, Bedford, MA). Analytical grade formic acid was obtained from *Sinopharm Chemical Reagent Co. Ltd.* 2-ml *SPE* Cartridges were obtained from *Alltech Co.*, USA. Cortex *Phellodendri* (No. ZM070605) was purchased

Scheme 7. *Proposed Mechanistic Pathway for Fragments Formed in MS² of* **7**



from *Shanghai Yanghetang Traditional Chinese Medicine Co., Ltd.*, Shanghai, P. R. China, in June 2007 and identified by *C.-G. H.* Voucher specimens were deposited with the Herbarium of Shanghai Institute of Materia Medica, CAS.

Preparation of Cortex Phellodendri *Decoction*. 200 g of Cortex *Phellodendri* was added to a bottle and extracted twice under reflux conditions with 2000 ml of H_2O (2 h per extraction). The H_2O phases were mixed and filtered. The filtrate was collected and concentrated to 200 ml by rotary vaporization at 50° under reduced pressure.

Instruments and Conditions. For HPLC separations, a *Waters 2690* system (*Waters*, Milford, MA) equipped with an automatic sample injector was used. For all separations, a *Diamonsil* C_{18} (5 μm, 4.6 × 200 mm, *Dikma Technologies*) analytical column equipped with a guard column containing the same stationary phase was used. HPLC Separation was performed using a linear gradient at r.t. (20°) and a flow rate of 1.0 ml/min. The mobile phase consisted of MeCN (*A*) and H_2O containing 0.1% HCOOH (*B*) using the elution of 15 – 27% *A* at 0 – 10 min and 27 – 70% *A* at 10 – 14 min. UV Spectra from 200 – 400 nm were recorded on a *Waters 2690 PDA* detector for peak identification. The decoction of Cortex *Phellodendri* was filtered through a 0.45 μm syringe filter and injected (10 μl) onto the column. MS: triple-quadrupole mass spectrometer (*TSQ*, *Finnigan MAT*, San Jose, CA) equipped with an ESI

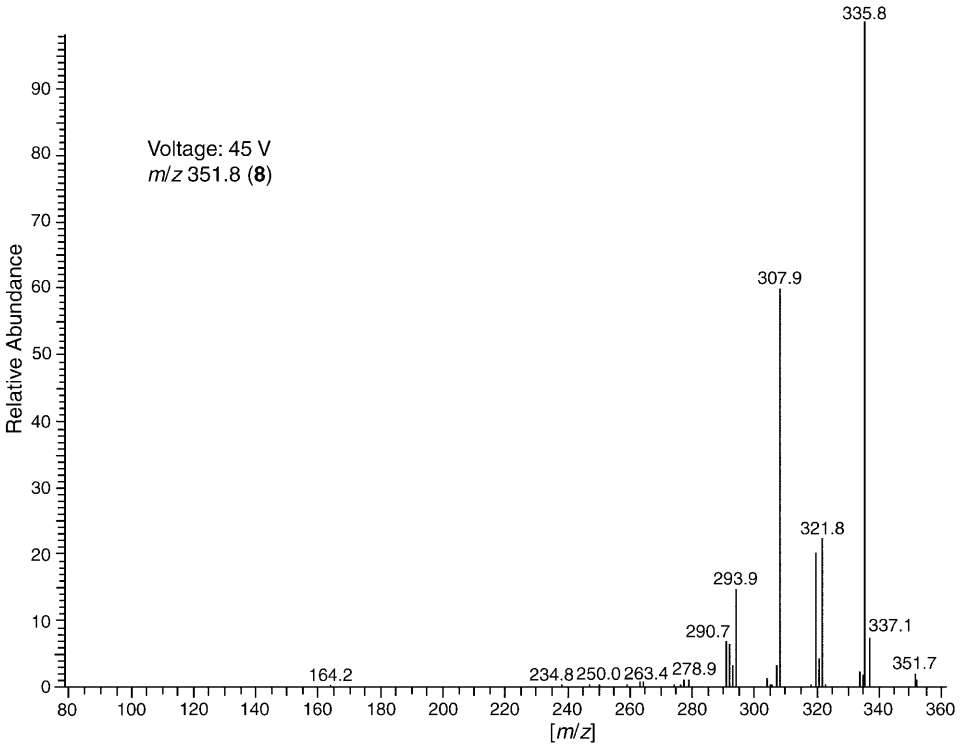
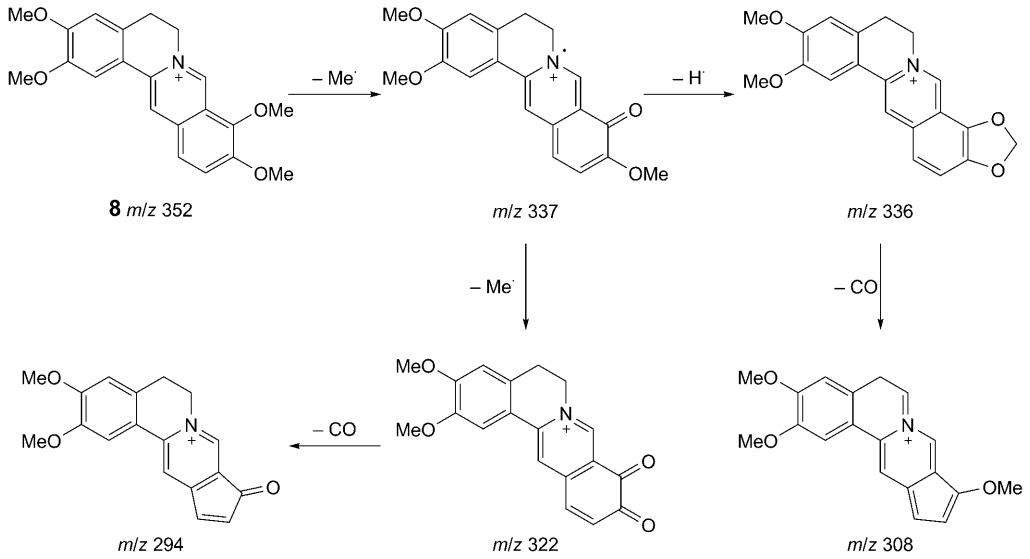


Fig. 10. *CID Spectrum of the ion at* m/z *351.8 of* **8**

Scheme 8. *Proposed Mechanistic Pathway for Fragments Formed in MS² of* **8**



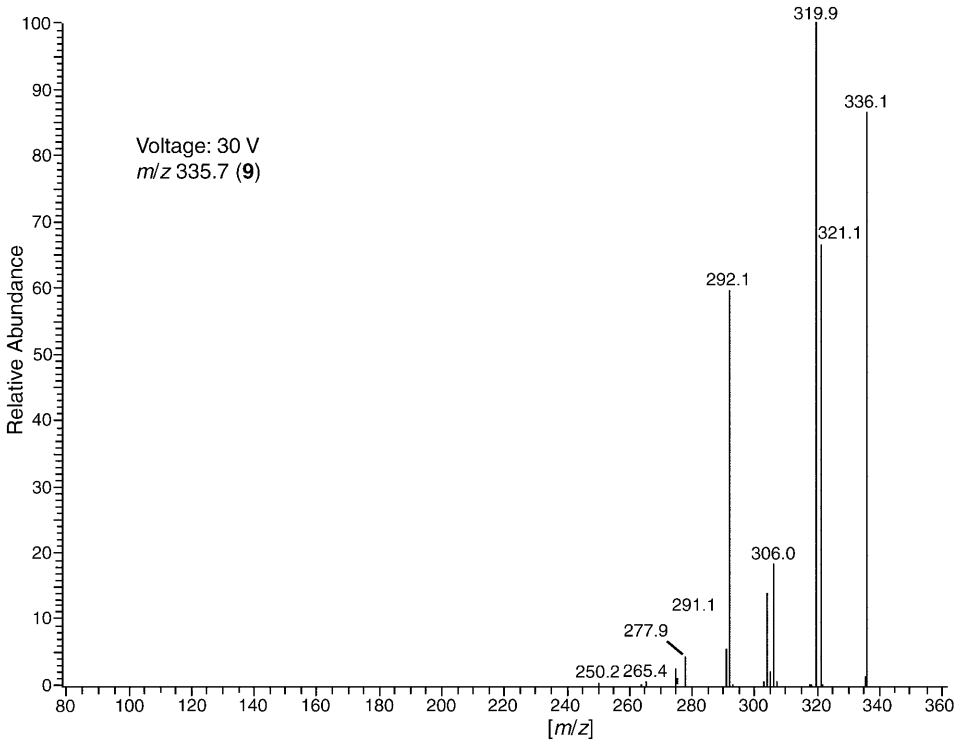
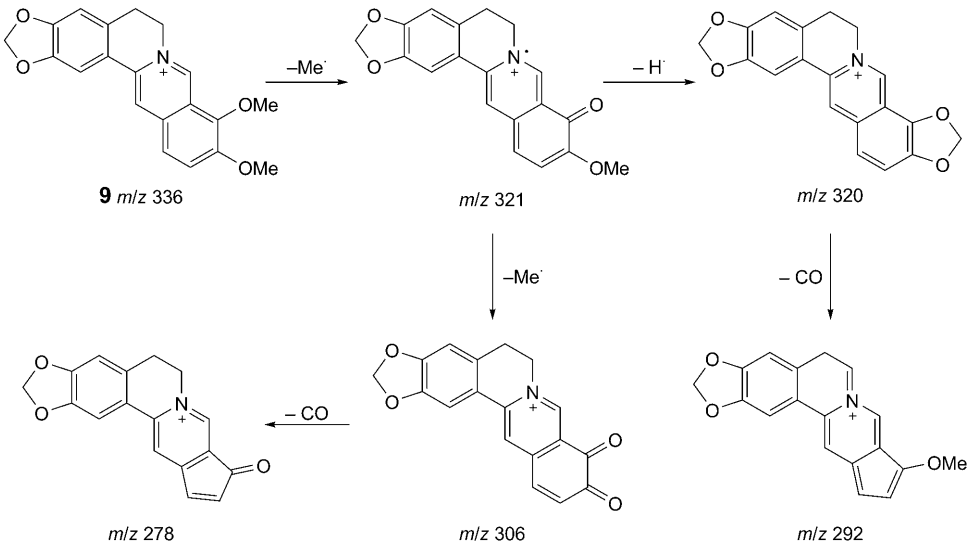


Fig. 11. *CID Spectrum of the ion at* m/z *335.7 of* **9**

Scheme 9. *Proposed Mechanistic Pathway for Fragments Formed in* MS^2 *of* **9**



interface. The ESI-MS^n spectra were acquired in both positive and negative ion modes. The mass spectrometry detector (MSD) parameters were as follows: nebulizer sheath gas, N_2 (20 uit); nebulizer auxiliary gas, N_2 (0 uit); collision gas, He; capillary temperature, 250°; spray voltage, 5000 V; capillary voltage, 24 V in (+) ESI; lens voltage, −16 V in (−) ESI; initially, the mass spectrometer was programmed to perform full scans between 100 and 1400 *m/z* in order to observe molecular ion signals as well as fragments or adducts in positive ion mode. Secondly, in MS/MS mode, CID of the protonated molecular species recorded in MS was carried out to identify the structure of constituents.

In vivo *Study.* Male *Sprague–Dawley* rats (200–220 g) were obtained from *Shanghai SLAC Laboratory Animal Co., Ltd.* (Shanghai, China). They were kept in metabolism cages and fasted for 15 h but with access to H_2O, and then administered 10 ml/kg doses of Cortex *Phellodendri* decoction. Urine samples were collected at different times up to 24 h and centrifuged at 3000 rpm for 10 min. The supernatant was stored at −20° until analyses.

Urine Extraction. An aliquot (1 ml) of mixed 0–24-h urine samples was loaded onto a *C18* solid-phase extraction cartridge, which was preconditioned with 2 ml of MeOH followed by 1 ml of *Milli-Q* water (18 M). The loaded *SPE* cartridge was washed with 2 ml of *Milli-Q* water (18 M), and the analytes were eluted with 1 ml of MeOH. The eluted soln. was filtered through a 0.45 μm filter and an aliquot (10 μl) was used for LC/MS^2 analyses.

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