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Metabolite Identification of Myricetin in Rats Using HPLC Coupled with ESI-MS

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Abstract Myricetin, a naturally occurring flavonol, shows multifarious pharmacological activities, e.g., antidiabetic, antioxidant, anti-inflammatory, antitumor, and liver protection effects. In order to obtain an understanding of the myricetin's metabolism in vivo, a rapid and sensitive method by high-performance liquid chromatography coupled with electrospray-ionization mass spectrometry (HPLC-MS^n) techniques was employed to investigate the biotransformation in rats after oral administration of myricetin. Recognition and structural exposition of the metabolites were operated by comparing the changes in molecular mass (ΔM) and MS^n spectra with the parent drug. As a result, the parent compound and seven metabolites were found in rat plasma, urine, and feces. In addition, besides 3,5-dihydroxyphenylacetic acid (**M1**) and 3,4,5-trihydroxyphenylacetic acid (**M2**), five other compounds were first discovered in the metabolite research of myricetin. These results indicated that, besides ring-fission, there were methylate (**M3**, **M4**, **M5**) and glucuronide (**M6**, **M7**) biotransformations of myricetin occurring in vivo.

Keywords HPLC-ESI-MS^n · Myricetin · Metabolites

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Introduction

Myricetin, a flavanoid compound with hydroxyl substitutions at the 3, 5, 7, 3′, 4′, and 5′ positions, is a major active ingredient of a Chinese traditional herb medicine *Portulaca oleracea* L., also named purslane or Machixian in Chinese. In our earlier research, we did a series of pharmacological experiments concerning *P. oleracea* and its major chemical compositions. We found that both *P. oleracea* and myricetin had significant anti-inflammatory activity and beneficial effects in the treatment of urinary tract infection. In fact, myricetin in nature is widespread among plants, including tea, berries, fruits, vegetables, and medicinal herbs [1, 2]. The present pharmacological studies have shown that myricetin possesses antioxidative, antiproliferative, and anticarcinogenic effects as major biological features [3]. Myricetin also possesses neuroprotective effects on the Parkinson models both in vivo and in vitro through its anti-oxidation and anti-apoptosis activity [4]. In addition, some preliminary work has shown that myricetin may be of benefit as an antithrombotic agent [5]. And Yan et al. [6] have demonstrated that myricetin possesses potent analgesic activity. Relative to the comprehensive study on its pharmacological activity, there is no recent report describing research on the metabolites of myricetin. Thus, in the present research, we use myricetin as the in vivo metabolic process for researching the subject to help discover the pharmacological action mechanisms.

Meanwhile, the metabolism study of drugs has become important in judging whether, at early stages of drug candidate screening, it is worth further development. And it allows a better understanding of the medical or toxic effects of the drug candidates. Drug metabolism is divided into phase I and phase II biotransformation methods. Phase I is the chemical modification, such as oxidation, reduction,

and hydroxylation. Phase II comprises the conjugations, such as glucuronidation and methylation of specific functional groups. Metabolites of drugs can be in three forms in vivo, that is deactivating, toxic or active compounds, from which we only want to seek out the biologically active metabolites [7]. Therefore, a thorough understanding of drug metabolites in vivo is very important.

As regards the means of detection, liquid chromatography coupled with mass spectrometry has been proven to be a powerful tool for the identification of substances in biofluids [8, 9]. Because of its high sensitivity and strong ability of structural exposition, it plays an important role in composition analysis of drug metabolites in the biological body [10, 11]. It is worth mentioning that using MS^n techniques we can acquire rich structural information from analyses of the interesting chemical compounds [12]. As we know, the metabolites can keep the basic structural features of the parent drug after biotransformation, so the product ions of parent drugz associated with their basic structural features obtained by MS^n techniques can be used as a substructural template of metabolite analysis even when there is no available standard for each metabolite [10].

In this research, we developed a more efficient method utilizing solid phase extraction (SPE) and HPLC-ESI-MS/MS techniques for the detection of potentially active metabolites in rat biological samples. Eventually, the parent compound and seven metabolites were identified in rat urine, plasma, and feces. This result will help to better understand the metabolic processes of myricetin in vivo, and the pharmacological action mechanisms of these metabolites.

Experimental Part

Materials and Reagents

Myricetin was purchased from Zelang Medical Technology (Nanjing, China), and the structures were identified on the basis of their spectral data (ESI-MS, ^1H NMR, ^{13}C NMR) by comparing with literature values [13]. Its purities were >95 % determined by HPLC-UV analysis. Before administration, myricetin was dissolved in a 0.5 % carboxy-methyl cellulose sodium salt (CMC-Na) aqueous solution. The voucher specimens of myricetin were deposited at Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China.

HPLC grade acetonitrile was purchased from Dikma (USA). Water was deionized and double distilled. All other analytical grade reagents, together with analytical grade formic acid, were from Sinopharm Chemical Reagent (Shanghai, China).

Instrument and Conditions

$LC-MS^n$ experiments were performed on the 6,300 Series Ion Trap LC/MS (Agilent Technology, Palo Alto, CA, USA). The hardware included an Agilent 1,200 Series LC, the ion trap mass spectrometer, and the data system. The software (v.6.1) included the trap control program for trap control, data acquisition, data analysis, quantitative analysis, and the Agilent ChemStation program for sample automation and LC control. A high-speed bench-top centrifuge (Sorvall ST16; Thermo Fisher Scientific, Germany) was used to separate the biological samples, which were extracted by a C18 solid phase extraction (SPE) cartridge (1 mL 100 mg^{-1}; Supelco, USA).

The Agilent 1200 HPLC was equipped with a reversed-phase column [Agilent ZORBAX Eclipse Plus C_{18} (2.1 × 150 mm, 3.5 μm)] and including an EasyGuard KitC_{18} (4 × 2 mm) guard column. The column was maintained at 25 °C. Detection wavelengths were set at 254 nm. The flow rate was 0.3 mL min^{-1}. A gradient elution of 0.5 % aqueous formic acid (A) and acetonitrile (B) was used as 5–10 % B at 0–10 min, 10–70 % B at 10–25 min, 70–100 % B at 25–30 min, 100 % B at 30–35 min. The mass spectra were recorded in negative modes, drying gas flow rate 10 L min^{-1}, drying gas temperature 35 °C, nebulizer 35 psig., capillary voltage 4,000 V, fragmentor 100 V, mass range 50–1,500 m/z. The HPLC system was controlled by an HPLC-MSD ChemStation software system.

Animals

All animal treatments were strictly in accordance with protocols approved by the review committee of animal care and use at the Shanghai Institute of Materia Medica (Shanghai, China). Twenty-four male Sprague–Dawley rats (210 ± 10 g; Shanghai SLAC Laboratory Animal, Shanghai, China) were acclimatized to the facilities for 1 week prior to experiments. These animals were randomly divided into four groups: a dosed urine collection group (group A, $n = 6$); a dosed plasma collection group (group B, $n = 6$); a dosed feces collection group (group C, $n = 6$); and a control group (group D, $n = 6$). All animals were fasted (but received water) for 12 h prior administration of the corresponding drug which have a single dose of 300 mg kg^{-1} body weight by oral gavage.

Sample Preparation

Samples of urine and feces were collected and combined, respectively, during 0–24 h post-administration. The blood samples were collected in a heparine-treated centrifuge tube (5 mL) at 1, 2, 4, 6, 8, and 10 h from the abdominal

aorta of the rats after administration. Then, the blood samples were combined and shaken. Finally, the samples were centrifuged at 10,000g for 10 min to obtain the plasma samples. All the samples were stored at −80 °C for future analysis. All the preparation procedures of plasma, urine, and feces samples were according to Liu et al. [14] and Chen et al. [15]. Then, 600-μL samples were loaded on a SPE column (1 mL capacity, 100 mg ODS; Supelco) which was subsequently preconditioned with 2 mL methanol and 2 mL of Milli-Q water (18 M) water. The cartridge was then washed with 2 mL of Milli-Q water (18 M) water. And the analytes were eluted with 1 mL methanol. The eluent was dried under nitrogen gas over a water bath of 37 °C. The residues were dissolved in 200 μL methanol, and centrifuged twice at 12,000g for 10 min. Then, 20 μL of the supernatants was injected into the HPLC-MS^n for analysis. Blank samples as control were prepared with the same method as drug containing samples.

Results and Discussion

In Vivo Metabolism Study

The in vivo metabolism data has been obtained by the full scan mass spectra of the compounds from dosed rat plasma, urine, and feces comparing with those of blank rat biological samples to explore the possible metabolites. Recognition and structural exposition of the metabolites were operated by comparing their changes in molecular mass (ΔM), retention times, and MS^n spectra with the parent drug.

The first step is to study the chromatography and mass spectrometry characteristics of the parent drug. The analysis of fragmentation patterns of the parent drug and conclusion of the rules have been used for subsequently elucidation of the structurally related metabolites. All these data, shown in Fig. 1, provide a structural 'template' for interpreting the structures of the metabolites. Thus, the parent compound and seven metabolites were detected in rat plasma, urine, and feces by using the negative ion mass spectra mode. Their MS^n data along with the retention times of myricetin and its metabolites are exhibited in Table 1. Meanwhile, in MS full scan, the extracted ion chromatographic peaks of these metabolites are shown in Fig. 2. All in all, our results indicated that methylation, glucuronidation, and ring-fission would be the major in vivo metabolic pathways of myricetin.

Identification of the Compounds Detected in Rat Biological Samples after Oral Administration of Myricetin

The Parent compound (M0) The retention time of **M0** was 10.10 min. By comparing the chromatographic behavior with published data, its MS^n spectra of the molecular ion at *m/z* 317 were consistent with those of the authentic standard myricetin [16]. In addition, the product ions *m/z* 179 (1,2 A$^-$) and 151 (1,3 A$^-$) are typical retro Diels–Alder (RDA) fragmentation of flavon-3-ols having the dihydroxylated A ring and trihydroxylated B ring [17]. That means that these two fragments together with the molecular ion *m/z* 317 are quite characteristic of myricetin. Therefore, **M0** has been confirmed as the parent drug myricetin that could be found in urine and feces.

Fig. 1 The MS/MS spectra of M0 couple with the putative fragmentation pathway of myricetin aglycone; annotation of fragments follows the nomenclature proposed [17]
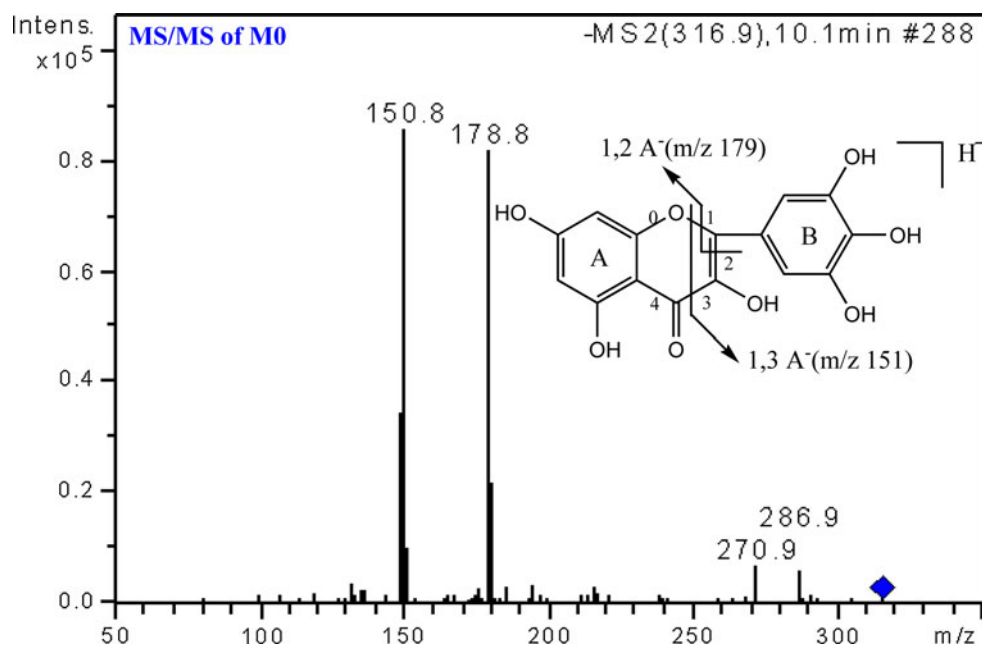
Table 1 Characterization of the compounds detected in rat after the oral administration of myricetin by HPLC–ESI-MS^n

No.	t_R (min)	(M–H) (m/z)	Detection in	HPLC/ESI-MS^n (m/z)
M0	10.10	316.9	Urine; feces	MS^2 [316.9]: 178.8, 150.8
M1	5.90	167.0	Urine; feces	MS^2 [167.0]: 122.5; MS^3 [122.5]: 108.4
M2	4.30	183.1	Feces	MS^2 [183.1]: 138.6; MS^3 [138.6]: 124.5
M3	13.40	331.3	Plasma; urine; feces	MS^2 [331.3]: 316.0; MS^3 [316.0]: 178.8, 150.8
M4	14.40	345.2	Urine	MS^2 [345.2]: 330.3; MS^3 [330.3]: 315.3
M5	21.70	373.0	Plasma; urine; feces	MS^2 [373.0]: 357.8; MS^3 [357.8]: 342.9
M6	8.70	492.8	Urine	MS^2 [492.8]: 316.8; MS^3 [316.8]: 178.9, 151.0
M7	6.40	668.9	Urine	MS^2 [668.9]: 493.0; MS^3 [493.0]: 316.9
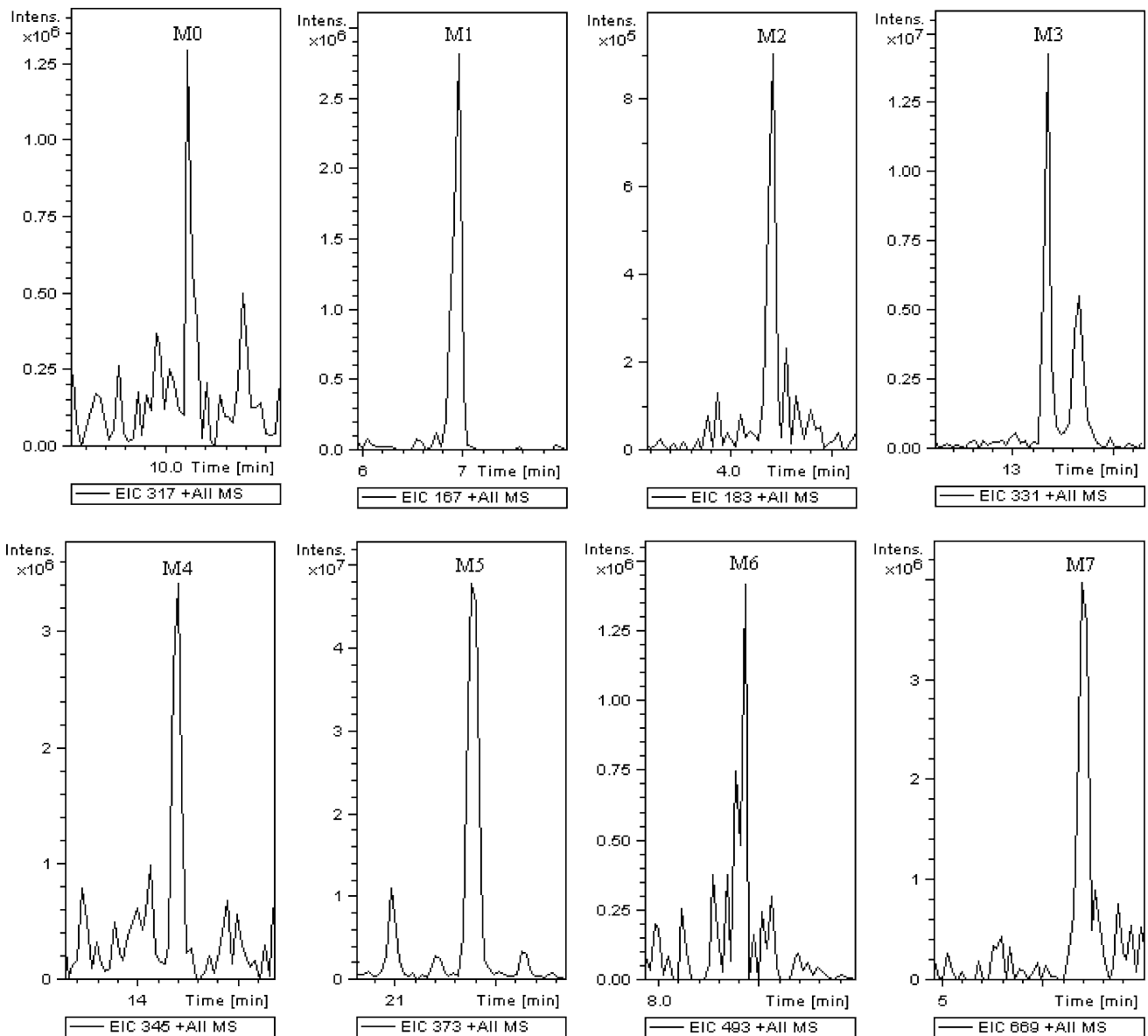
Fig. 2 Extracted ion chromatography from MS full scan of myricetin and its metabolites

The ring-fission product of myricetin (M1, M2) **M1** was observed as an [M–H]$^-$ ion at m/z 167 in the negative electrospray mass spectrum with a retention time of 5.90 min. Additionally, two product ions at m/z 122 [M–CHO$_2$]$^-$ and 108 [M–CHO$_2$–CH$_2$]$^-$ were detected in the collision-induced dissociation (CID) spectrum of the ion at m/z 167. By comparing the chromatographic behavior with published data, the molecular ion m/z 167 of **M1** corresponds to the reported metabolite [18, 19] of myricetin. And its reported fragmentation patterns [20] with a 44 amu loss, yielding a MS/MS ion at m/z 123, have been detected in the **M1** mass spectra. Therefore, **M1** was tentatively identified as 3,5-dihydroxyphenylacetic acid that could be found in urine and feces.

M2 was observed as a deprotonated molecule [M–H]$^-$ ion at m/z 183 with a retention time of 4.30 min. Additionally, two product ions at m/z 138 [M–CHO$_2$]$^-$ and 124 [M–CHO$_2$–CH$_2$]$^-$ were detected in the collision-induced dissociation (CID) spectrum of the ion at m/z 183. Similar to the resolution process with **M1**, the molecular ion m/z 183 of **M2** corresponds to the reported metabolite [18, 19] of myricetin. And its reported fragmentation patterns [20] have been detected in the **M2** mass spectra. Therefore, **M2** was tentatively identified as 3,4,5-trihydroxyphenylacetic acid that could only be found in feces.

The methylation product of myricetin (M3, M4, M5) **M3** was observed as an [M–H]$^-$ ion at m/z 331 in the negative electrospray mass spectrum with a retention time of 13.4 min. The mass spectra of **M3** (m/z 331) were 14 Da (CH$_2$) more than **M0**, and its product ions at m/z 179 and 151 were similar to **M0**, respectively. According to the metabolism rule of phase II in vivo, the methylation reactions may occur at the skeleton of myricetin. Hence, **M3** was presumed to be the methylate product of myricetin. Similarly, **M4** (m/z 345) and **M5** (m/z 373) were 28 Da (2CH$_2$) and 56 Da (4CH$_2$) more than that of **M0**, indicating that these two compounds should be dimethyl and tetramethyl metabolites of the parent drug. Additionally, **M3** and **M5** could be found in plasma, urine, and feces, while **M4** could only be found in urine.

The glucuronidation product of myricetin (M6, M7) The MS^2 spectrum of m/z 493 (**M6**) gave an abundant daughter ion at m/z 317 by neutral loss of 176 Da. The MS^3 spectrum of m/z [317]: 179, 151 was the same as the MS^2 spectrum of myricetin. Based on the metabolism rule of phase II in vivo and the above MS^n spectrum data, **M6** should be the monoglucuronide product of myricetin which could only be found in urine. Similarly, **M7** (m/z 669) was 352 Da higher than that of myricetin, implicating that two
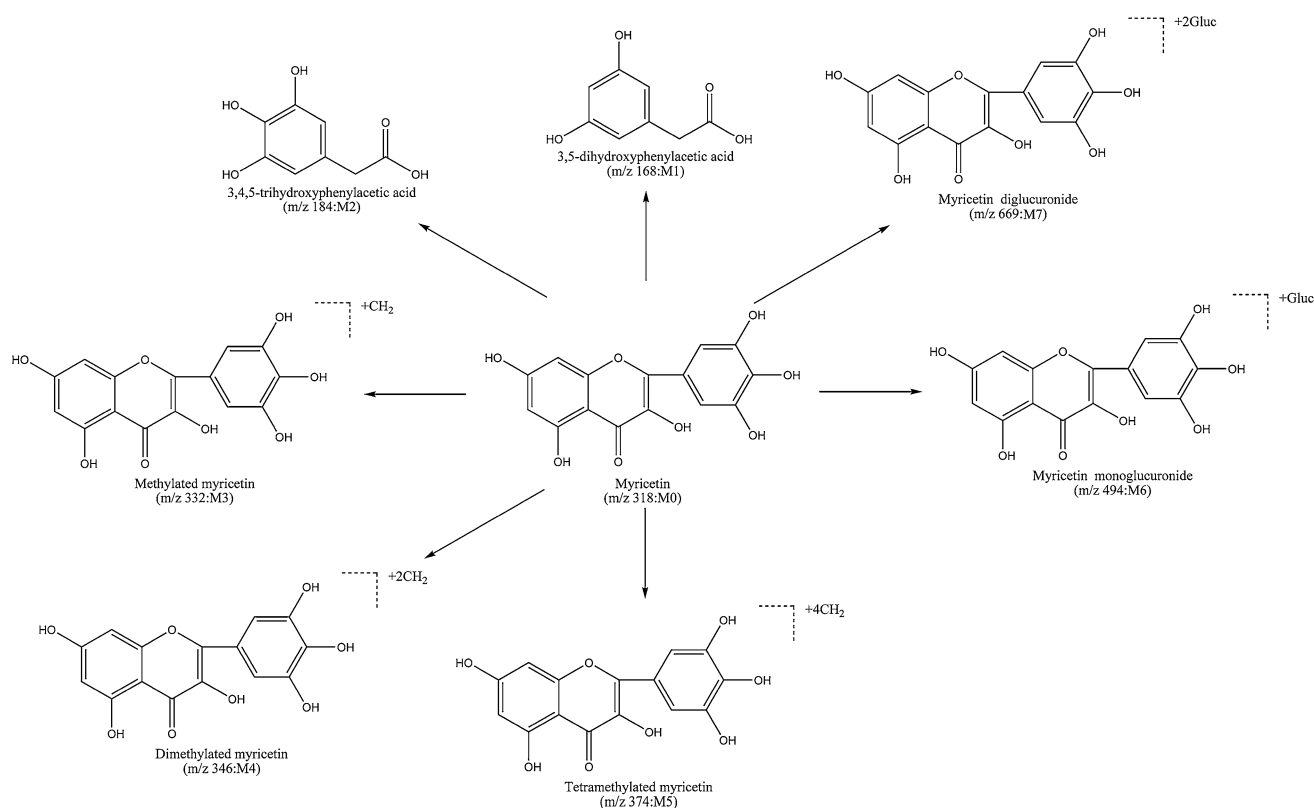

Fig. 3 Proposed major metabolic pathways of myricetin in rats

glucuronyl units were attached on the myricetin skeleton to form the diglucuronide conjugate of myricetin. Therefore, **M7** was tentatively proposed to be the diglucuronide product of myricetin which could only be found in urine.

Conclusion

After oral administration of myricetin, the rat urine, plasma, and feces samples have been received. By combined HPLC-ESI-MS^n techniques, a total of eight compounds were detected in these rat biological samples. Among them, seven metabolites were identified. In rat urine sample, the parent compound and seven metabolites have been found, while two and four metabolites were characterized in rat plasma and feces, respectively. In addition, the in vivo metabolic pathways of myricetin in the present study are shown in Fig. 3, from which it can be seen that the phase I metabolites (**M1**, **M2**) were the ring-fission products of myricetin, and the phase II metabolites were the methylate (**M3**, **M4**, **M5**), and glucuronide (**M6**, **M7**) products of myricetin. All in all, these results give us important information for future pharmacological research and subsequent metabolite action mechanism research on myricetin. In the meantime, our work for structural confirmation and pharmacological activity testing of these metabolites is in progress.

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