Characterization of Flavonoid Metabolites in Rat Plasma, Urine, and Feces after Oral Administration of Semen Ziziphi Spinosae Extract by HPLC-Diode-Array Detection (DAD) and Ion-Trap Mass Spectrometry (MS^n)

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A highly specific and sensitive method using high performance liquid chromatography coupled with diode-array detection and ion-trap mass spectrometry (HPLC-DAD-MS^n) was developed for study of the constituents of flavonoid extract of jujube seeds and the metabolites in rat plasma, urine, and feces samples after oral administration of flavonoid extract of jujube seeds. Two major flavonoids (spinosin and 6‴-feruloylspinosin) with content >60% in the flavonoid extract of jujube seeds were detected and confirmed by comparison with the reference standards. Furthermore, five metabolic components in plasma, seven in urine, and four in feces were detected and elucidated. The scientific and plausible biotransformation pathways of the main components in flavonoid extract of jujube seeds were also proposed, together with presentation of clues for potential bioactive mechanisms. This convenient HPLC-DAD-MS^n method could be used to identify the chemical components of flavonoid extract of jujube seeds as well as their metabolites, and to reveal their possible metabolic mechanism of action *in vivo*.

Key words Semen Ziziphi Spinosae; jujube seed; flavonoid extract; HPLC-diode-array detection and ion-trap mass spectrometry; original component; metabolic component

Semen Ziziphi Spinosae (jujube seeds), the dried semen of *Ziziphus jujuba* Mill. var. *spinosa* (Bunge) Hu ex H. F. Chou, is one of the most famous traditional Chinese medicines (TCMs) with hypnosis, sedation, and anticonvulsivant effects.[1] It has been widely used for the treatment of insomnia in clinical practice.[2] Chemical investigations on jujube seeds resulted in the discovery of several groups of bioactive components including flavonoids, saponins, and fatty acids.[3,4] Modern pharmacological studies have demonstrated that flavonoids are the main bioactive compounds responsible for the sedative and hypnotic effects of jujube seeds.[2,5] Hence it is important to explicate the biotransformation of flavonoids *in vivo* so as to clarify the mechanism of pharmacological action and to promote its availability as well. To date, there are few reports regarding the physiological disposition of flavonoids after oral administration of jujube seeds-containing TCMs[6,7]; however, the biotransformation of flavonoids *in vivo* remains unknown. The challenges for the metabolic study *in vivo* of TCMs extract lie in two aspects: one is the complexity of TCMs containing hundreds of constituents, and the other is the lack of proper analytical methods for the identification of the metabolites even in trace amount.[8]

A major goal of this study was to develop a highly specific and sensitive high-performance liquid chromatography method coupled with diode-array detection and ion-trap mass spectrometry (HPLC-DAD-MS^n) for the study of the constituents and metabolites in rat plasma, urine, and feces after oral administration of flavonoid extract of jujube seeds, providing scientific plausibility for the activity of this extract. These achievements would support preclinical pharmacokinetic studies and achieve a better understanding of the pharmacological action mechanism of flavonoid extract of jujube seeds.

Experimental

Materials Jujube seeds were purchased from a genuine area Xintai (Hebei, China) and identified by one of the authors, Dr. Ping Li. Spinosin (with a purity of 99.0%), and 6‴-feruloylspinosin (with a purity of 99.0%) (Fig. 1) was prepared in our laboratory. Male Sprague-Dawley (SD) rats were acquired from the Laboratory Animal Center of China Pharmaceutical University (Nanjing, China). HPLC grade acetonitrile was purchased from Merck KGaA Company (Merck, Germany). Deionized water was purified by Milli-Q system (Millipore, U.S.A.). Physiological saline was purchased from Shijiazhuang Pharmaceutical Corporation (Shijiazhuang, China). Heparin sodium, obtained from Hanbang Science & Technology (Nanjing, China), was dissolved in physiological saline at a concentration of 1250 U/ml and used to rinse the test tubes prior to blood collection for plasma. Glacial acetic acid, as well as the other reagents, was of analytical grade.

Preparation of Flavonoid Extract from Jujube Seeds Jujube seeds (3.0 kg) were ground into powder (particle size 20—40 mesh) and refluxed with 24 l of petroleum ether (60—90 °C) three times (each for 12 h). After removing the petroleum ether under reduced pressure, the residue was refluxed with 24 l of 70% ethanol twice to obtain the total extract of jujube seeds with a yield of 11.3%. The total extract of jujube seeds was separated by macroporous resin column chromatography (D101, Tianjin, China) and eluted with 30 l of water, 30 l of 25% ethanol, 30 l of 45% ethanol, and 30 l of 70% ethanol, successively. The part of 45% ethanol eluent was collected and refluxed under reduced pressure to remove the ethanol. The residue was then lyophilized and the yield was 5.86 g for every 1 kg of crude dried herb.

Preparation of Standard and Flavonoid Extract Solutions The standard stock solution was prepared by dissolving 1.0 mg spinosin and 1.0 mg 6‴-feruloylspinosin successively in a 1-ml volumetric flask, diluting with 70% methanol to obtain a nominal concentration of 1.0 mg/ml. The

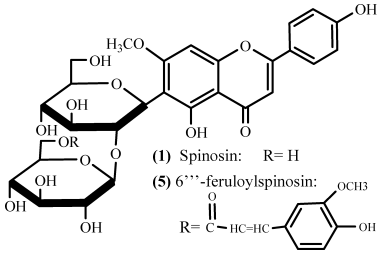

Fig. 1. Chemical Structure of Spinosin and 6‴-Feruloylspinosin

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flavonoid extract of jujube seeds solution was prepared by dissolving 2.0 mg flavonoid extract in a 1.0-ml volumetric flask, diluting with 70% methanol to obtain a nominal concentration of 2.0 mg/ml. All the stock solutions were kept at 4 °C before HPLC-DAD-MS^n analysis.

Animal and Biological Sample Collection Ten male SD rats (body weight, 240±20 g) were divided into a blank group (two rats) and drug group (eight rats). Prior to oral administration, each rat was fasted in a metabolic cage with free access to water for 12 h. To rats of the drug group, a dose of 180 mg/kg of flavonoid extract of jujube seeds, which is equivalent to 30 g crude dried herb/kg, was dissolved in 0.9% sodium chloride solution and administered orally. Equal dose of 0.9% sodium chloride solution was given to the rats of the blank group. For each group, the urine of all rats was collected and admixed at 4 h, 6 h, 12 h, and 24 h, while the feces was collected at 6, 12, and 24 h. The samples were stored instantly at −70 °C prior to analysis. Five days (more than five half-life periods) later, the rats of the two groups were administered orally with the same dosage of flavonoid extract and 0.9% sodium chloride solution as mentioned above. After superalimentation, a blood sample (1 ml) was collected from each rat by puncture of the retro-orbital sinus at 0, 1, 2, 4, 6, 8, 10, 16, and 24 h and deposited in clean heparinized glass tubes. The blood was centrifuged at 3500 rpm for 15 min to separate plasma, then the plasma of each group was mixed together as the sampling schedule and stored at −70 °C before analysis.[9—14)]

Bio-Samples Preparation To release the protein-binding medicinal composition, 50 μl of 20% acetic acid was added into 2 ml plasma sample and mixed by vortex, followed by 8 ml methanol added and vortexed again. Then, the solution was centrifuged at 3500 rpm for 15 min. The supernatant was evaporated to dryness at 40 °C in vacuum, and the residue dissolved in 0.5 ml of 70% methanol. After centrifugation at 15000 rpm for 15 min, 20 μl of the supernatant was introduced into the HPLC system for HPLC-DAD-MS^n analysis.

Urine samples (5 ml) were dissolved in 20 ml of methanol, followed by filtration. The filtrate was evaporated to dryness at 40 °C in vacuum, and the residue dissolved in 1 ml of 70% methanol. After filtering through a 0.22-μm membrane filter, 10 μl of the filtrate was inoculated into the HPLC system for HPLC-DAD-MS^n analysis.

Feces samples (3.0 g), stirred into powder, were extracted undisturbed with 30 ml of 70% ethanol and 30 ml of distilled water successively, followed by filtration. The filtrate was evaporated to dryness at 40 °C in vacuum, and the residue dissolved in 1 ml of 70% methanol. The solution was filtered through a 0.22-μm membrane filter. Five microliters of the filtrate was injected into the HPLC system for HPLC-DAD-MS^n analysis.[9—14)]

The blank samples of feces, urine, and plasma were processed with the same methods as described above. All samples were stored at −70 °C until analysis.

Assay Condition The HPLC-DAD-MS^n analysis was carried out on Agilent Series 1100 HPLC-DAD-MSD-Trap SL (Agilent Technologies, U.S.A.), equipped with a bibasic pump and an autosampler. A CLC-ODS reversed-phase C_{18} column (150×4.6 mm, i.d. 5 μm; Shimadzu, Japan) with an ODS C_{18} guard column (4.6×12.5 mm, i.d. 5 μm; Shimadzu, Japan) was maintained at 25 °C. Detection wavelengths were set at 335 nm. A gradient elution was adopted by water of 0.1% acetic acid aqueous (A) and acetonitrile (B) was used as 19—21% B at 0—9 min, 21—29% B at 9—20 min, 29—45% B at 20—35 min, 45—70% B at 35—40 min, 70—100% B at 40—45 min for flavonoid extract of jujube seeds, plasma samples, and urine and feces samples with the flow rate of 1.0 ml/min. The re-equilibration time of gradient elution was 15 min.

The mass spectra were recorded in positive and negative modes. Ultrahigh pure helium (He) was used as the collision gas and high purity nitrogen (N_2) as nebulizing gas. The optimized parameters in the negative ion mode were as follows: drying gas flow rate, 10 l/min; drying gas temperature, 350 °C; nebulizer, 40 psig; HV capillary voltage, 3500 V. For full scan MS analysis, the spectra were recorded in the range of *m/z* 50—1200. The isolation width of precursor ions was 3.0 U. The relative collision energy was adjusted to 45% of maximum. MS^n data were acquired in the automatic data-dependant mode.

Results and Discussion

Analysis of Chemical Components of Flavonoid Extract from Jujube Seeds by HPLC-DAD-MS^n Commonly, curative effects of TCMs result from integrative contributions of a number of bioactive compounds, including natural components from the extract of TCMs and their bioactive metabolites in the course of physiological disposition. Therefore study on the natural constituents of the extract of TCMs lays the basis for further pharmacokinetic research of TCMs *in vivo*. Before studying the metabolites of flavonoid extract of jujube seeds in rat after oral administration, the constituents in flavonoid extract were identified by HPLC-DAD-MS^n. The chromatogram of flavonoid extract on HPLC-DAD, together with the extract spectra on LC-MS[1] in negative mode, is shown in Fig. 2. By comparing UV spectra, MS data, and retention time with those of standards, two major chromatographic peaks of flavonoid extract of jujube seeds were undoubtedly identified as spinosin and 6‴-feruloylspinosin, which act as the main bioactive ingredients of jujube seeds.[2,3,14)] The contents of spinosin and 6‴-feruloylspinosin in flavonoid extract of jujube seeds were calculated as 47.3% and 14.8%, respectively, according to our preliminary experiments.

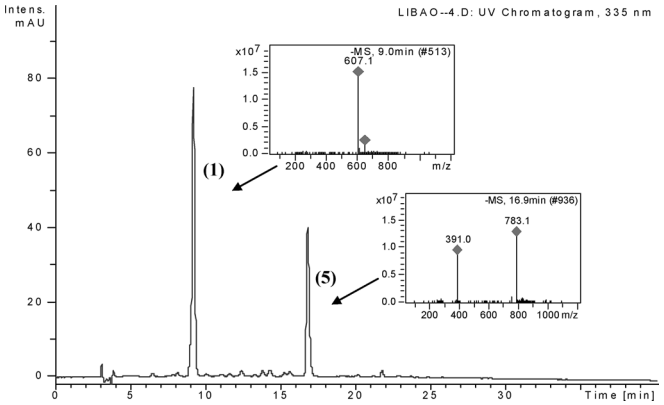

Fig. 2. HPLC-DAD Chromatogram and Extract Spectra in LC-MS^n of the Main Components in Flavonoid Extract of Jujube Seeds

Metabolic Study of Flavonoid Extract of Jujube Seeds in Rat Plasma, Urine, and Feces by HPLC-DAD-MS^n To elucidate the active constituents responsible for the pharmacological action, it is necessary to perceive the metabolic changes *in vivo* and chemical constituent profile in biological system. Therefore rat plasma, urine, and feces samples after oral administration of flavonoid extract of jujube seeds were detected and compared with blank samples by HPLC-DAD-MS^n in negative mode. The results show that the total peaks and corresponding peak areas in metabolic chromatograms were different when collected at different periods, and the most abundant metabolites were found at 4, 12, and 12 h in plasma, urine, and feces samples, respectively, corresponding to the cases of components during physiological disposition.[9—13)] To obtain further metabolic information, the biological samples with predominant metabolic compounds were further analyzed and elucidated by HPLC-ESI-MS^n. The base peak chromatograms of plasma (A), urine (C), and feces (E) and UV chromatograms of plasma (B), urine (D), and feces (F) with the most abundant metabolites are presented in Fig. 3. Five metabolic components in plasma, seven in urine, and four in feces were detected by HPLC-DAD-MS^n. The retention time of each peak on HPLC, proposed compound names, $[M-H]^-$, MSD Trap fragment ions (*m/z*), and location of the metabolites are listed in Table 1. A total of 10 flavone-*C*-glucoside-based compounds, including two original components and eight secondary metabolites of

Table 1. HPLC-MS^n Identification of Flavonoid Metabolites Detected in Rat Plasma, Feces and Urine after Oral Administration of Flavonoid Extract of Jujube Seeds

Peak	t_R (min)	Compound	$[M-H]^-$ (m/z)	MSD trap fragments ions (% base peak)	Location
1	9.1	Spinosin (**1**)	607	MS^2 [607]: 427 (100); MS^3 [427]: 307 (80)	Plasma
2	10.4	Chalcone-glycoside (**2**)	623	MS^2 [623]: 563 (80)	Plasma
3	11.9	Swertisin (**3**)	445	MS^2 [445]: 325 (100)	Plasma
4	13.9	Phenantithetical-furane-glycoside (**4**)	741	MS^2 [741]: 681 (100); MS^3 [681]: 663 (90)	Plasma
5	16.9	6‴-Feruloylspinosin (**5**)	783	MS^2 [783]: 607 (90); MS^3 [607]: 487 (60), 427 (40)	Plasma
6	9.2	Spinosin (**1**)	607	MS^2 [607]: 427 (100); MS^3 [427]: 307 (80)	Urine
7	9.8	Phenantithetical-furane-glycoside (**6**)	579	MS^2 [579]: 403 (90); MS^3 [403]: 385 (80)	Urine
8	11.5	Swertisin (**3**)	445	MS^2 [445]: 325 (100)	Urine
9	14.0	Phenantithetical-furane-glycoside (**4**)	741	MS^2 [741]: 681 (100); MS^3 [681]: 663 (90)	Urine
10	16.9	Phenolic-glycoside (**7**)	477	MS^2 [477]: 301 (100)	Urine
11	27.1	Phenolic glycoside (**8**)	461	MS^2 [461]: 285 (100)	Urine
12	28.8	Phenantithetical-furane-glycoside (**9**)	413	MS^2 [413]: 201 (75)	Urine
13	9.2	Spinosin (**1**)	607	MS^2 [607]: 427 (100); MS^3 [427]: 307 (80)	Feces
14	11.6	Swertisin (**3**)	445	MS^2 [445]: 324.8 (100)	Feces
15	13.6	Phenantithetical-furane-feruloyl-glycoside (**10**)	755	MS^2 [755]: 579.1 (90), MS^3 [579]: 459.0 (80)	Feces
16	16.8	6‴-Feruloylspinosin (**5**)	783	MS^2 [783]: 607 (90); MS^3 [607]: 487 (60), 427 (40)	Feces

Peak numbers and HPLC retention times refer to HPLC trace in Figs. 4B, D, F. t_R: retention time; $[M-H]^-$: negatively charged molecular ion.

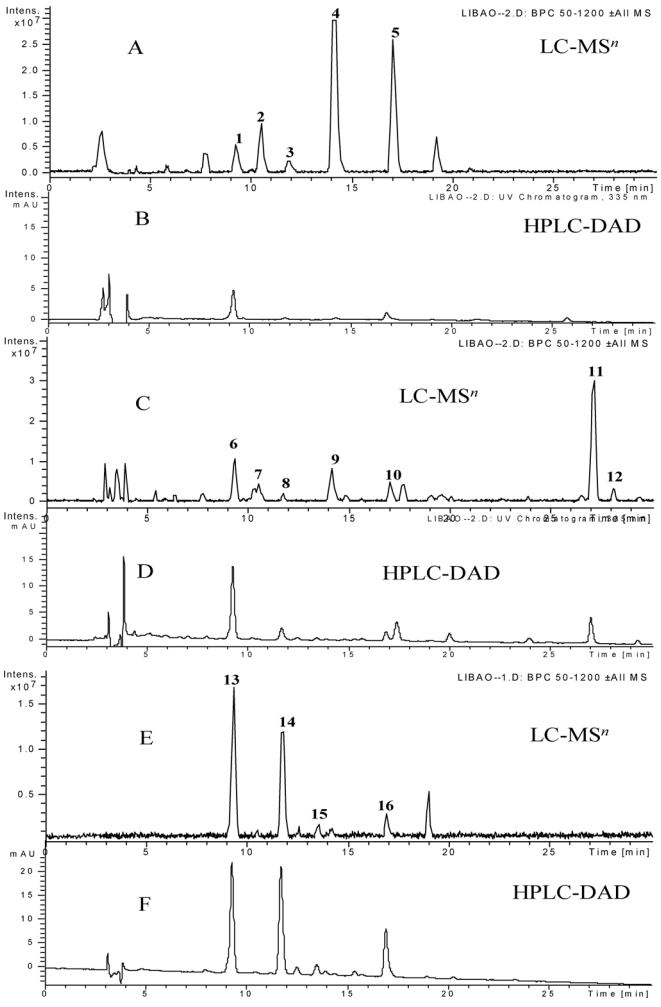

Fig. 3. Base Peak Chromatograms A (Serum), C (Urine), and E (Feces) and UV Chromatograms B (Serum), D (Urine), and F (Feces) of Typical Samples by HPLC-DAD-MS^n

flavonoid extract of jujube seeds, were characterized.

Metabolic Study in Plasma Sample Five peaks related to flavonoid extract of jujube seeds were detected in plasma sample by HPLC-DAD-MS^n. Co-chromatograms with the reference compounds were established. Peaks 1 and 5 were confirmed as original components, spinosin (**1**) and 6‴-feruloylspinosin (**5**). Peak 1 showed a $[M-H]^-$ m/z 607.1, with retention time at 9.1 min on HPLC. In the MS^2 spectrum of m/z 607.0, the fragment ion of 427.0 was generated by natural loss of a glucose unit, and the predominant product ion at m/z 307.0 was produced in MS^3 by successive loss of a fragment of 120 Da (4-ethynylphenol), resulting from the cleavage of the flavonoid skeleton at position C-3 of spinosin, according to the Retro Diels Alder cleavage (RDA) fragmentation pathway of flavonoid in MS with ESI resource.[15,16] It may bring a daughter ion $[M-H]^-$ m/z 486.7 directly by loss of 120 Da (4-ethynylphenol) in MS^2 as well. Peak 5 with retention time at 17.1 min on HPLC, showed a $[M-H]^-$ ion of m/z 783.1. In its MS^2 spectrum, the fragment ion of m/z 607.0 was generated by natural loss of a feruloyl group. The predominant product ion at m/z 486.8 was formed by sequential loss of a fragment of 120 Da (4-ethynylphenol) at position C-3 of 6‴-feruloylspinosin in MS^3, and it may also degrade into a fragment with $[M-H]^-$ m/z 426.0 by loss of a feruloyl group and a glucose unit at position C-3″ of 6‴-feruloylspinosin, successively, in MS^3.

With a retention time at 10.4 min on HPLC, peak 2 generated a $[M-H]^-$ at m/z 623.3 in MS spectrum and a $[M-H]^-$ at m/z 563.3 ($[M-H]^- - 60$, loss of CH_3COOH) in MS^2. According to the MS spectrum and references,[3,14—16] peak 2 was presumed as chalcone glycoside and was detected only in plasma. Peak 3 presenting at 11.9 min on HPLC created a $[M-H]^-$ at m/z values 445.1. Collision-induced dissociation (CID) of this compound produced an ion of m/z 324.8 in MS^2, which resulted from the neutral loss of 4-ethynylphenol (120 Da). It was easily identified as swertisin (**3**) derived from spinosin ($[M-H]^-$ m/z 607.1) by natural loss of a glucosyl unit (162 Da), and confirmed by the data of HPLC-DAD-MS^2 and reference.[3,14]

Peak 4, another secondary metabolite of 6‴-feruloylspinosin in plasma, produced a $[M-H]^-$ at m/z 741.3. Its MS^2 and MS^3 spectra gave ions at m/z 681.4 and 663.3 in negative mode, suggesting sequential losses of CH_3COOH (60 Da) and H_2O (18 Da) residues. It was tentatively identified as phenantithetical-furane glycoside (**4**), according to the

UV spectrum and molecular ion peak in MS^3 spectra, which acts as one of the major 6‴-feruloylspinosin metabolites in plasma.

Metabolic Study in Urine Sample Seven relevant peaks of flavonoid extract of jujube seeds were found in urine samples by LC-MS^n. Peaks 6, 8, and 9 in urine sample had got similar retention times on HPLC, $[M-H]^-$, and MSD Trap fragment ions (*m*/*z*) (listed in Table 1) to those in plasma samples, under the same LC-MS^n condition. These compounds were tentatively identified as compounds (**1**), (**3**), and (**4**), respectively.

With the extremital glucosyl group aldehydic-acidized by the enzymes *in vivo*, peak 7, appearing at 9.8 min on HPLC, had a $[M-H]^-$ at *m*/*z* 579.1 and yielded a major ion at *m*/*z* 403.0 ($[M-H]^- - 176$ Da, loss of a glucuronic acid unit) in MS^2 and a MS^3 fragment $[M-H]^-$ at *m*/*z* 385.0 ($[M-H]^- - 176$ Da − 18 Da, loss of a glucuronic acid unit and one molecule of H_2O). Therefore peak 7 was presumed as phenantithetical-furane glycoside (**6**), which acts as one of the major spinosin metabolites in urine.

Similarly, peaks 10 and 11 metabolites were derived from spinosin, followed by the glucuronidation of extremital glucosyl group *in vivo*. Peak 10 with a retention time at 16.9 min on HPLC, yielded MS fragments at *m*/*z* 477.2 and $[M-H]^-$ *m*/*z* 300.9 ($[M-H]^- - 176$ Da, loss of a glucuronic acid unit) in MS^2. This compound could be confirmed as phenolic glucoside (**7**), for the reason of construction of flavone-*C*-glucoside[3,14] and metabolic pattern of flavonoid.[9,15,16] Peak 11 had a retention time at 27.1 min on HPLC with the highest level in urine sample and generated a $[M-H]^-$ at *m*/*z* 460.9 in MS^1. Its MS^2 spectrum displayed a $[M-H]^-$ at *m*/*z* 284.9 ($[M-H]^- - 176$ Da, loss of a glucuronic acid unit). On the basis of the construction of flavone-*C*-glucoside[3,14] and metabolic pattern of flavonoid,[9,15,16] this compound was tentatively identified as phenolic glycoside (**8**). For peak 12 with HPLC retention time at 28.8 min, it exhibited a $[M-H]^-$ at *m*/*z* 413.1 and yielded MS^2 fragments at *m*/*z* 201.2 ($[M-H]^- - 176$ Da − 36 Da, losses of a glucuronic acid unit and two molecules of H_2O) for its glucosyl group glucuronidated by the enzymes *in vivo*. As a result, peak 12 could be concluded as phenantithetical-furane glycoside (**9**), according to the construction of flavone-*C*-glucoside[3,14] and metabolic pattern of flavonoid.[9,15,16]

Metabolic Study in Feces Sample Four relevant peaks of flavonoid extract of jujube seeds were identified in feces samples by LC-MS^n. Peaks 13, 14, and 16 in feces samples possessed similar retention times on HPLC, $[M-H]^-$, and MSD Trap fragments ions (*m*/*z*) (listed in Table 1) to those in plasma samples, under the same LC-MS^n condition. Peaks 13, 14, and 16 were therefore presumed as compounds (**1**), (**3**), and (**5**), respectively.

With a remarkable level in feces samples, peak 15 performed at 13.6 min on HPLC and generated a $[M-H]^-$ *m*/*z* 755.0 on MS, which yielded MS^2 fragment at *m*/*z* 579.1 ($[M-H]^- - 176$ Da, loss of feruloyl moiety) and $[M-H]^-$ *m*/*z* 459.0 ($[M-H]^- - 176$ Da − 120 Da, losses of feruloyl moiety and 4-ethynylphenol) in MS^3. Peak 15 was tentatively identified as phenantithetical-furane feruloyl-glycoside (**10**).

Elucidation of the Potential Flavonoid Metabolic Pathways in Rat Figure 4 shows the structural skeleton of flavonoids and their potential metabolic pathways. The parent compound (6‴-feruloylspinosin) was found in both plasma and feces samples but not in urine. It was supposed that 6‴-feruloylspinosin was deferuloylized into spinosin, even deglucosed into swertisin or other metabolites easily *in vivo*, and produces effects with its secondary metabolites and/or influence the endogenous ingredients.[2—5,14] Since spinosin was found as a significant component in rat plasma, urine, and feces samples, it could be tentatively concluded that spinosin could perform bioactivity not only with its original form but also with its transformations.[2—7,14] To our excitement, swertisin, which was deglucosed from spinosin and/or 6‴-feruloylspinosin by hepatic metabolism or enteral germs, was detected in all kinds of bio-samples and in particular with a larger amount in feces for the first report. This compound, reaching the systemic circulation, is therefore correlated to drug bioactivities[2—7,14] and suggests that TCMs have characteristics of transformation *in vivo*. The extremital

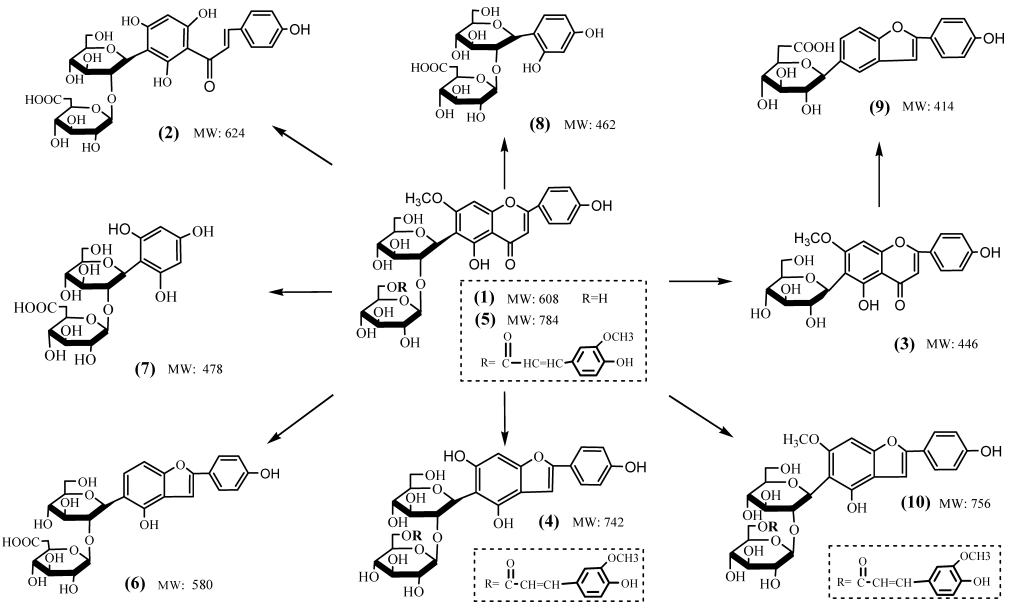

Fig. 4. Structural Skeleton of Flavonoids and Their Potential Metabolic Pathways

glucosyl groups in most of the metabolites could be glucuronidated by the enzymes *in vivo*. All of them were flavonoid metabolites from phase I metabolism. The results are quite consistent with previous publications in which flavonoids are reported to be extensively degraded by the intestinal microflora or enzymes *in vivo*.[6—11,17—19)]

Conclusions

An HPLC-DAD-MS^n investigation on chemical and metabolic information of flavonoid extract of jujube seeds was carried out for the first time. The metabolites of flavonoid extract of jujube seeds in rat plasma, urine, and feces were identified by comparing their chromatographic retention time, changes in observed mass (Δm), and ion-trap MS spectra with those of the parent compounds. Five metabolic components in plasma, seven in urine, and four in feces were characterized. The results indicate that eight secondary metabolites are degraded from spinosin and/or 6‴-feruloylspinosin through various transforming approaches, such as deglucosing hydroxylation, deferuloylizing glucuronidation, ring-opening of the flavone skeleton, and so forth. This is of great importance in understanding the molecular mechanism of action and clinical effects of flavonoid extract of jujube seeds, and facilitating further quantitative studies as well.

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