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Identification of Phenolics and Nucleoside Derivatives in *Gastrodia elata* by HPLC-UV-MS

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An HPLC-UV-MS method for simultaneous identification of predominant phenolics and minor nucleoside derivatives in *Gastrodia elata* was developed, which was based on their UV and MS characteristics summarized thorough a series of home-made reference standard experiments. Phenolics showed characteristic UV λ_{max} at 267nm, $[M+NH_4]^+$ base peak in positive mode and $[M-H]^-$ base peak in negative mode while nucleosides exhibited UV λ_{max} at 255nm, $[M+H]^+$, $[M-H+2H_2O]^-$ or $[M-H+CH_3COOH]^-$. Phenolics conjugates mainly underwent the consecutive loss of gastrodin residue (-268u) and the combined loss of H_2O and CO_2 from the citric acid unit under negative MS/MS conditions whereas nucleosides simply lost the ribose (-132u) under positive MS/MS conditions. According to these characteristics, special pattern under MS/MS and reported compound data for *Gastrodia elata* in the literatures, not only 15 phenolics were identified but also 6 nucleoside derivatives were identified. Among these compounds, seven phenolics and three nucleoside derivatives have not been reported yet from *Gastrodia elata*.

Keywords: *Gastrodia elata* / LC-MS / Nucleoside / Phenolic

1 Introduction

Gastrodia elata Blume (Tianma in China), a popular Chinese herb medicine, has been used for many years as an anticonvulsant, an analgesic and a sedative against vertigo, general paralysis, epilepsy and tetanus [1-2]. Phytochemical researches on this herb have revealed the existence of abundant phenolics [3-10] and fewer nitrogenous compounds such as nucleoside [11] and amino

acid [12-14]. In addition, some organic acids including citric acid, succinic acid and palmitic acid, as well as sterols and sugars, were also isolated from this herb [3-4].

Phenolics in *Gastradia elata* consist of monomer phenolics such as p-hydroxybenzyl alcohol, p-hydroxybenzaldehyde and gastrodin (4-(β-D-glucopyranosyloxy)-benzyl alcohol) and gastrodin conjugates with several citric acids like parishin (tris[4-(β-D-glucopyranosyloxy -D-glucopyranosyloxy) benzyl] citrate), parishin B (1,2-bis[4-(β-D-glucopyranosyloxy) benzyl] citrate) and parishin C (1,3-bis[4-(β-D-glucopyranosyloxy) benzyl] citrate). Monomer phenolics are normally regarded as active components of *Gastrodia elata* [15-17] and selected as marker compounds for quality control during HPLC [18-19] and CE analysis [20]. The determination of monomer phenolics together with sugars was also reported. Cao et al. reported a CE method for determination of five phenolics and three sugars in *Gastrodia elata* [21] and Li et al. reported a GC-MS method for determination of three phenolics and three sugars after derivatization [22]. Only a few publication on the determination of gastrodin conjugates such as parishin, parishin B and parishin C due to lack of commercial standards. Ku et al. developed a CE method for determination of three parishins by homemade reference compounds [23]. Recently, Shi et al. developed a HPLC fingerprint chromatogram consisted of 16 common peaks for better controlling of the quality of *Gastrodia elata* [24], which is the most comprehensive analysis of *Gastrodia elata* components but limited information is available about the peak assignments except for two major peak gastrodin and p-hydroxybenzyl alcohol.

Nucleoside, a group of highly active compounds with widespread effects [25-26], has attracted much attention on quality control of *Cordyceps sinensis.* But the determination of nucleosides in *Gastrodia elata* has not been reported.

In the present paper, we attempt to develop an HPLC-UV-MS method for simultaneous identification of predominant phenolics and attractive minor nucleoside derivatives because adenosine and uridine have been isolated from *Gastrodia elata* in our previous works (not published yet), which indicated more nucleoside derivatives might be existed in lower content.

2 Experimental

2.1 Materials and chemicals

The rhizoma of *Gastrodia elata* was collected from Sichuan and identified as *Gastrodia elata*

Blume by Professor Suiqing Chen (Henan College of Traditional Chinese Medicine). The voucher specimen (no. 050528) was stored in our laboratory.

Reference compounds gastrodin, p-hydroxybenzyl alcohol, p-hydroxybenzaldehyde, 4,4' -dihydroxydibenzyl ether, parishin, parishin B, parishin C, uridine, adenosine were isolated from *Gastrodia elata* Bl. in our laboratory and unequivocally elucidated by NMR and MS data. Analytical grade citric acid was obtained from Shenyang (Shenyang Reagent Corporation, Liaoning, China). HPLC grade methanol and acetate acid were purchased from Yuwang (Yuwang Co. Ltd, Shandong, China) and Tedia (Fairfield, OH, USA), respectively. Water was prepared with a Milli-Q water purification system (Millipore, Bedford, USA).

2.2 Sample preparation

Eight gram of the rhizoma of *Gastrodia elata* were dried, grounded and extracted ultrasonically with 80ml methanol in a 150ml triangle bottle for 30min. The ultrasonic procedure was repeated after the extract was kept overnight at room temperature. The supernatant of the extract was concentrated to dryness under reduced pressure and the residue was dissolved in 10ml water and filtered through 0.45 μm membrane prior to HPLC-UV-MS analysis.

2.3 HPLC-ESI-MS/MS analyses

HPLC experiment was carried out on a HPLC system consisted of a Waters 2690 separation model and a 996 diode-array detector (DAD) (Waters, Milford, MA). The column used was an Hypersil C_{18} column (4.6×250mm, 5 μm, Dalian Elite Co, Dalian, China). The mobile phases consist of methanol (A) and water (B) each containing a volume fraction of 0.5% acetic acid. A gradient program was adopted as follows: started with 2% A (0-10 min), linearly changed to 40%A (10-60 min), held at 40%A for 15 min. The temperature of the column oven was set to 35℃. The flow rate was set to 0.8ml/min but only 163 μl/min eluent was transferred to mass spectrometer by a split T piece at a ratio of 1:3.9. The UV spectrum was scanned from 200nm to 400nm at 1Hz frequency and the HPLC chromatogram was extracted at 270nm.

The mass spectrometer used was a TSQ triple quadrupole (Thermo Finnigan, San Jose. CA, USA) equipped with an ESI interface. The ionization parameter were as follows: spray voltage 4.0kv; heated capillary temperature 325℃; Sheath gas of nitrogen at 20 arbitrary units and auxiliary gas at 40 p.s.i.. For MS/MS, argon at a pressure of 3mTorr was used as the collision gas and the collision

energy was set at 8-40V. Initially, the mass spectrometer was programmed to perform full scans ranging from m/z 100 to 1100 for observing molecular ion signals as well as adducts in positive and negative ionization modes. Secondly, the collision-induced dissociation (CID) spectra of $[M+H]^+$ or $[M-H]^-$ at compound dependent scan range were recorded to elucidate the structures. For phenolics, negative ion mode was used, while positive ion mode was used for nucleosides.

3 Results and discussion

3.1 UV and ionization characteristics of reference phenolics and nucleosides

The UV spectra of reference phenolics and nucleosides, which were obtained under HPLC-DAD condition, showed distinctive characteristics for phenolics and nucleosides, i.e., UV λ_{max} were around at 267nm and 255nm, respectively (Table 1). Thus, other peaks with similar UV feature in the *Gastrodia elata* could be preliminarily classified.

In present HPLC and MS conditions, characteristic MS adducts ions were also found for these two structural types (Table 1). In positive ionization mode, nucleosides mainly showed the protonated molecular ions $[M+H]^+$, whereas phenolics mainly showed ammonia adduct ions $[M+NH_4]^+$ though ammonia is not existent at all in our mobile phase. In negative ionization mode, adduct ions $[M-H]^-$, $[M-H+2H_2O]^-$ and $[M-H+CH_3COOH]^-$ were observed for both phenolics and nucleosides, but phenolics mainly showed the abundant $[M-H]^-$ ions while nucleosides mainly showed the abundant $[M-H+2H_2O]^-$ or $[M-H+CH_3COOH]^-$ ions. These series characteristic ions would be beneficial to determine the molecular masses in *Gastrodia elata* extract.

3.2 Fragmentation characteristics of reference phenolics and nucleosides

The negative CID spectra of monomer phenolics mainly showed the characteristic ions originated from functional group such as hydroxyl and carboxyl group (Table 2). For example, the negative CID spectrum of p-hydroxybenzyl alcohol showed the product ions at m/z 105 $[M-H-H_2O]^-$, m/z 95 $[M-H-CO]^-$ and m/z 77 $[M-H-H_2O-CO]^-$, corresponding to alcoholic hydroxyl and phenolic hydroxyl; the negative prominent product ions of citric acid were at m/z 173 $[M-H-H_2O]^-$, m/z 155 $[M-H-2H_2O]^-$, m/z 129 $[M-H-H_2O-CO_2]^-$ and m/z 111 $[M-H-2H_2O-CO_2]^-$ originated from carboxyl and hydroxyl group (data not shown); gastrodin, the glucoside of p-hydroxybenzyl alcohol, in addition to characteristic ions of p-hydroxybenzyl alcohol, its negative CID spectrum mainly

showed the abundant product ions at m/z 123 and m/z 161 corresponding to the elimination a molecule of glucose and a molecule of p-hydroxybenzyl alcohol, respectively.

The negative CID spectra of gastrodin with citric acid conjugates are more complicated, which exhibit not only the fragment of conjugate bond but also the characteristic ion originated from citric acid and gastrodin. For example, parishin, the conjugate of one citric acid and three gastrodins, mainly showed the fragmentation procedure of ester glucoside bond and exhibited consecutive loss of gastrodin residue (-268u) corresponding to ions at m/z 727 and 459 (see CID spectrum in Fig. 2a). Ions at m/z 441, 423, 397, 379 and 369 were also observed, which were related to elimination of H_2O and CO_2 from the tertiary alcoholic hydroxyl group and the new freely carboxylic group produced by the broken of ester glucoside bond. In addition, ions at m/z 263 and 161 owning to gastrodin concerned ions, as well as lower abundance of citric acid ions at m/z 173, 129 and 111 were also observed (detailed interpretation was shown in Fig. 3).

Reference Nucleosides, uridine and adenosine, their positive CID spectra simply showed the loss of ribose moiety (-132u) yielding an $[BH]^+$ (protonated heterocyclic base units) ions at m/z 113 and m/z 136 only (respective spectrum for adenosine was seen in fig. 2b), which is identical to literature report [27].

3.3 Identification of phenolics and nucleoside derivatives in *Gastrodia elata*

For the peaks shown in Figure 4, the molecular masses of over thirty compounds were determined based on the series adduct ions such as $[M+H]^+$/$[M+NH_4]^+$ in positive MS spectra and $[M-H]^-$/$[M-H+2H_2O]^-$/$[M-H+CH_3COOH]^-$ in negative MS spectra. However, some peaks were too weak to get suitable MS/MS spectra for identification, and some were out of the structural type concerned in this paper. So only 21 peaks were assigned in this study. Their individual MS adduct ions, retention time and UV λ_{max} were shown in Table 1; their UV trace at 270nm together with MS total ions chromatograms were shown in figure 4; their product ions were shown in Table 2.

By comparison the retention times and MS spectra of 21 peaks with those of reference compounds, peak **1**, **2**, **4**, **5**, **9**, **14**, **17**, **20** and **21**, were ascribed to uridine, gastrodin, p-hydroxybenzyl alcohol, adenosine, p-hydroxybenzaldehyde, parishin B, parishin C, parishin and 4,4'-dihydroxydibenzyl ether, respectively. Other peaks were identified by comparing their UV feature, MS/MS spectra with corresponding reference phenolic or nucleoside analogues.

Based on the fragmentation mechanism of parishin, the structures of other phenolics were

dertermined. For example, Peak **15** and **16**, both showed molecular mass of 890u, i.e., 162u larger than 728 of di-substituted parishin (parishin B and parishin C). Their main fragment ions included the characteristic parishin ions at m/z 423, 397, 379 and 369, disaccharide residue ions at m/z 323 as well as ions at m/z 621 (459+162), 603 (441+162), 585 (423+162, 559 (397+162), 541 (379+162) and 531(369+162) corresponding to parishin linking with an additional hexose (see fig. 5a for MS spectrum and see fig. 3 for comparison). Thus, their structures were assigned to di-substituted parishin glucoside and its positional isomer because glucose residue was the only hexose substituted in *Gastrodia elata.* Peak **8**, with the same molecular mass 286 as gastrodin, its MS/MS spectrum mainly showed the predominant ion at m/z 179 $[M-H-106]^-$ and 161$[M-H-124]^-$. The loss of 124u was ascribed to p-hydroxybenzyl alcohol just like ion in gastrodin, and the other loss of 106u was related to the neutral loss of a molecule of 4-methyleneclohexa-2,5-dienone that derived from p-hydroxybenzyl group, a common residue of phenolics in *Gastrodia elata*. Therefore, the likely structure of peak 8 is p-hydroxybenzyl alcohol glucoside. Similarly, Peak **10** and **11**, with identical molecular mass of 460, were assigned to mono-substituted parishin and its positional isomer; Peak **6**, **12** and **18** were assigned to mono-substituted parishin-H_2O, di-substitued parishin isomer and methyl di-substituted parishin, respectively (Table 2). Except for peak **8**, other seven compounds, peak **6**, **10**, **11**, **12**, **15**, **16** and **18**, have not been reported.

For peak **8**, **10** and **11**, these compounds have been isolated from *Gastrodia elata* and their structures were verified by NMR data. The NMR data indicated that peak **8**, **10** and **11** could be identified as p-hydroxybenzyl-β-D-glucoside, 2-mono-[4-(β-D-glucopyranosyloxy) benzyl] citrate and 1-mono-[4-(β-D-glucopyranosyloxy) benzyl] citrate unambiguously. To some extent, this case checked the validity of the method mentioned above.

In addition to reference nucleosides uridine and adenosine, four other nucleoside derivatives were found in *Gastrodia elata* extract. Peak **3** showed characteristic UV λ_{max} at 255nm, prominent $[M+1-132]^+$ ion at m/z 152 and short retention time in RP-HPLC. All these information indicated that this compound was guanosine. The MS/MS spectrum of peak **7** mainly showed the loss of 162u giving an ion at m/z 268 and the following loss of 132u giving an ion at 136 (Table 2). A weak ion at m/z 298 corresponding to loss of 132u from protonated ion indicates that the ribose is the terminal sugar residue. Thus this compound was assigned to adenosine glucoside. In MS/MS spectrum of peak **13**, besides characteristic guanosine ions (m/z 284 to 152) (Fig. 5b), another characteristic fragmentation is loss of 106u (m/z 390 to 284) corresponding to

4-methyleneclohexa-2,5-dienone. Therefore, this compound was assigned to p-hydroxybenzyl guanosine and the possible conjugated position is N^2- by referring to the analogue in literature (see fig. 1 for structure) [28]. Similarly, peak **19** was assigned to p-hydroxybenzyl adenosine and the likely structure was N^6-substituted (see fig. 1 for structure) [29]. These three nucleosides analogues (peaks **7**, **13** and **19**) have not been reported yet from *Gastradia elata*.

4 Concluding remarks

In the study, an HPLC-ESI/MS method was developed for simultaneous identification of predominat phenolics and minor nucleosides derivatives in *Gastrodia elata* extract. Based on the nine reference compounds isolated by our laboratory, we found that, phenolics presented a characteristic UV λ_{max} at around 267nm and an abundant $[M+NH_4]^+$ ion in positive mode, whereas nucleosides showed a characteristic UV λ_{max} at around 255nm and a prominent $[M+H]^+$ ion. Although negative ions $[M-H]^-$, $[M-H+2H_2O]^-$ and $[M-H+CH_3COOH]^-$ were observed for both phenolics and nucleosides, these series adduct ions together with characteristic positive ions allowed the correct determination of the molecular masses of unknowns. In addition to loss of H_2O and CO_2, the negative MS/MS spectra of gastrodin conjugates normally show the consecutive loss of gastrodin residue (-268u), while loss a ribose was usually observed for nucleosides. According to the characteristics and referring to the literature data, 15 phenolics and 6 nucleoside derivatives in *Gastrodia elata* were identified. The procedure mentioned above was verified by further NMR structure determination of peaks **7**, **13** and **19** after purification. Among these compounds, seven phenolics and three nucleoside derivatives have not been reported yet from *Gastrodia elata*. The suggested new compounds will be beneficial for further isolation.

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Figure Legends

Figure 1. Representative chemical structures studied in this paper and the dotted arrow showed the likely conjugated position.

Figure 2. ESI-MS/MS spectra of representative reference phenolics and nucleoside. (a) parishin in negative mode, collision energy 40V; (b) adenosine in positive mode, collision energy 15V.

Figure 3. The proposed fragment patterns of parishins and characteristic fragmentation ions, carboxyl and tertiary alcoholic hydroxyl concerned ions, gastrodin concerned ions and citric acid ions.

Figure 4. HPLC/UV Chromatogram and HPLC/ESI-MS total ion chromatogram (m/z 100-1100) of *Gastrodia elata*. (a) UV chromatogram recorded at 270nm; (b) positive ion chromatogram; (c) negative ion chromatogram. LC conditions: column 4.6×250mm, 5μm particle size; mobile phase: solvent A（0.5% acetic acid in methanol）, solvent B (0.5% acetic acid in water), gradient, 0-10min 2% A, 10-60min linearly changed to 40%A, 60-75min held at 40% A. Flow rate 0.8ml/ml.

Figure 5. ESI-MS/MS spectra of representative peaks in *Gastrodia elata*. (a) peak 16 in negative mode, collision energy 4 0V; (b) peak 13 in positive mode, collision energy 15V.

Table 1. The retention time (t_R), UV λ_{max} and characteristic MS adduct ions of twenty-one compounds in *Gastrodia elata* extract.

Peak No.	t_R (min)	UV λ_{max} (nm)	Positive ESI-MS		Negative ESI-MS			Molecular mass
			$[M+1]^+$	$[M+18]^+$	$[M-1]^-$	$[M-1+36]^-$	$[M-1+60]^-$	
1[a)]	6.64	260[b)]	245 (100)		243 (71)	279 (100)	303 (54)	244
2[a)]	12.23	267		304 (100)	285 (3)	321 (20)	345 (100)	286
3	13.33	255[b)]	284 (100)		282 (91)	318 (69)	342 (100)	283
4[a)]	16.92	272		142 (13)[c)]	123 (27)		183 (60)	124
5[a)]	19.95	255[b)]	268 (100)		266 (4)	302 (70)	326 (100)	267
6	21.89	267		460 (79)[c)]	441 (100)			442
7	25.20	-[b)]	430 (100)					429
8	25.40	-		304 (100)		321 (85)	345 (100)	286
9[a)]	31.27	284	123 (100)		121 (100)		181 (11)	122
10	31.55	-		478 (100)	459 (100)			460
11	32.31	267		478 (100)	459 (100)			460
12	41.00	269		746 (100)	727 (100)	763 (18)		728
13	41.55	-[b)]	390 (52)[c)]					389
14[a)]	45.28	267		746 (100)	727 (100)			728
15	45.69	-		908 (38)[c)]	889 (100)			890
16	46.66	-		908 (100)	889 (100)			890
17[a)]	47.14	267		746 (100)	727 (100)			728
18	48.11	267		760 (100)	741 (26)	777 (100)	801 (56)	742
19	48.93	-[b)]	374 (100)		372 (9)	408 (100)	432 (33)	373
20[a)]	53.28	267		1014 (100)	995 (100)	1031 (39)	1055 (28)	996
21[a)]	64.87	274		248 (100)	229 (13)	265 (45)	289 (100)	230

[a)] Reference compounds.
[b)] Nucleoside derivatives.
[c)] The base peak ion was the fragment ion or background ion that was hard to subtract due to weak target ion.

Table 2. Peak assignments and ESI-MS/MS product ions for 21 compounds in *Gastrodia elata* extract.

Peak No.	$[M+1]^+$ (m/z)	$[M-1]^-$ (m/z)	CID product ions (m/z)	Assignment
1[a)]	245		***113*** [b)]	Uridine
2[a)]		285	161, ***123***, 105	Gastrodin
3	284		***152***	Guanosine
4[a)]		123	105, 95, ***77***	P-hydroxybenzyl alcohol
5[a)]	268		***136***	Adenosine
6		441	235, 173, 155, ***111***	Mono-substituted parishin-H_2O
7	430		298, 268, ***136***	Adenosine glucoside
8[a)]		285	***179***, 161	P-hydroxybenzyl- β -D- glucoside
9[a)]		121	***93***	P-hydroxybenzaldehyde
10[a)]		459	***173***, 111	2- [4-(β -D-glucopyranosyloxy) benzyl] citrate
11[a)]		459	397, 173, 129, ***111***	1- [4-(β -D-glucopyranosyloxy) benzyl] citrate
12		727	459, ***441***, 423, 397, 235, 173, 111	Di-substituted parishin
13	390		284, 258, ***152***	P-hydroxybenzyl guanosine
14[a)]		727	459, 441, ***423***, 397, 369, 263, 161,129	Parishin B
15		889	621, 603, 585, 559, 541, 531, ***423***, 397, 323, 161	Di-substituted parishin glucoside
16		889	621, 603, 585, 559, 541, 531, ***423***, 397, 323, 161	Di-substituted parishin glucoside isomer
17[a)]		727	459, 441, ***423***, 397, 369, 263, 161, 129	Parishin C
18		741	***473***, 441	Methyl di-substituted parishin
19	374		***242***, 136	P-hydroxybenzyl adenosine
20[a)]		995	***727***，459, 423，441，397，369，263, 161	Parishin
21[a)]		229	***123***, 107, 93	4,4'-dihydroxydibenzyl ether

[a)] Positively identified via comparison with authentic reference compounds.

[b)] Black and italic font shows the base peak ion.

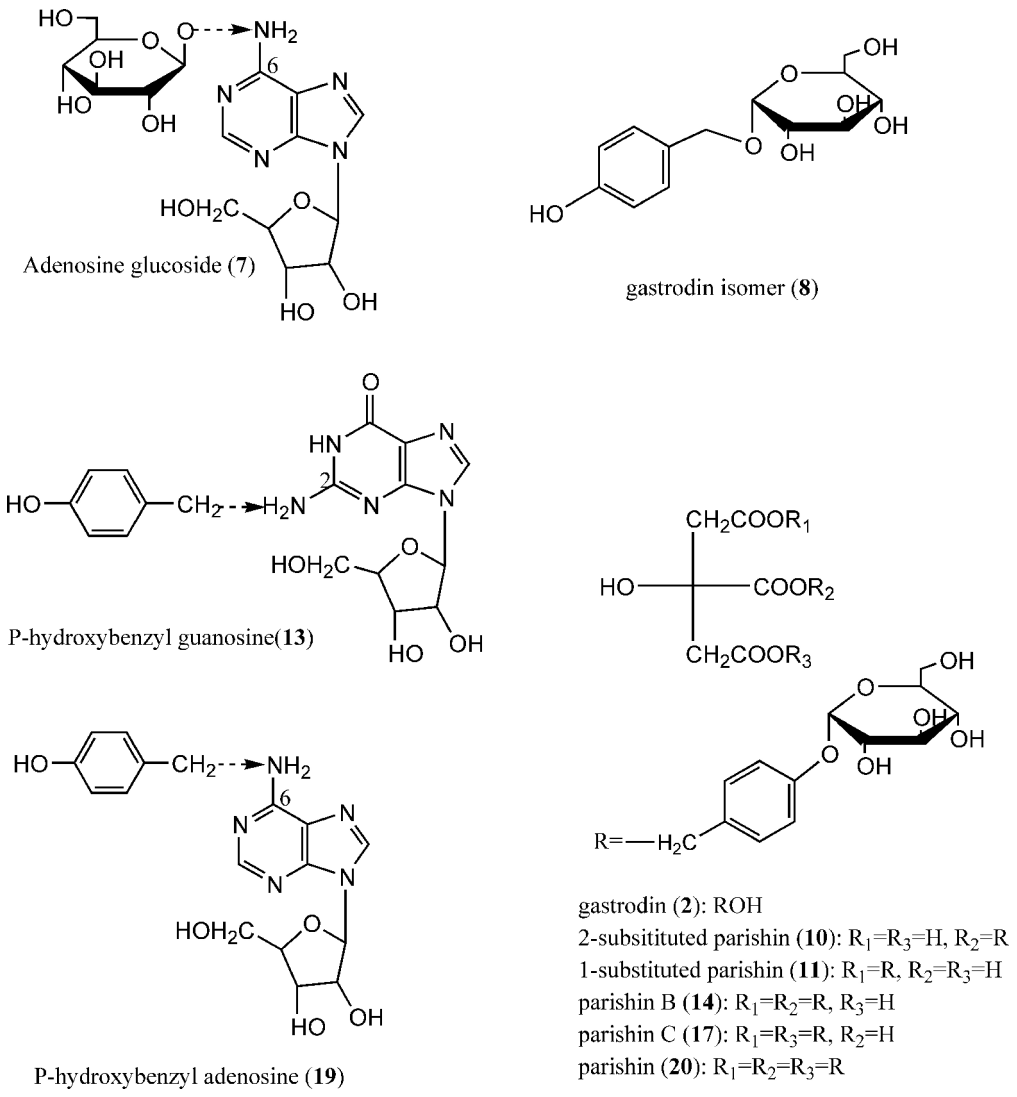

Figure 1. Representative chemical structures studied in this paper and the dotted arrow showed the likely conjugated position.

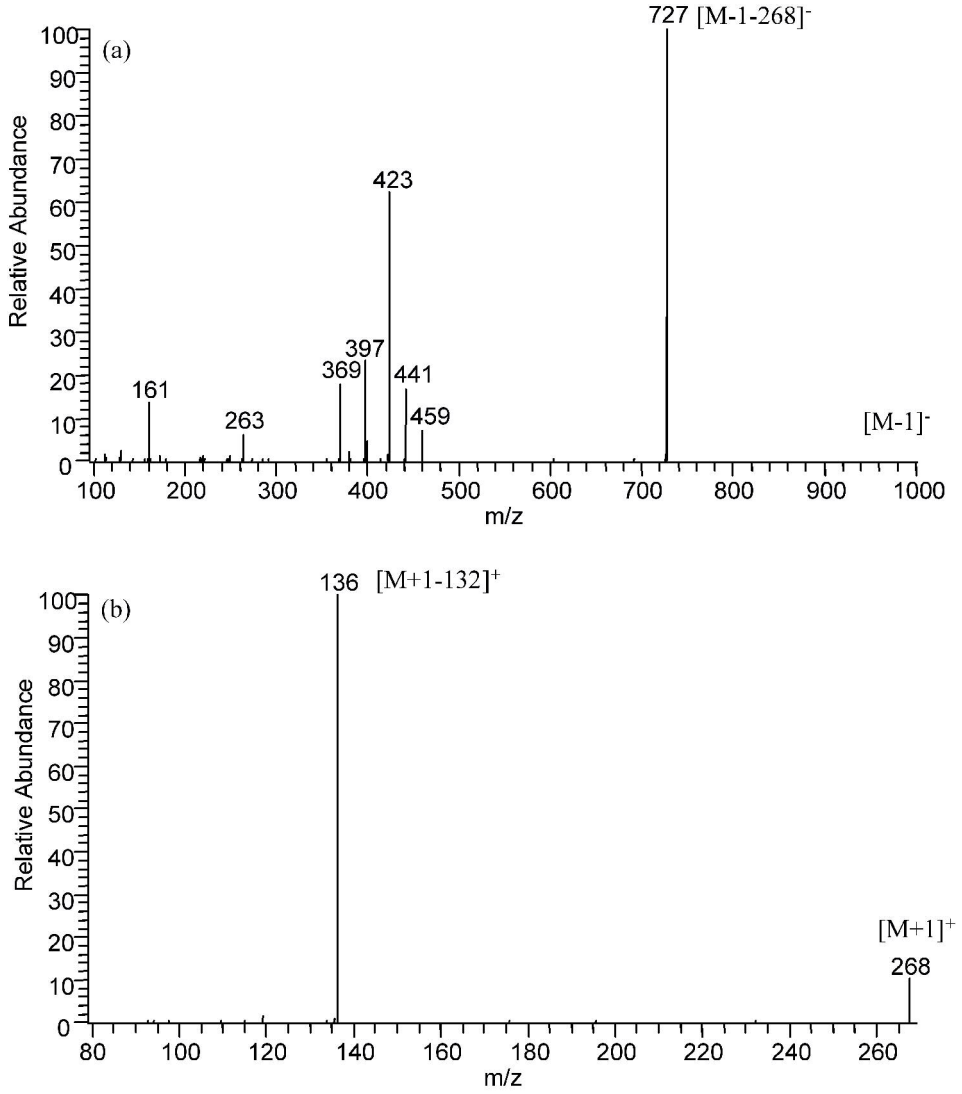

Figure 2. ESI-MS/MS spectra of representative reference phenolics and nucleoside. (a) parishin in negative mode, collision energy 40V; (b) adenosine in positive mode, collision energy 15V.

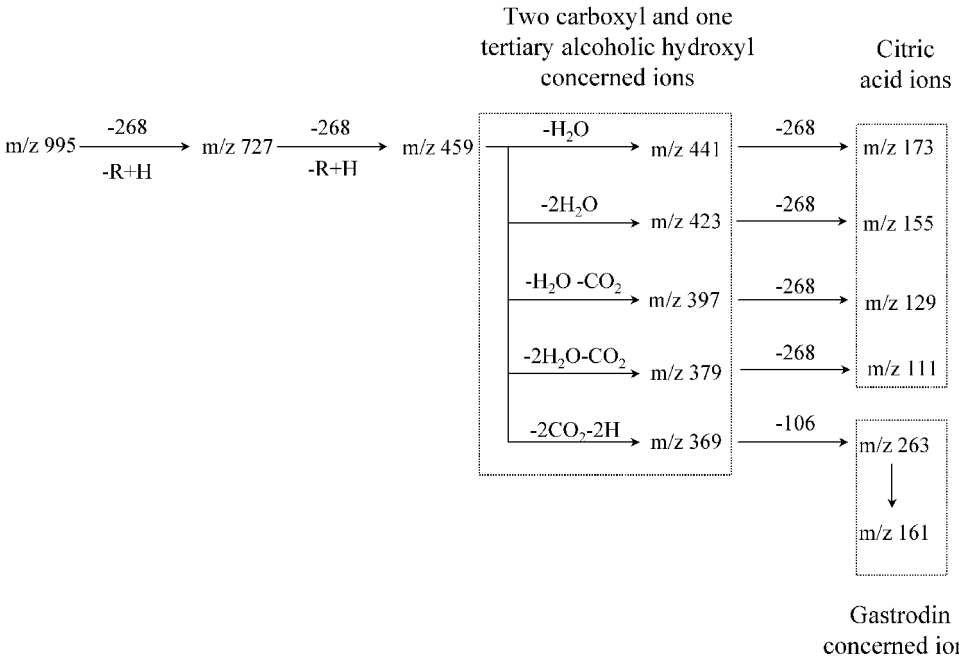

Figure 3. The proposed fragment patterns of parishins and characteristic fragmentation ions, carboxyl and tertiary alcoholic hydroxyl concerned ions, gastrodin concerned ions and citric acid ions.

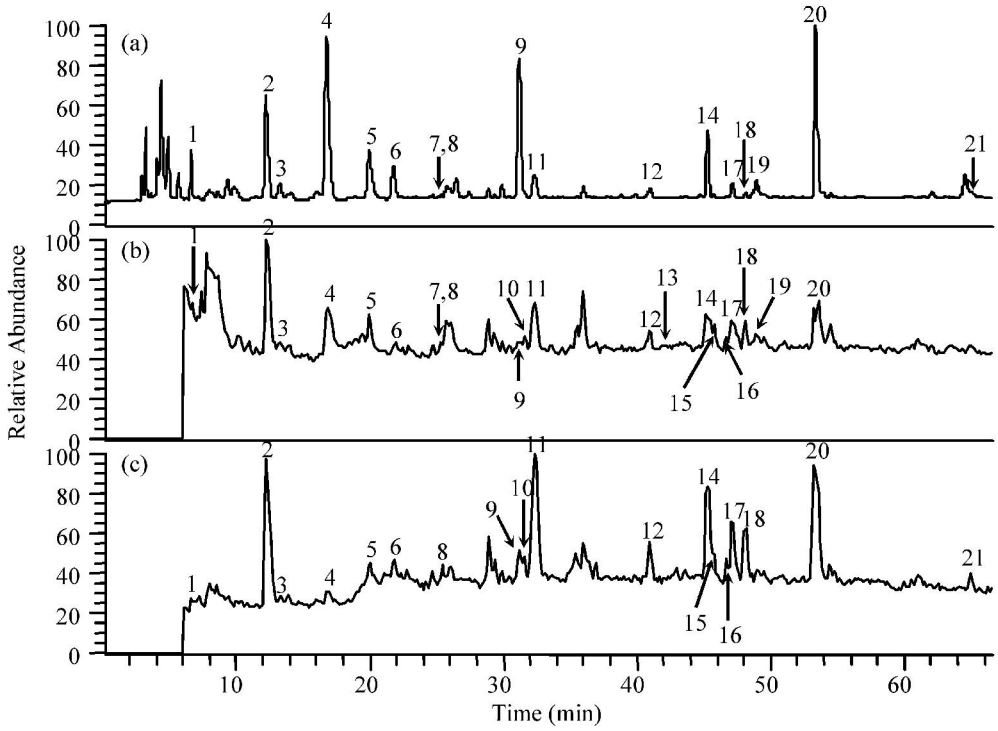

Figure 4. HPLC/UV Chromatogram and HPLC/ESI-MS total ion chromatogram (m/z 100-1100) of Gastrodia elata. (a) UV chromatogram recorded at 270nm; (b) positive ion chromatogram; (c) negative ion chromatogram. LC conditions: column 4.6iÂ250mm, 5¦Ìm particle size; mobile phase: solvent A £¨0.5% acetic acid in methanol£©, solvent B (0.5% acetic acid in water), gradient, 0-10min 2% A, 10-60min linearly changed to 40%A, 60-75min held at 40% A. Flow rate 0.8ml/ml.

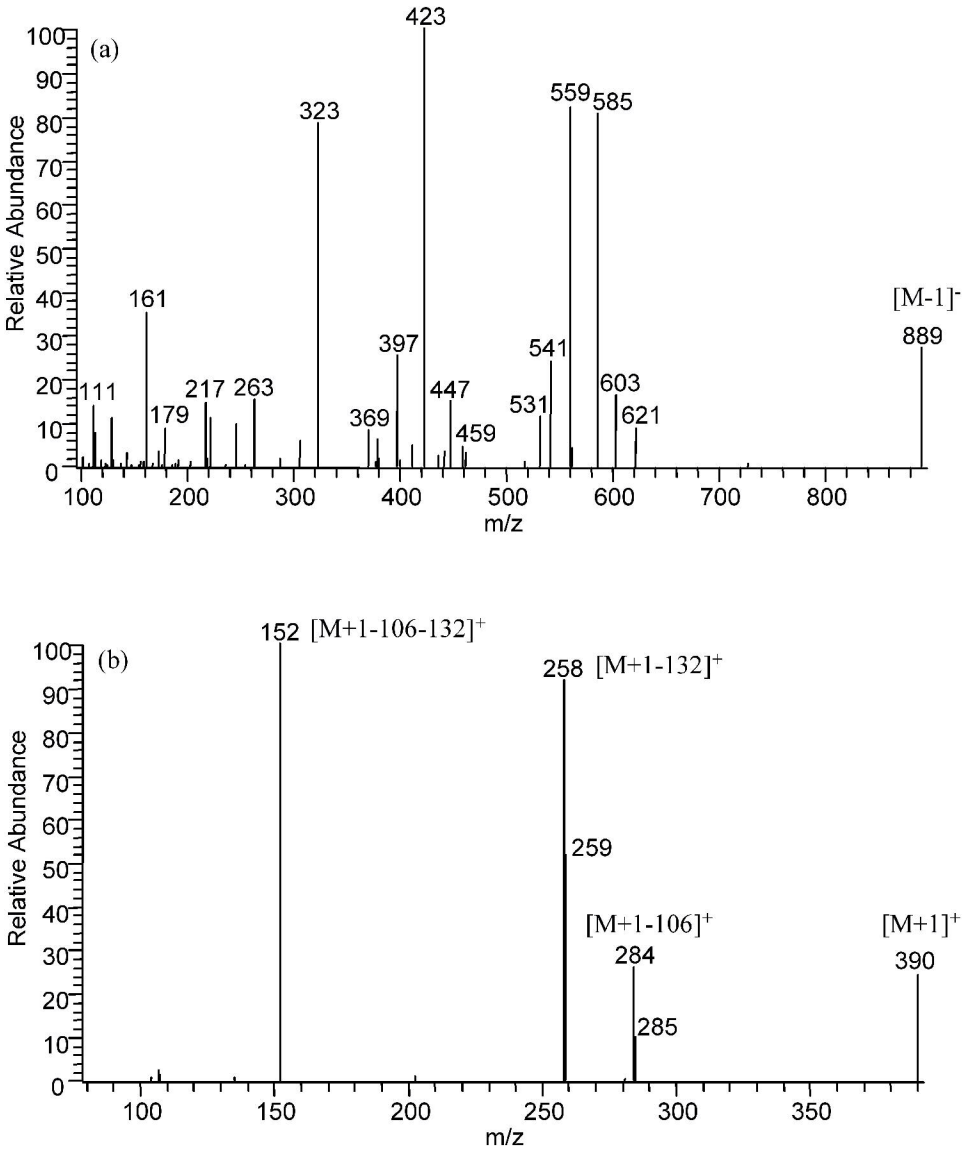

Figure 5. ESI-MS/MS spectra of representative peaks in Gastrodia elata. (a) peak 16 in negative mode, collision energy 4 0V; (b) peak 13 in positive mode, collision energy 15V.