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Running title: Metabolite profiling of Plantaginis Semen in three species

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Abstract

Plantaginis Semen is commonly used in traditional medicine to treat edema, hypertension, and diabetes. The commercially available Plantaginis Semen in China mainly come from three species. In order to clarify the chemical composition and distinct different species of Plantaginis Semen, we established a metabolite profiling method based on ultra high performance liquid chromatography with electrospray ionization quadrupole time-of-flight tandem mass spectrometry coupled with elevated energy technique. A total of 108 compounds,including phenylethanoid glycosides, flavonoids, guanidine derivatives, terpenoids, organic acids, and fatty acids, were identified from *Plantago asiatica* L., *P. depressa* Willd., and *P. major* L.. Results showed significant differences in chemical components among the three species, particularly flavonoids. This study is the first to provide a comprehensive chemical profile of Plantaginis Semen, which could be involved into the quality control, medication guide, and developing new drug of *Plantago* seeds.

Keywords: mass spectrometry; metabolite profiles; Plantaginis Semen; traditional Chinese medicines; ultra high performance liquid chromatography;.

Abbreviations

FAs, fatty acids; MS^E, Elevated Energy mass spectrometry; PAL, *Plantago asiatica* L.; PDW, *Plantago depressa* Willd.; PhG, phenylethanoid glycoside; PML, *Plantago major* L.; RDA, retro-Diels–Alder

1 Introduction

Plantaginis Semen has been traditionally used as medicines and supplements to treat antipyretic, diuretic, and expectorant, etc, in many countries [1]. Previous studies show that Plantaginis Semen contains phenylethanoid glycosides (PhGs), iridoids, flavonoids, and triterpenes [2,3], which account for a variety of pharmaceutical functions such as immunomodulatory, antioxidant, reducing blood lipids and sugar, facilitating defecation, and decreasing blood pressure [4–7]. The sources of commercially available Plantaginis Semen in China mainly consist of *Plantago asiatica* L. (PAL), *Plantago depressa* Willd. (PDW), and *Plantago major* L. (PML) [8]. Among them, PAL and PDW are the official sources of Plantaginis Semen in Chinese Pharmacopoeia to treat acute glomerulonephritis, hypertension and liver injury [9]. PML, one of the most abundant and widely distributed medicinal herb in the world, is recorded to exhibit wound healing, anti-inflammatory, antioxidant and antibiotic activities [10, 11]. Identification of Plantaginis Semen from different species is significant for guiding medicine use. However, the authentication is fairly complicated and time-consuming because the seeds are extremely small and have subtle differences on morphological characteristics. As the result, the explanation of chemical constitutions and differences is an effective way to distinguish different species of Plantaginis Semen.

Chromatographic fingerprint is a powerful technique and has been widely applied in origin authentication and QC of traditional Chinese medicines. The metabolite profiles reveal chemical compositions of the herbal medicines and contribute to elucidate the pharmacodynamic substance. Various techniques such as photocolorimetry, spectrophotometry, HPLC, GC–MS,and LC–MS have been applied to analyze chemical constituents of herbal medicines [12–14]. With recent advancements, UHPLC–ESI-QTOF-MS is considered as a powerful and reliable tool to identify compounds [15–17]. It can provide accurate ion mass values and molecular formulas to elucidate the compound structures [18, 19]. Recently, a novel elevated energy mass spectrometry (MS^E) technique has been utilized to obtain the parent and fragment ion information in a single run by simultaneously acquiring the accurate mass values at high and low collision energies [20, 21]. Combined with MS^E, UHPLC–ESI-QTOF-MS can rapidly provide outstanding chromatographic separation, accurate MS

and MS/MS data, and greatly increases the efficiency for compound identification in herbal medicines [22–24].

In the present work, a metabolite profiling was established by UHPLC–ESI-QTOF-MS coupled with MS^E technique to clarify the chemical composition Plantaginis Semen from species of PAL, PDW, and PML. Compounds were identified using accurate masses of pseudo-molecular and fragment ions and chromatographic behavior data. Differences of chemical components among the three species were also analyzed, which could be the guidance for medical applications of Plantaginis Semen.

2 Materials and methods

2.1 Reagents and chemicals

HPLC-grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (Santa Clara, USA). Ultrapure water was prepared using a Millipore Alpha-Q water system (Millipore, USA). All other reagents and solvents were of analytical or HPLC grade. Aucubin, geniposidic acid, plantamajoside, rhoifolin, luteolin, and isoquercitrin-7-*O*-gentiobioside were obtained Shanghai R&D Center for Standardization of Chinese Medicines (Shanghai, China). Acteoside, isorhamnetin-3-*O*-glucoside, caffeic acid, eriodictyol,kaempferol, and isorhamnetin were purchased from Meilun Biotech (Dalian, China). Linolenic acid, linolic acid, palmitic acid, and oleic acid were procured from Sigma–Aldrich (St. Louis, USA). Isoacteoside, 2-hydroxyacteoside, plantagoguanidinic acid, and plumbagine D were isolated from PAL in our laboratory. The structure was confirmed through MS, 13C NMR, and 1H NMR methods, and purity was over 95% after determined by HPLC–UV.

2.2 Plant materials

A total of 18 batches of Plantaginis Semen were acquired from different provinces in China (Table S1). All of the voucher specimens were authenticated as PAL, PDW, or PML by Professor Li-hong Wu (Shanghai R&D Center for Standardization of Chinese Medicines).

2.3 Sample preparation

All of the samples were pulverized into fine powder. Three hundred mg of powder samples were accurately weighed, dispersed in 20 mL of 60% v/v methanol/water solution, and ultrasonically extracted in a water bath for 30 min at room temperature.

The mixtures were then centrifuged at 6000 × *g* for 10 min and the supernatants were filtered through a 0.22 μm filter membrane for analysis.

2.4 Chromatographic conditions

Chromatographic separations were performed on an Acquity UPLC system (Waters, USA) equipped with a binary solvent delivery system and an autosampler. The extracts were separated using an Acquity UPLC BEH C_{18} RP column (1.7 μm, 100 mm × 2.1 mm i.d.; Waters, USA) in which the column temperature was maintained at 45°C to avoid excessive column pressure. The mobile phase consisted of 0.1% formic acid in deionized water (mobile phase A) and acetonitrile (mobile phase B). Separation was conducted with the following gradient elution at a flow rate of 0.3 mL/min: 0–1 min, 5% B; 1–4 min, 5–15% B; 4–5 min, 15–17% B; 5–7 min, 17% B; 7–9 min, 17–23% B; 9–14 min, 23–50% B; 14–23 min, 50–65% B; 23–28 min, 65–95% B, and 28–30 min, 5% B for equilibration of the column. The injection procedure was carried out for 1 min. An aliquot of 5 μL of sample solution was injected for analysis.

2.5 Mass spectrometric conditions

MS detection was performed using Acquity Synapt G2 QTOF tandem mass spectrometer (Waters, UK) connected to the UHPLC system by an ESI interface and controlled by MassLynx version 4.1 (Waters, UK). The ESI source was operated in both positive (ESI+) and negative (ESI−) ionization modes. The optimized conditions to trigger maximum response of metabolites were listed as follows: capillary voltage, −2.5 kV (ESI−) or +3 kV (ESI+); sample cone, −25 V (ESI−) or +30 V (ESI+); extraction cone, −4.0 V (ESI−) or +4.0 V (ESI+); source temperature, 120°C; desolvation temperature, 350°C; cone gas (nitrogen) flow, 50 L/h; and desolvation gas (nitrogen) flow, 600 L/h. Argon was used as collision gas. Leucine-enkephalin (2 ng/mL) was used as the lock mass generating a reference ion at m/z of 554.2615 (ESI−) or 556.2771 (ESI+) by a lockspray at 5 μL/min to acquire accurate mass during analysis.

Data were collected in a centroid mode. MS^E approach was conducted with two scan functions. In function 1, the following parameters were set: *m*/*z* 50–1500; scan duration, 0.3 s; interscan delay, 0.024 s; and collision energy ramp, 4 V. In function 2, the following parameters were set: *m*/*z* 50–1500; scan duration, 0.3 s; interscan delay, 0.024 s; and collision energy ramp, 10–30 V. In MS^E, MS and MS/MS data can be

acquired almost simultaneously in a single analytical run. Data acquisition and processing were conducted using Waters MassLynx version 4.1.

3 Results and Discussion

3.1 Sample preparation and metabolite profiling

Ultrasonication was selected for the extraction for its time-saving, convenient, and reproducible. Methanol/water ratio was optimized to 60% v/v to achieve the maximum extraction efficiency of compounds with different polarities. In UHPLC–ESI-QTOF-MS analysis, gradient elution, flow rate, and column temperature were evaluated for an optimized chromatographic condition. For a comprehensive analyses of the metabolite compositions of the extracts, positive and negative ionization ESI modes were used to verify the molecular formula of online MW assignments of the unknown substances.

3.2 Identification strategy

A total of 108 compounds belonging to phenylethanoid glycosides (PhGs), guanidine derivatives, flavonoids, terpenoids, organic acids, fatty acids (FAs) were identified (Table S2). Chromatograms of three different species of Plantaginis Semen in negative ion mode are shown in Fig. 1. The UHPLC–ESI-QTOF-MS coupled with MS^E technique could simultaneously afford accurate mass values of precursor and fragment ions in a single injection. The detected peaks were then identified by their elemental compositions or comparing MS^Edata to the literatures or public online databases, including PubChem (http://pubchem.ncbi.nlm.nih.gov), ChemSpider (http://www.chemspider.com), Kegg Ligand Database (http://www.genome.jp/kegg/ligand.html), SciFinder Scholar (https://scifinder.cas.org), Metlin (http://metlin.scripps.edu), and Riken (http://spectra.psc.riken.jp/menta.cgi/index). Mass errors between measured and calculated values were <1.2 mDa or 5 ppm to guarantee high resolution and good accuracy. We also use 20 standard compounds, including one organic acid, two iridoids, two guanidine derivatives, four fatty acids, seven flavonoids, and four phenylethanoid glycosides to confirm the identification results. Furthermore, these standards were applied to evaluate the proposed method which was proved to be stable and reliable.

3.3 Chemical constituents in Plantaginis Semen

Phenylethanoid glycosides

PhGs are natural products widely distributed in the plant kingdom and isolated from many medicinal plants [25]. PhGs exhibit verifiable therapeutic effects, including neuroprotection, antioxidation, anti-metastasis and cytotoxiy [26–28]. In this study, 11 PhGs were characterized in Plantaginis Semen, shown in Table 1. Among them, 2-hydroxyacteoside, plantamajoside, acteoside, and isoacteoside were identified and confirmed by comparing with standards. According to the PhGs determined in this experiment, the central glucose always connects with rhamnose by Rha (1→3) Glu linkage. Glucose is also directly attached to aglycone, and caffeoyl is usually located at C_4 or C_6 position of glucose. The detected PhGs produced similar fragmentation patterns in the MS^E spectra. The neutral losses of 162, 152, or 146 Da were related to caffeic acid, glucose, phenethanol aglycone, and rhamnose. H_2O or CO_2 is frequently eliminated in the fragmentations. Identical product ions at *m*/*z* 179.0369, 161.0235, and 135.0457 were observed, whichrespectively indicated the presence of caffeoyl, anhydroglucose, and anhydrophenylethanol. We take plantamajoside to illustrate the fragmentation pathway of PhGs (Fig. 2). The molecular formula of plantamajoside was $C_{29}H_{36}O_{16}$ with *m*/*z* 639.1944 $[M–H]^–$. In the MS^E spectrum, the fragment ion at *m*/*z* 477.1632 was formed by eliminating caffeoyl residue from the precursor ion. The fragment ion at *m*/*z* 315.1056 was from the group of central glucose with phenethanol aglycon. Furthermore, two fragment ions at *m*/*z* 179.0369 and 161.0235 could be served as characteristic ions to identify PhGs, which are respectively produced from the dehydrogenation and the dehydration of caffeic acid.

Flavonoids

Flavonoids are a chemically and structurally diverse group of polyphenolic compounds widely distributing in all parts of the plant [29]. Flavonoids are recognized as pigments responsible for leaf colors and are mainly involved in biological processes, such as the protection of plant tissues against UV radiation [30, 31].

A total of 23 flavonoids, including ten flavonols, four flavones, two flavanonols, six flavanones, and one aurone, were identified in this study (Table 2); most of these compounds are present in the form of glycosides with sugars attached to flavonoid aglycone by C–O bonds. Among them, isoquercitrin-7-*O*-gentiobioside, isorhamnetin-3-*O*-glucoside, rhoifolin, eriodictyol, luteolin, kaempferol, and isorhamnetin were verified using reference standards. Flavonoids mainly consist of two aromatic

benzene rings separated by an oxygenated heterocyclic ring. The characteristic fragmentation of flavonoid aglycones involves RDA cleavage of ring C and multiple neutral loss pathways, such as sequential elimination of sugar residues [32]. Compound **37** with quasi-molecular ion at *m*/*z* 465.1040 $[M–H]^-$indicated the molecular formula of $C_{21}H_{22}O_{12}$. In the MS^E spectrum, compound **37** displayed fragment ions at *m/z* 313.0919, 303.0500 and 151.0021, suggesting the loss of glucose and the RDA cleavage of ring C. Compound **37** was finally identified as plantagoside. Compound **47** was identified as pentahydroxyflavanone. The $[M–H]^-$ ion ofcompound **47** was *m*/*z* 303.0500 with the elemental composition of $C_{15}H_{11}O_7$. The MS^E result showed that the product ions at *m*/*z* 151.0023 and 107.0120 respectively corresponded to the RDA cleavage of ring C and the loss of CO_2, indicating that compound **47** was the aglycone of plantagoside (Fig. 3). Plantagoside and pentahydroxyflavanone were first isolated from *P. asiatica* var. *japonica* [33] and *Helichrysum bracteatum* [34]. These compounds inhibit the formation of advanced glycation end products of proteins under physiological conditions and impede protein cross-linking glycation [35].

Compounds **19** and **27** (ampelopsin glucoside) are the characteristic components of PDW. Ampelopsin is one of the most common flavonoids involved in multiple biological activities, such as antimicrobial and antioxidant effects, as well as antihypertension and hepatoprotection [36, 37]. Ampelopsin glucosides exhibit more efficient antioxidant properties than ampelopsin alone [38]. Compounds **19** and **27** were tentatively identified as ampelopsin-4′-glucoside and ampelopsin-3′-glucoside, respectively, based on the MS^Efragmentation pattern.

Guanidine derivatives

Guanidine derivatives are a group of infrequent alkaloids which are mostly reported in marine organisms [39]. This group of compounds is widely applied in medicine, chemistry, and other industries because of their strong alkalinity, high stability, and good biological activities [40, 41]. Guanidine derivatives ionize more efficiently in the positive ion mode. In this study, we characterized 18 guanidine derivatives which contained similar imidazoline skeletons (Table 3). The structures of plantagoguanidinic acid and plumbagine D were confirmed by standard compounds. Plantagoguanidinic acid was first isolated from PAL seeds [42]. The fragment ion of plantagoguanidinic

acid at *m*/*z* 208.1445 was generated by the elimination of H_2O from the carboxyl (Fig. 4). The product ion at *m*/*z* 84.0562 wasfrom the cleavage between the imidazoline ring and the side chain. Plumbagine D, first discovered from *Plumbago zeylanica* [43], has one more butanediol group replaced on the imidazoline ring than plantagoguanidinic acid. Plumbagine D then yielded fragment ions at *m/z* 226.1567 and 172.1093 from the side-chain cleavage and the specific product ion at *m*/*z* 84.0567 from imidazoline ring (Fig. 4). These fragmentation pattern can be applied to characterize the remaining unknown guanidine derivatives. For example, the $[M+H]^+$ ion of compound **11** with the molecular formula of $C_{15}H_{28}N_3O_5$ was detected at *m*/*z* 330.2028. The MS^E spectrum showed that a H_2O loss was generated at *m*/*z* 312.1938. Compound **11** also displayed the same fragment ions at *m/z* 172.1082 and 84.0576 as plumbagine D. The results demonstrated that compound **11** was hydroxy-substituted plumbagine D, and was tentatively recognized as plumbagine E [43].

Terpenoids

Five iridoids and four triterpenoids were characterized from Plantaginis Semen (Table 4). Iridoids are a group of terpene-derived compounds which have structural similarity and biosynthetic relationship to iridodial and iridomyrmecin [44]. Plenty of iridoids have been isolated from the genus *Plantago*. Iridoids in this experiment displayed strong MS response in negative ion mode. In the spectrum of geniposidic acid, fragment ion at *m*/*z* 211.0607 was obtained after the elimination of glucose residue. Further fragment ions at *m*/*z*123.0443 and 89.0236 were also exhibited because of $^{1,4}F$ cleavage. Furthermore, the neutral loss of CO_2 occurred in this compound to indicate thepresence of a carboxyl functionality. Aucubin generated specific solvent adducts ion *m/z* 391.1245 $[M+HCOO]^-$ with high intensity and underwent similar cleavage pathways as geniposidic acid. The other three iridoids (8-epiloganic acid, gentiopicroside, and catalposide) were also identified by similar cleavage pathways.

Triterpenoids have been recognized to have hepatoprotective, antihyperlipidemic, anticancer, and anti-inflammatory effects. These compounds are synthesized from isopentenyl pyrophosphate by the 30-carbon intermediate squalene. In this study, four triterpenoids (sumaresinol, oleanolic acid, ursonic acid, and oleanolic acid acetate) were identified by comparing with those described in previous studies [45, 46].

Organic acids and amino acids

Four organic acids (gluconic acid, citric acid, ferulic acid, and caffeic acid) and one amino acid (tryptophan) were identified in Plantaginis Semen (Table S2). Organic acids and amino acids are a widespread primary metabolites that play essential roles in plant growth processes, including respiration, photosynthesis, and hormone and protein syntheses [47]. In the MS/MS fragmentation, organic acids generate the neutral losses of CO_2 from the carboxylic group or H_2O, and amino acids generate neutral losses of CO_2 and the amino group NH_3.

Fatty acids

FAs are essential macromolecules present in all living organisms. FAs consist of long hydrophobic, often unbranched chains of hydrocarbons, with hydrophilic carboxylic acid groups at one end [48]. FAs and their derivatives also function as signaling molecules that modulate normal and disease-related physiological characteristics in microbes, insects and other animals, and plants [49].

We identified 28 FAs in the extracts of Plantaginis Semen (Table S2). Among them, linolenic acid, linolic acid, palmitic acid, and oleic acid were previously reported in PAL and then confirmed by comparing with authentic standards. The other detected FAs were C16 and C18 hydroxy FAs, which are presumably produced by oxidative metabolism of polyunsaturated FAs. However, the exact structures of four trihydroxyoctadecadienoic acids and three hydroxyoctadecatrienoic acids could not be defined for the uncertain locations of hydroxy groups and double bonds.

3.4 Comparison of the metabolite profiles of three *Plantago* species

We had collected 18 batches of Plantaginis Semen in species of PAL, PDW and PML from different places in China, in which PAL and PDW are the official sources recorded in Chinese Pharmacopoeia. The UHPLC–ESI-QTOF-MS Chromatograms revealed obvious diversities in chemical composition of three Plantago species (Fig. S1). PhGs and iridiods are primary bioactive components of Plantago species and acteoside and geniposidic acid are recognized as the QC markers of Plantaginis Semen in Chinese Pharmacopoeia [6, 9]. In our study, acteoside displayed similar levels in PAL and PDW (Table 1), while the amount of geniposidic acid in PAL is higher than in PDW (Table 4). Moreover, PML displayed low levels of PhGs and terpenoids. The contents of acteoside and geniposidic acid in PML were significantly lower than PAL and PDW and did not meet the quality standards for Plantaginis Semen. The results indicated that

according to the test standards for Plantaginis Semen in Chinese Pharmacopoeia, the quality of the seeds from PAL was superior to those from PDW and PML was unqualified to serve as the source of Plantaginis Semen.

Different flavonoid levels were also found in PAL, PDW and PML (Table 2). In General, PML contains the greatest amount of flavonoids followed by PDW and PAL. Meanwhile, PML had more widely spread of flavonoids. Therefore the seeds of PML probably have advantages in anti-oxidative, anti-inflammatory and anti-bacterial effects [35–38]. In addition, the three species of Plantaginis Semen could be differentiated by several flavonoids. Plantagoside, isoquercitrin, and pentahydroxyflavanoneare were much higher in PML than in PAL and PDW. Ampelopsin glucoside and its isomer showed high concentrations in PDW while could not be determined in PML.

Guanidine derivatives are a group of novel alkaloids which have potential hypoglycemic effect [42]. The three species of Plantaginis Semen showed similar contents of Plantagoguanidinic acid and Plumbagine D, while PAL and PML displayed higher amounts of total guanidine derivatives (Table 3). In that case, PAL and PML could be used as the resources for guanidine separation, which is greatly helpful for the development of new glucose-lowering drugs.

Furthermore, PAL contained higher amounts of FAs than PDW and PML. Previous reports showedthat FAs display antioxidation, immunoregulation, vascular-protection, and cholesterol-reducing effects [50]. For that reason, the seeds of PAL might be the first choice to treat metabolic diseases.

4 Conclusions

In the present study, the metabolite profiling based on UHPLC–ESI-QTOF-MS combined with MS^E technique was applied to evaluate the chemical variation of three species of Plantaginis Semen, PAL, PDW, and PML. In this experiment, MS^E technique provided accurate mass values of precursor and fragment ions in a single injection. Positive and negative ionization modes were applied, and the chemical formula was obtained based on the errors <1.2 mDa or 5 ppm between measured and calculated mass values. Twentystandard compounds were collected to validate the reliability of the proposed method. Using this approach, we identified 108 compounds and most of them were first detected in Plantaginis Semen. Our results indicated significant differences

in chemical compositions among the three species. PAL showed higher levels of PhGs, terpenoids, guanidine derivatives and FAs, while PML contained the greatest amount of flavonoids. This study provides a comprehensive chemical profile of Plantaginis Semen, which could be involved into the QC, medication guide, and developing new drug of Plantago seeds.

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Figure captions

Fig. 1 Base peak ion chromatograms of three species of Plantaginis Semen obtained by UHPLC-ESI-QTOF-MS in the negative ion mode. (a) PAL, (b) PDW and (c) PML.

Fig. 2 Proposed fragmentation pathway of plantamajoside.

Fig. 3 Proposed fragmentation pathways of plantagoside (a) and pentahydroxyflavanone (b).

Fig. 4 Proposed fragmentation patterns of plantagoguanidinic acid (a) and plumbagine D (b).

Table 1 Contents of PhGs in three species of Plantaginis Semen (mg/g crude drug, mean ± SE)

No.	Compound	R_1	R_2	R_3	R_4	R_5	PAL	PDW	PML
12	Decaffeoylacteoside	OH	H	0.037±0.004	–*	-			
34	2-Hydroxyacteoside [a]	Rha	Caffeoyl	H	OH	H	0.056±0.006	-	-
38	Plantamajoside [a]	Glu	Caffeoyl	H	H	H	0.491±0.079	-	0.193±0.074
44	Acteoside [a]	Rha	Caffeoyl	H	H	H	6.334±0.827	6.423±1.043	3.606±0.776
45	Isoplantamajoside	Glu	H	Caffeoyl	H	H	0.183±0.034	-	-
48	Isoacteoside [a]	Rha	H	Caffeoyl	H	H	1.433±0.243	0.67±0.181	0.11±0.081
	Total content						8.924±1.198	8.114±1.037	4.361±1.087

[a] This compound is confirmed by standards.

* (-) indicates absence of the compound.

Table 2 Contents of Flavonoids in three species of Plantaginis Semen (mg/g crude drug, mean ± SE)

No.	Compound	PAL	PDW	PML
15	Isoquercitrin-7-O-gentiobioside [a]	–*	-	0.459±0.021
16	Luteoloside dihexose	-	-	0.206±0.04
19	Ampelopsin glucoside	0.121±0.022	4.121±0.450	-
20	Isorhamnetin triglucoside	-	-	0.364±0.035
21	Isorhamnetin triglucoside isomer	-	-	0.124±0.028
26	Plantagoside-hexoside I	-	-	0.432±0.114
27	Ampelopsin glucoside isomer	0.089±0.017	3.603±0.259	-
30	Plantagoside-hexoside II	-	-	0.269±0.057
35	Quercetin 3,7-dihexoside/ Quercetin 3-sophoroside	0.059±0.008	-	-
37	Plantagoside	0.139±0.013	0.130±0.038	9.520±0.948
39	Hyperoside	-	-	0.063±0.003
42	Kaempferol rhamnoside hexoside	0.073±0.007	-	-
43	Isoquercitrin	-	-0	
41	Plantagoguanidinic acid [a]	2.885±0.238	1.513±0.075	2.079±0.386
54	Unknown	0.054±0.009	0.022±0.005	0.016±0.001
57	Unknown	0.03		

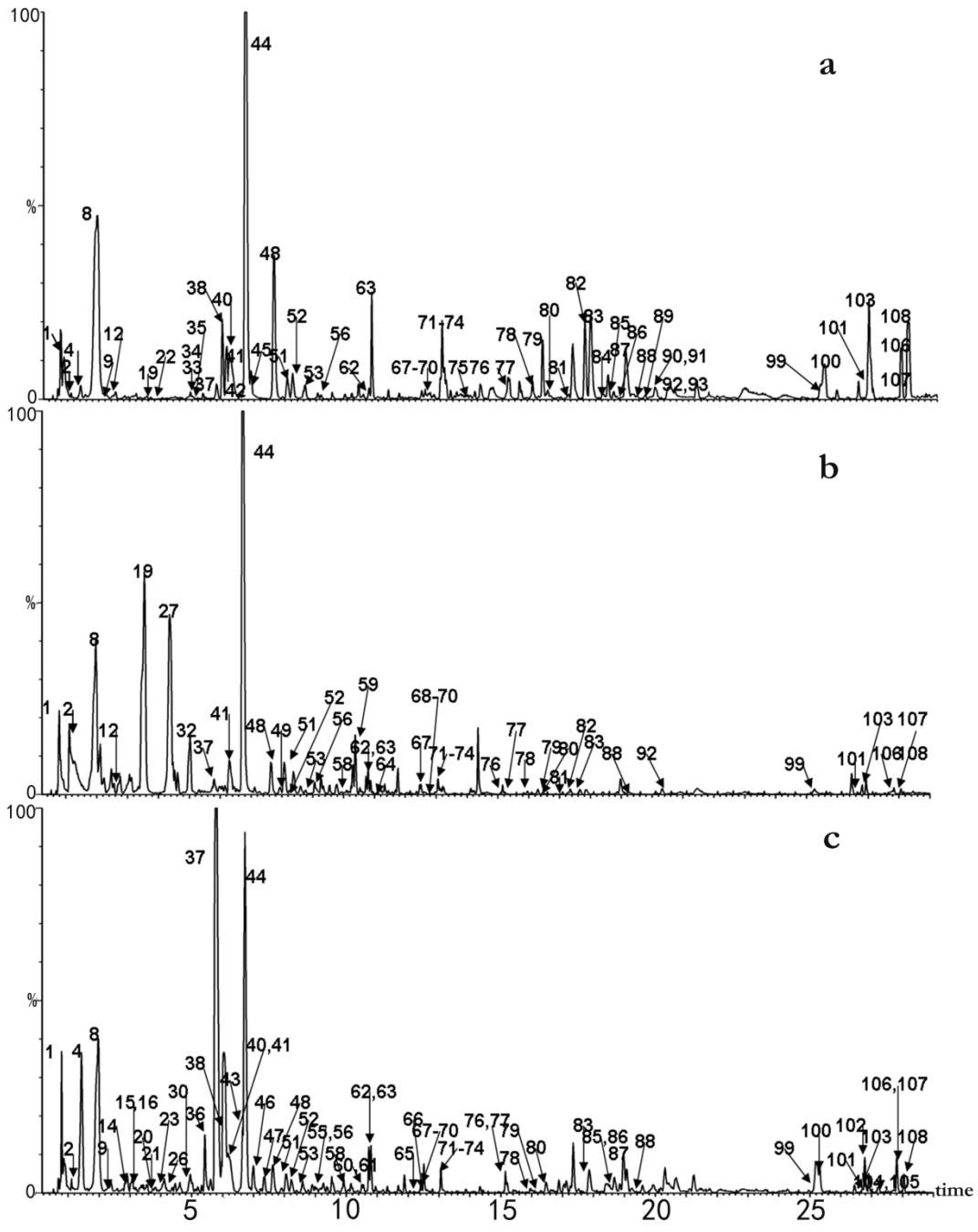

Figure 1 Base peak chromatograms of Plantaginis Semen obtained by UPLC/ESI-QTOF-MS. (a) *Plantago asiatica* L. in the negative ion mode. (b) *Plantago depressa* Willd. in the negative ion mode. (c) *Plantago major* L. in the negative ion mode.

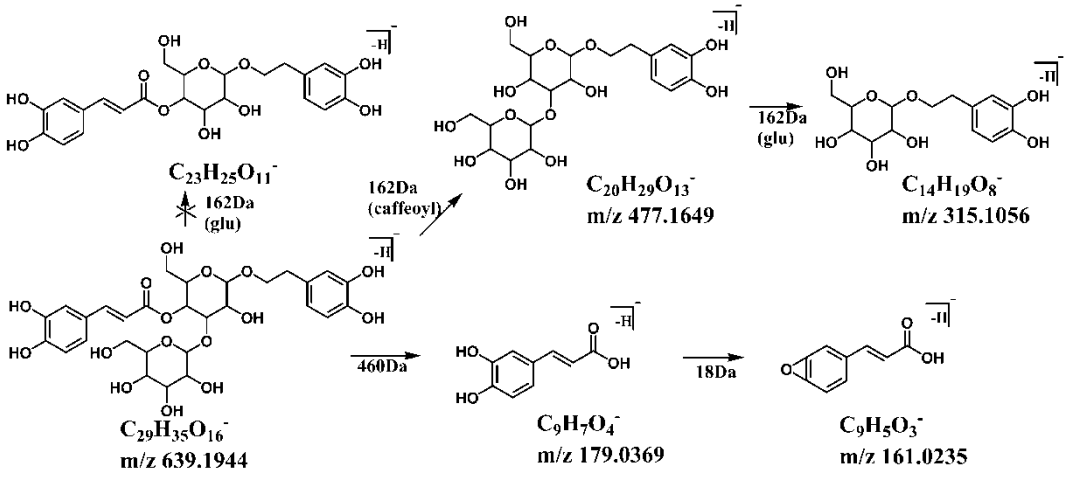

Figure 2 The proposed fragmentation pathway of plantamajoside.

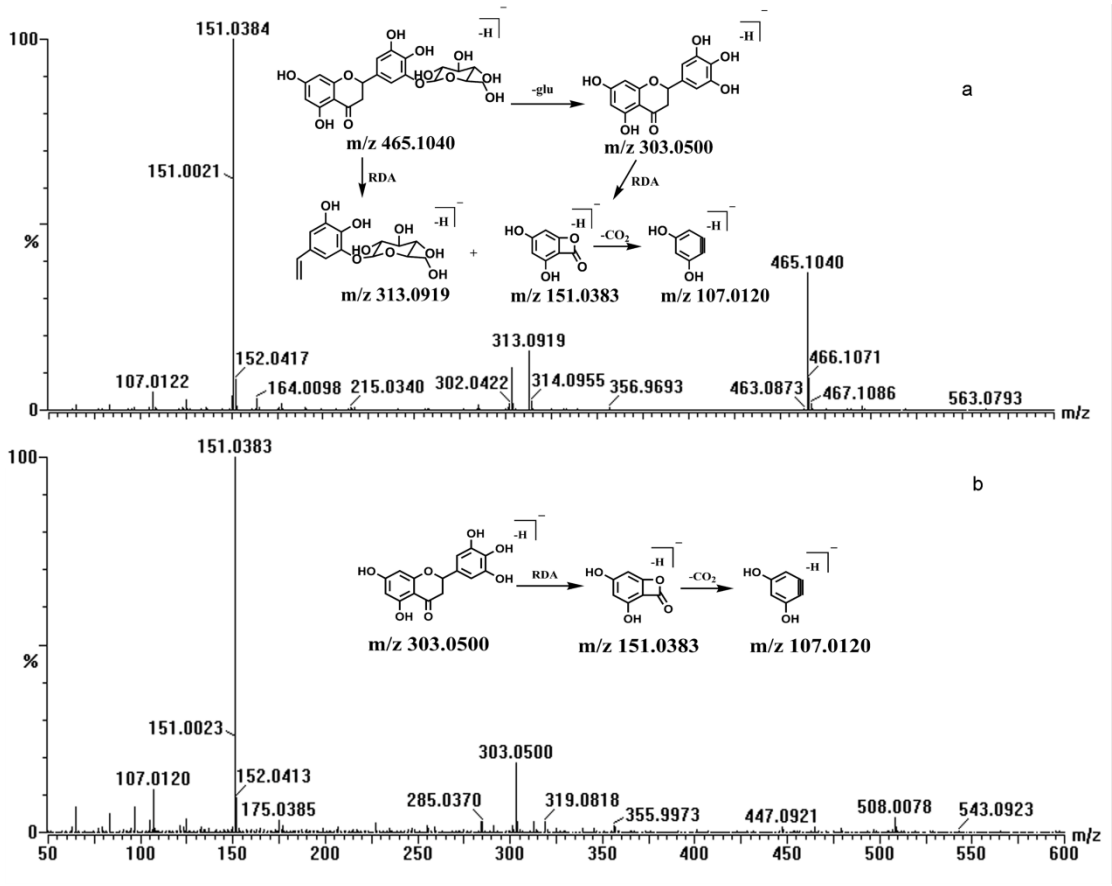

Figure 3 Fragmentation reactions of flavonoids. (a) plantagoside and (b) pentahydroxyflavanone.

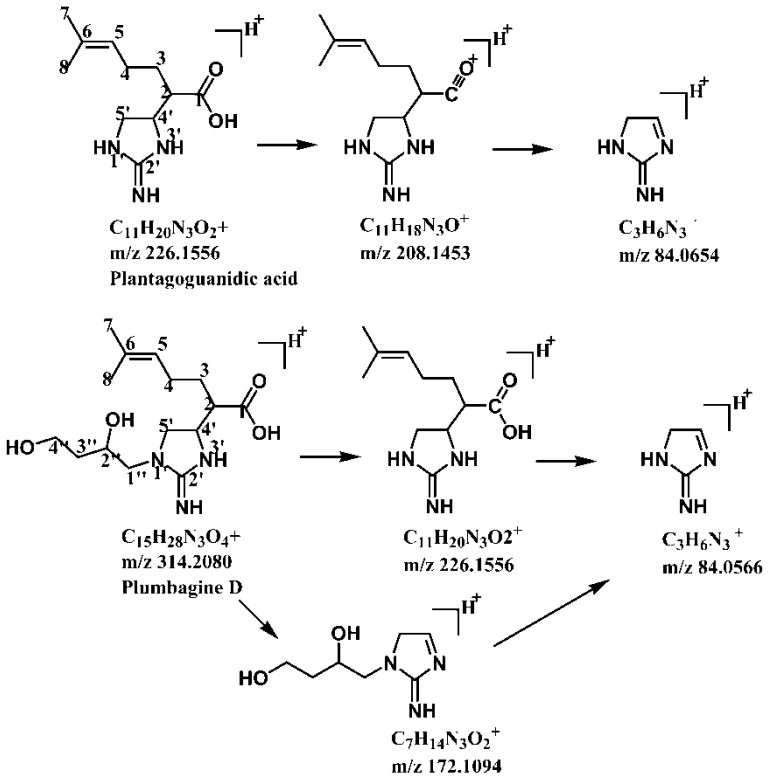

Figure 4 Fragmentation patterns of plantagoguanidinic acid and plumbagine D.