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ORIGINAL ARTICLE

Ultra-performance Liquid Chromatography/Quadrupole Time-of-flight Mass Spectrometry Analysis of *In vitro* Metabolites of Lignans from Fructus Forsythiae by Human Fecal Flora

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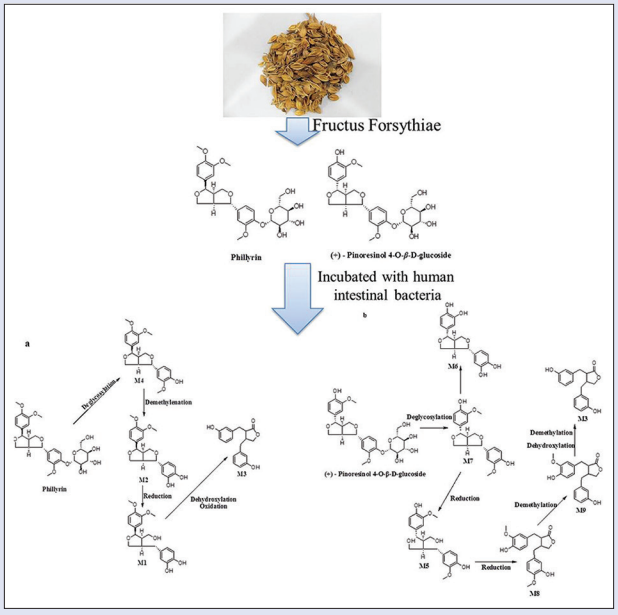
ABSTRACT

Background: Phillyrin and (+)-pinoresinol 4-O-β-D-glucoside are the major active furofuran-type lignans in Fructus Forsythiae. The metabolic routes and metabolites of these two lignans are not well understood yet. **Objective:** In this study, we attempted to identify the human-intestine bacterial metabolites of lignans from Fructus Forsythiae. **Materials and Methods:** Two natural compounds, phillyrin and (+)-pinoresinol 4-O-β-D-glucoside were incubated with human fecal microflora in an anaerobic incubator for 72 h and the metabolites with highly sensitive ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) were analyzed. **Results:** As a result, nine metabolites were determined using a Syncronis™ C_{18} column (particle size 1.7 mm) in a gradient elution system. These metabolites were then identified according to the mass fragmental mechanism, MS/MS fragment ions, and previous publications. The results of this study indicated that the major metabolites of furofuran-type lignans are through the processes of hydrolysis, demethylation, reduction, dehydroxylation, and oxidation. **Conclusions:** Lignans can be metabolized by intestinal microbiota and the intestinal bacteria play a critical role in the metabolism of components administered orally.

Key words: Furofuran lignan, human fecal flora, metabolism, ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry

SUMMARY

- Ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry was used to analyze the metabolic process of phillyrin and (+)-pinoresinol 4-O-β-D-glucoside in human fecal flora
- Nine metabolites were identified by MS/MS fragmentation
- The metabolic pathways of phillyrin and (+)-pinoresinol 4-O-β-D-glucoside induced by human intestinal bacteria were also investigated.



Abbreviations used: GAM: General anaerobic medium; UPLC/Q-TOF-MS: Ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry; ESI: Negative ion electrospray; RT: Retention time; TCM: Traditional Chinese Medicine.

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INTRODUCTION

Fructus Forsythiae ("Lianqiao" in Chinese) is a kind of well-known traditional Chinese medicine (TCM). It is the fruit of *Forsythia suspense* (Thunb.) Vahl (Oleaceae) and widely used for the treatment of infections such as acute nephritis, pharyngitis, pyrexia erysipelas, ulcer, tonsillitis, and gonorrhea.[1] Phytochemical investigations have elucidated that the bioactive components of Fructus Forsythiae are lignans, phenylethanoid glycosides, and flavonols.[2] Lignans in Fructus Forsythiae include phillyrin, matairesinol, (+)-pinoresinol 4-O-β-D-glucoside, matairesinol-4′-O-glucoside, and arctigenin.[3] As one of the main active components of Fructus Forsythiae, phillyrin is thus selected as the quality control index of "Lianqiao," which is shown to have the effects of antioxidation and blood–fat repression.[4,5] (+)-pinoresinol 4-O-β-D-glucoside is another one of the

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most abundant lignan glucosides in Fructus Forsythiae with antioxidant, blood pressure-reducing, and cyclic adenosine monophosphate (cAMP) phosphodiesterase inhibitory effects.[6] The herbal medicines like "Lianqiao" are orally administered, their active components are then brought into contact with intestinal microflora, mostly composed of anaerobes, in the alimentary tract.[7] Studies have shown that lignans can be metabolized by intestinal microbiota and such transformation can subsequently affect the absorption and biological activity.[8-10] Therefore, the intestinal bacteria may play a critical role in the metabolism of components administered orally or excreted into bile.[11] However, the metabolic routes and metabolites of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside are not well understood yet.

In our previous study, we incubated water extract of Fructus Forsythiae with human intestinal bacteria, some prototype compounds metabolized to their relative metabolites during the incubation time. We isolated some of the main prototype compounds from Fructus Forsythiae. Two of them were identified as phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside according to the authentic standard. Profiling the metabolites of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside is crucial to further understand their pharmacological mechanisms of actions. However, little is known about the metabolic reactions of these two lignans following ingestion in human. Therefore, we incubated the lignans anaerobically with human fecal microflora and analyzed the metabolites by ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UPLC/Q-TOF-MS) with automated data analysis (MetaboLynx™). To our knowledge, this is the first study that identifies the human-intestine bacterial metabolites of lignans from Fructus Forsythiae. The results probably provided helpful information for further *in vivo* metabolism and active mechanism research on furofuran-type lignans [Figure 1].

Experiment

Reagents and Materials

Anaerobic glove box was purchased from Plas-Labs Lansing Co., (MI. USA). General anaerobic medium broth (GAM broth) was purchased from Shanghai Kayon Biological Technology Co., Ltd (Shanghai, China). Phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside (purity >98%) were purchased from Shanghai Winherb Medical Technology Co., Ltd (Shanghai, China). HPLC-grade acetonitrile and methanol were purchased from Merck. Ultrapure water was prepared with Milli-Q water purification system (Millipore). All other chemicals and reagents were of analytical grade.

Standard solutions

The standard solutions of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside were individually and accurately weighed and dissolved in water to reach a final concentration of 5.0 mg/mL. The solution was stored in refrigerator at 4°C before analysis.

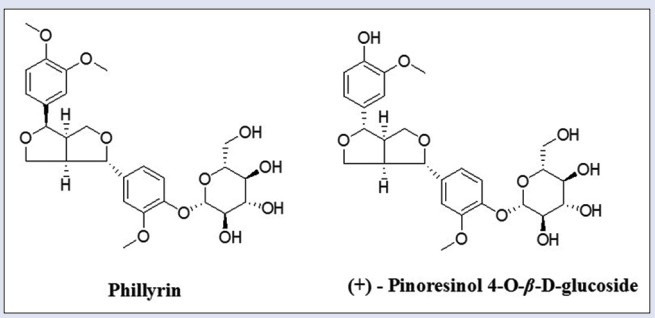

Figure 1: Chemical structures of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside

Preparation of fecal suspensions

Fresh fecal samples were obtained from ten healthy volunteers (five males, five females, 22 to 50 years of age), who gave informed written consent to the study protocol. The volunteers had no history of gastrointestinal disorders and had not taken antibiotics for at least 3 months prior to the study. The fresh fecal mixture samples were immediately homogenized with 25 volumes of GAM broth. The sediments were removed by filtration through three pieces of gauze, and the filtrate was used as human intestinal bacteria mixture. The fecal suspension was incubated at 37°C in an anaerobic incubator in which the air had been replaced with a gas mixture (H_2 5%, CO_2 10%, and N_2 85%).

Sample preparation

The standard solution was added to human fecal suspension containing 0.1 mM phillyrin or (+)-pinoresinol 4-O-*β*-D-glucoside, and the mixture was incubated at 37°C in an anaerobic incubator for 72 h. The cultured mixture was sampled when incubated at 24, 48, and 72 h and extracted with water-saturated n-butanol. The extract was evaporated, and then the residue was dissolved in methanol (0.5 ml) and analyzed with ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS).

Ultra-performance liquid chromatography

Analysis was performed on a commercially available ACQUITY UPLC system with a conditioned autosampler at 4°C, connected online to an ACQUITY TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). All data were collected using MassLynx software. The separation was performed on a Syncronis C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm; Thermo, USA) with the column temperature set at 35°C. The mobile phase consisted of (A) ultra-pure water containing 0.1% formic acid and (B) acetonitrile, and the gradient elution conditions were 0–1.5 min, 5%–10% B; 1.5–8 min, 10%–25% B; 8–13 min, 25%–45% B; 13–18 min, 45%–100% B; 18–21 min, 100% B; and 21–24 min, 100%–5% B. The flow rate was 0.4 ml/min and the sample injection volume was 5 μl.

Waters Synapt™ mass spectrometer (Waters Corp., Milford, MA, USA) was used for the mass spectrometry analysis. Negative ion electrospray (ESI) mode was adopted to perform the ionization with the capillary value of 2.0 kV. The cone gas was set to 50 L/h, and the source temperature was set at 120°C. The desolvation gas was set to 700 L/h at a temperature of 350°C. The scan range was selected from m/z 50 to 1000. The data acquisition rate was set to 0.28 s. Data were centroided during acquisition using an external reference (LockSpray™), which comprised of 2 μg/ml leucine-enkephalin (J and K Chemical Ltd.), and were continuously infused into the ESI source at a rate of 400 μl/min via a syringe pump. Data were processed using MassLynx™ 4.1 software (Waters Corp., Milford, MA, USA).

RESULTS AND DISCUSSION

Ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry optimization

To achieve better resolution, greater baseline stability, and higher ionization efficiency of the lignans and major metabolites, the chromatographic conditions were optimized. A solvent system consisting of 0.1% formic acid in water/acetonitrile was selected as the mobile phase for gradient elution. According to the results from optimization experiments, negative ion mode was employed and the collision energy was set to 6–50 V.

Analysis of metabolites of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside

Identification of bioactive compounds in TCM is one of the important drug discovery paths, especially for the ones that can be successfully absorbed and utilized after oral administration. Thus, the purpose of using UPLC-Q-TOF/MS technique in this study was to investigate the metabolic pathways of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside. To eliminate the effects of impurity compounds that may elute together with the target compounds in the GAM broth, we used MetaboLynx™ software to identify the metabolites by comparing the retention time, MS spectra, and peak area of the peaks present in the analytes to that in the control samples. Compared with the blank sample that only contained GAM broth, metabolites were found. Representative typical chromatograms of the samples are shown in Figure 2. The constituents were well separated using the developed UPLC method. Table 1 shows the retention time, ion mass peaks in m/z, and fragment ions in the MS/MS stage of the metabolites. These metabolites were numbered M1–M4 for phillyrin and M5–M9 for (+)-pinoresinol 4-O-*β*-D-glucoside according to their retention time.

Table 1: Retention time, ion mass peaks and peak area of lignans, and their metabolites

Number	RT (min)	$[M-H]^-$	Ion mass peaks (m/z)	Metabolite name	Formula
M1	8.69	359.1509	359, 344, 329, 315	1,2-Benzenediol, 4-[(2S,3R,4R)-4-[(3,4-dimethoxyphenyl) methyl] tetrahydro-3-(hydroxymethyl)-2-furanyl]	$C_{20}H_{24}O_6$
M2	10.24	357.1336	357, 342, 327, 151	Lantibetin	$C_{20}H_{22}O_6$
M3	10.57	297.1140	297, 253, 121, 107	Enterolactone	$C_{18}H_{10}O_4$
M4	11.76	371.1506	371, 356,341, 151	Phillygenin	$C_{21}H_{24}O_6$
M5	7.77	361.1621	361, 346, 331, 313, 121	Secoisolariciresinol	$C_{20}H_{26}O_6$
M6	8.10	329.1388	329, 299, 175, 137	(-)-3,3'-Bisdemethyl-pinoresinol	$C_{18}H_{18}O_6$
M7	9.56	357.1324	357, 342, 327, 151	Pinoresinol	$C_{20}H_{22}O_6$
M8	10.30	357.1361	357, 342, 327, 313,175, 137	(-)-3-(3″-hydroxy-4″-methoxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl) butyrolactone	$C_{20}H_{22}O_6$
M9	10.42	327.1229	327, 312, 283, 161, 121	(-)-3-(3″-hydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl) butyrolactone	$C_{19}H_{20}O_5$

RT: Retention time

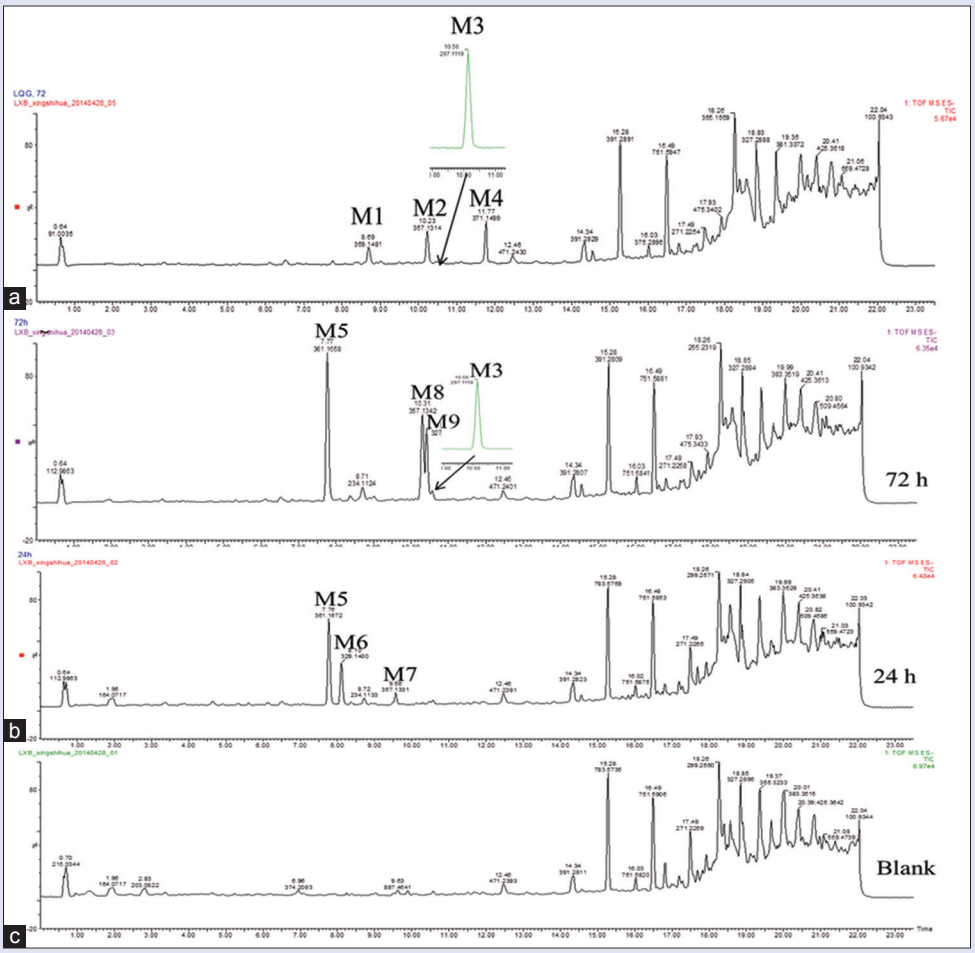

Figure 2: Total Ion Chromatography chromatograms of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside and their metabolites, (a) phillyrin, (b) (+)-pinoresinol 4-O-*β*-D-glucoside, and (c) the blank sample

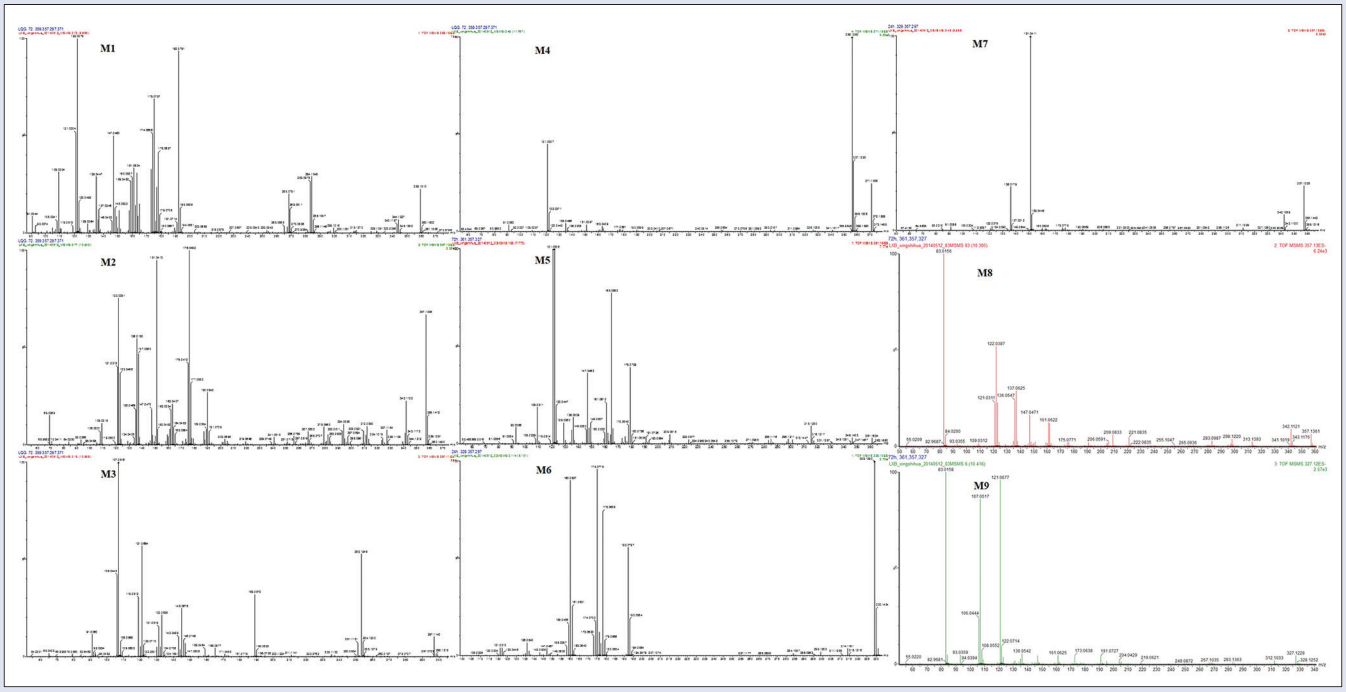

Figure 3: Ultra-performance liquid chromatography mass spectrometry/mass spectrometry spectra of M1–M9

Identification of the metabolites of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside

Metabolite M4

As illustrated in Figure 3, the negative electrospray mass spectrum of M4 showed a $[M-H]^-$ ion at m/z 371, which was generated due to deglycosylation from phillyrin. The fragment ion at m/z 357 was produced by the loss of a methyl group. The MS/MS spectrum of the deprotonated molecule obtained from M3 gave a product ion at m/z 341, which was obtained by the loss of CH_2O from m/z 371. Tetrahydrofuran cleavage generated the characteristic fragment ion at m/z 151. Then, m/z 151 was further fragmented to m/z 136 and m/z 121 by sequential loss of CH_3. Furthermore, the major metabolite M4 was confirmed based on the identical same retention time (11.77 min) with authentic standard and matching MS spectra. Therefore, M4 was identified unequivocally as phillygenin.

Metabolite M2

Metabolite M2 demonstrated a retention time of 10.23 min. MS/MS spectrum showed a protonated molecular ion $[M-H]^-$ at m/z 357 which was 14 Da lower than the protonated ion of M4, indicating the removal of one methyl group. The product ion at m/z 357 then yielded two fragments at m/z 342 and 327, respectively, indicating the successive loss of two methoxyl groups (15 Da). The prominent ion at m/z 151 was considered to be generated through the cleavage of the tetrahydrofuran ring, as reported in the literature.[12] Based on the above observations and the published data, M2 was tentatively characterized as lantibetin.[13]

Metabolite M1

Metabolite M1 was detected as deprotonated molecular ion $[M-H]^-$ at m/z 359 with a retention time of 8.69 min, which was 2 Da higher than the protonated ion of M2, indicating that M2 was either hydrogenated or cleaved through a single bond. Product ions at m/z 359, 344, and 329 were observed, which again suggests the consecutive loss of the 15 Da methoxyl group. The presence of a minor fragment m/z 315 implies that M1 could be a 2,3-dibenzyl butyrolactone-type lignan through the loss of H_2O and CO.[14] Given the findings from the previous report,[13] M1 was considered as 1,2-Benzenediol, 4-[(2S,3R,4R)-4-[(3,4-dimethoxyphenyl) methyl] tetrahydro-3-(hydroxymethyl)-2-furanyl].

Metabolite M3

Metabolite M3 exhibited an $[M-H]^-$ ion at m/z 297. It produced signals at m/z 297, 253, 121, and 107. M/z 297 first lost one CO_2 to form m/z 253, and m/z 253 was then further fragmented to m/z 121 and m/z 107 by dropping C_7H_7O and C_8H_9O, respectively [Figure 4]. Based on these results, M3 was identified as enterolactone. The identification of M3 was also confirmed by its identical retention time to the reference standard.

Metabolite M7

M7 was observed at m/z 357 and was also considered as a product through the loss of glucose as well as a characteristic fragmentation of furofuran lignans [Figure 3]. The MS/MS spectrum of M7 showed a product ion at m/z 327, which was generated from m/z 357 by loss of CH_2O. The MS/MS spectrum of the deprotonated molecule obtained from M3 gave a product ion at m/z 136. The loss of 192 Da can be explained by a heterolytic cleavage of the bond involving the benzoic carbon, which formed the dimethoxybenzyl product ion at m/z 136 [Figure 4]. Then, m/z 136 lost a CH_3 to form the product ion at m/z 121. Based on the above findings and characteristics of the standard sample, we concluded that M7 was pinoresinol.

Metabolite M5

Another major component M5 (t_R = 7.76 min) gave an $[M-H]^-$ ion at m/z 361, which was 4 Da higher than the protonated ion of M7. MS^n of m/z 361 yielded ions at m/z 346 and 331, corresponding to $[M-H-15]^-$ and $[M-H-30]^-$, suggesting the presence of two methoxyl groups. The product ion at m/z 313 was formed by the elimination of OH and following condensation from m/z 361 [Figure 4]. The

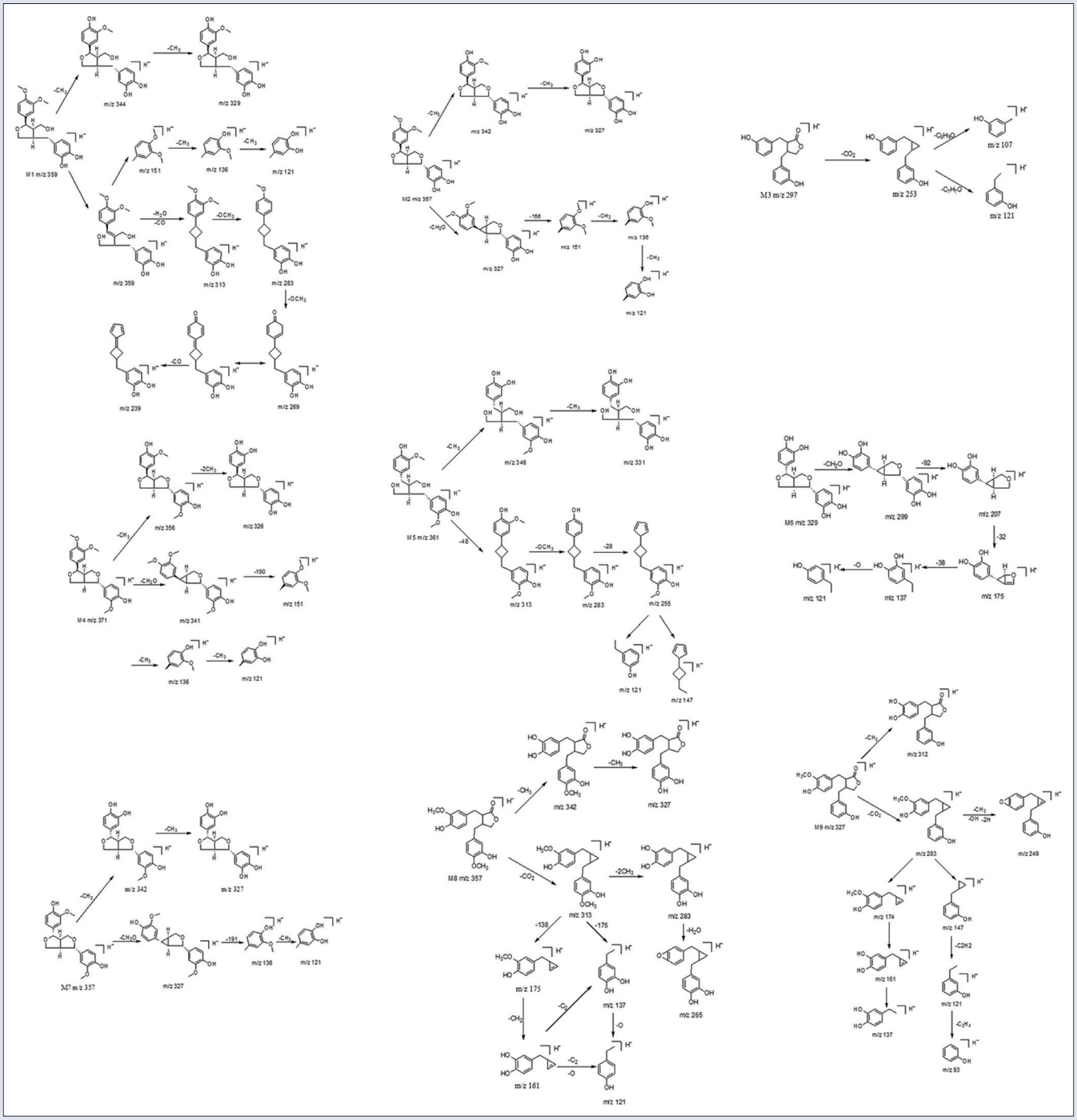

Figure 4: Proposed fragmentation pathways of M1–M9

characteristic fragment ion was m/z 121 which shared similar fragmentation pathway to that of M3. Thus, M5 was considered as secoisolariciresinol, the reductive cleavage product of furofuran ring hydrolysis of M7.

Metabolite M6

The metabolite M6 at m/z 329 eluted at 8.10 min. It was 28 Da lower than the protonated ion of M7, indicating that the metabolite might be the demethylation product of M7. On the basis of the MS/MS spectrum in Figure 3, M6 had product ions at m/z 299, 175, and 137. We concluded that the product ion at m/z 299 was formed by the elimination of CH_2O from m/z 329, and m/z 121 was formed by loss of O from m/z 137 [Figure 4]. Therefore, M6 was identified as (-)-3,3'-bisdemethylpinoresinol.

Metabolite M8

Metabolite M8 was detected as the deprotonated molecular ion $[M-H]^-$ at m/z 357 with a retention time of 10.30 min. It gave signals at m/z 357, 342, and 327, indicating the successive loss of 15 Da, and thus the presence of two methoxyl groups. m/z 357 dropped one CO_2 molecule to form m/z 313. Then, m/z 313 was further fragmented to m/z 175 and m/z 137 [Figure 4]. M8 was identified as (-)-3-(3″-hydroxy-4″-methoxy benzyl)-2-(4′-hydroxy-3′-methoxybenzyl) butyrolactone.

Metabolite M9

Metabolite M9 was eluted with a retention time of 10.42 min. Its deprotonated molecular ion at m/z 327 was 30 Da lower than that of M8, indicating that M9 might be generated from M8 through the loss of CH_2O group. M9 then produced signals at m/z 327, 312, 283, and 121. The product ion at m/z 312 was shaped by the elimination of CH_3 from m/z 327 [Figure 4]. The characteristic product ion at m/z 283 was formed by the elimination of CO_2 from m/z 327, and then m/z 327 was further fragmented to m/z 161 and m/z 121. Based on these results, metabolite M9 was tentatively identified as (-)-3-(3″-hydroxybenzyl)-2-(4′-hydroxy-3′-methoxybenzyl) butyrolactone.

As previously described, M3 was identified as enterolactone. It should be noted that natural furofuran lignans may exist as different stereoisomers due to their chiral carbon centers. Therefore, the configurations of the intestinal bacterial metabolites could not be established solely by MS, and could only be plausibly identified by their initial structures.

Analysis of the metabolic pathway of phillyrin and (+)-Pinoresinol 4-O-*β*-D-glucoside by human intestinal microflora

In summary, nine metabolites were detected in total. We confirmed three of them (M3, M4, and M7) by comparing their reference compounds, but we could only tentatively identify the rest six compounds. According to our results, the metabolic pathways of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside by human intestinal bacteria were proposed as displayed in Figure 5. The results indicated that the biotransformation of furofuran-type lignans by human intestinal microbiota included four types of reaction: hydrolysis of lignan glucoside, demethylation of a methoxy group, reductive cleavage of furofuran ring hydrolysis dehydroxylation of hydroxyl groups, and oxidation of dibenzylbutanediol to dibenzylbutyrolactone. This finding agrees with previous studies about intestinal bacterial metabolism of plant lignans.[8,15,16]

In the present study, we proved that phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside, two major furofuran-type lignans from Fructus Forsythiae, were metabolized to mammalian lignan enterolactone. This result also agrees with the previous finding that lignans (including furofuran lignans) are precursors of the mammalian lignans.[17-20] A study about phillyrin metabolism in rat intestinal bacteria has demonstrated that phillyrin was transformed to its metabolites during the incubation.[13] However, M3 and M4 observed in our study were not detected in that study. It is because of either the difference between human and rat or the low content in specimens. Among nine metabolites detected in our study, six of them were just tentatively identified. To confirm our results, metabolites with low content will be isolated and identified from the cultured mixture.

The presence of intestinal bacteria plays an important role in TCM metabolism after oral administration. Some of the metabolites show similar or higher pharmacological activities compared with that of the parent drugs, which can be dedicated to the treatment of disease as well.[9,21,22] Phillygenin (M4), one of the major metabolites of phillyrin, actually has higher antioxidative activity than its glycoside form phillyrin.[23,24] Furthermore, pinoresinol (M7) was proved to have stronger anti-inflammatory[25,26] and antifungi[27] properties than that of (+)-pinoresinol 4-O-*β*-D-glucoside. Enterolactone (M3) has been reported to have estrogenic, antiestrogenic,[28] and antioxidative[29] activities. The study has also revealed that phillyrin cannot be absorbed, and its excrete level in fecal is low. This further supports that the intestinal bacterial metabolites of plant lignans are the actual active ingredients after oral administration of TCM.[30] Once the low content of metabolites were isolated, some relevant pharmacological activities between the prototype compounds and metabolites will be further studied to understand the role of gastrointestinal tract conditions on pharmacological effects of Fructus Forsythiae.

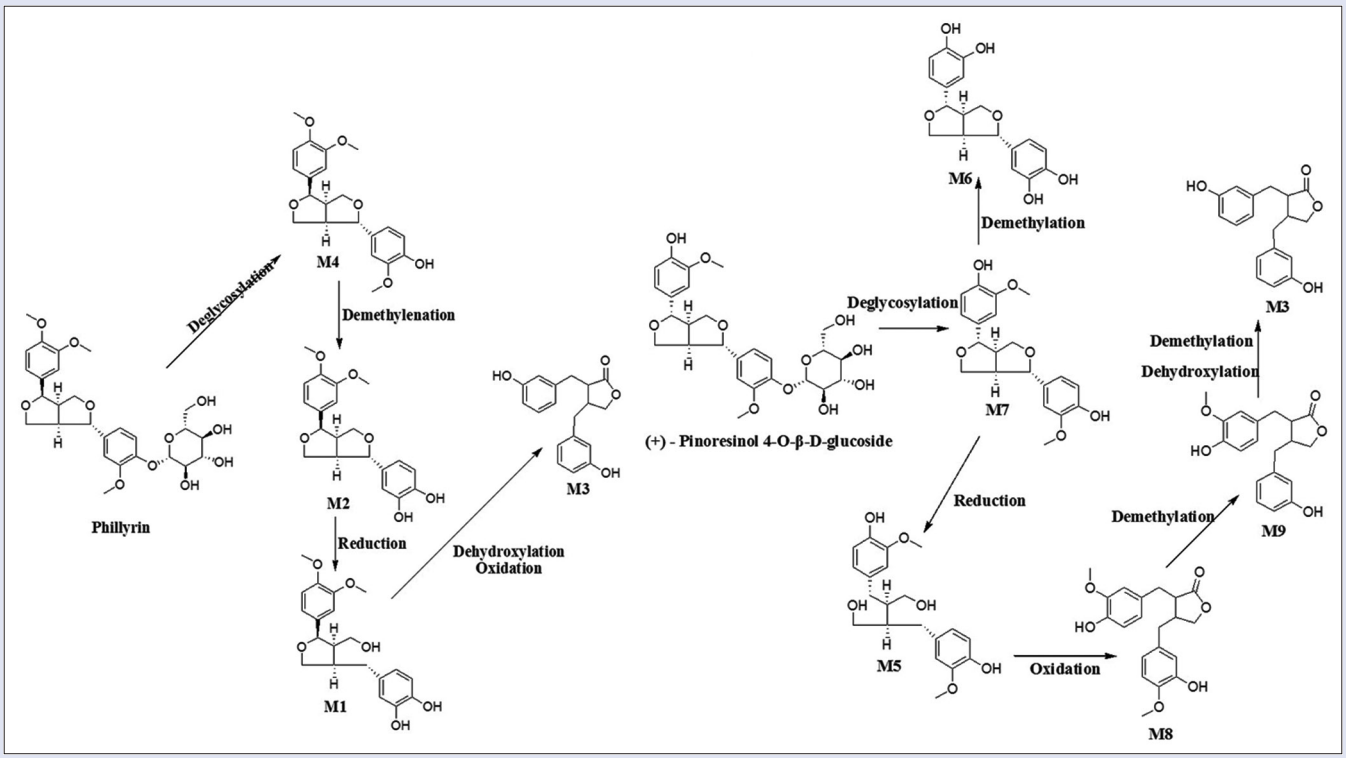

Figure 5: Proposed metabolic pathways of phillyrin and (+)-pinoresinol 4-O-*β*-D-glucoside incubated with human intestinal bacteria

Phenylethanoid glycosides were also identified as the main bioactive components of Fructus Forsythiae besides lignans.[31] Forsythoside A is one of the major phenylethanoid glycosides and is used as a phytochemical marker for the TCM quality control in Chinese Pharmacopoeia.[4] Our previous study has shown that Forsythoside A can be metabolized to its active metabolites (hydroxytyrosol and 3,4-dihydroxybenzenepropionic acid) under the human intestinal bacterial environment.[32] It is obvious that the pharmacological effects of main ingredients from Fructus Forsythiae are highly dependent on intestinal bacterial metabolism.

CONCLUSIONS

This investigation was to get further insight into the biotransformation of furofuran-type lignans by the incubation with human intestinal bacterial mixture. Finally, nine metabolites were detected during the incubation process. Results also indicated that hydrolysis, demethylation, reduction, dehydroxylation, and oxidation were the major metabolic pathways of furofuran-type lignans. These metabolites were analyzed by UPLC/Q-TOF-MS combined with automated data analysis (MetaboLynx™). These metabolites could be used to improve the bioavailability and bioactivity of furofuran lignans. However, *in vivo* studies will be required to better understand the metabolic mechanism by the intestinal bacteria, which is currently ongoing in our laboratory.

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Nil.

Conflicts of interest

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