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Structure elucidation of *in vivo* and *in vitro* metabolites of mangiferin

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ABSTRACT

The *in vivo* and *in vitro* metabolism of mangiferin was systematically investigated. Urine, plasma, feces, contents of intestinal tract and various organs were collected after oral administration of mangiferin to healthy rats at a dose of 200 mg/kg body weight. For comparison, mangiferin was also incubated *in vitro* with intestinal flora of rats. With the aid of a specific and sensitive liquid chromatography coupled with electrospray ionization tandem hybrid ion trap mass spectrometry (LC–ESI–IT–MS^n), a total of thirty-three metabolites of mangiferin were detected and their structures were tentatively elucidated on the basis of the characteristics of their precursor ions, product ions and chromatographic retention times. The biotransformation pathways of mangiferin involved deglycosylation, dehydroxylation, methylation, glycosylation, glucuronidation and sulfation.

1. Introduction

Mangiferin, a natural bioactive xanthone C-glycoside, was originally isolated from Mangifera indica L. (Anacardiaceea) [1]. Though unmetabolised mangiferin cannot be recovered in large rates according to a previous bioavailability study [2], a wide spectrum of pharmacological activities including antioxidant [3], antidiabetic [4], anti-HIV [5], antitumor [5], hepatoprotective [3], antiviral [5,6] and anticancer [7] actions attributed to this compound have been reported in recent years. So it can be assumed that some metabolites exert stronger effects. Therefore, investigation of the metabolic fate of mangiferin is of great significance in elucidation of its pharmacological mechanisms and discovering and developing novel drugs from the metabolites.

Previous reports involving metabolism investigation of mangiferin conducted on different species [8–15] provided some important information. Nevertheless, knowledge about its metabolism behavior to date is still limited and no report has been seen in the literature that comprehensively and comparatively investigated the *in vivo* and *in vitro* metabolic profile of this compound.

In the present study, metabolism of mangiferin was investigated systematically using the specific and sensitive LC–ESI–IT–MS^n. Structures of its metabolites were unambiguously identified or tentatively proposed by comparing their fragmentation patterns with those of the standards. The transformation pathways of mangiferin in rats were elucidated based on these results, which will help us better understand the *in vivo* metabolic fate of this compound.

2. Experimental

2.1. Chemicals and reagents

Mangiferin (95%, purity) for dosing was purchased from Tianjin Zhongxin Pharmaceutical Group Co., Ltd. (Tianjin, China). The standard compound mangiferin for reference was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Norathyriol and 1,7-dihydroxyxanthone (99%, purity) were isolated and identified by our laboratory from rat urine post-dosing of mangiferin [11]. Acetonitrile and formic acid (HPLC grade) were purchased from Fisher Scientific (Tustin, CA, USA). All other analytical grade reagents were from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Triple deionized water was prepared from a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Instrumentation

LC–MS^n experiments were performed on the 6300 Series Ion Trap LC/MS (Agilent technology, Palo Alto, CA, USA). The hardware included an Agilent 1200 Series LC, the ion trap mass spectrome-

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ter and the data system. The software (version 6.1) included the TrapControl program for trap control, data acquisition, data analysis, quantitative analysis, and the Agilent ChemStation program for sample automation and LC control. Pre-treatment of samples by solid phase extraction (SPE) was performed on Waters Oasis HLB cartridges (60 mg, 3 cm^3) purchased from Waters (Milford, MA, USA). The intestinal incubation experiments were carried out in anaerobic bags (AnaeroPouch™-Anaero, Mitsubishi Gas Chemical Company Inc., Tokyo, Japan)

2.3. Sample preparation

2.3.1. Animals

Thirty male Sprague–Dawley rats (250 ± 20 g; Shanghai SLAC Laboratory Animal Co., Shanghai, China) were divided into three drug-treated groups (six rats in group A for urine and feces collecting; six rats in group B for blood withdrawn (with three rats at each time point); twelve rats in group C for organs and intestinal contents obtained (with three rats at each time point)) and one control group with six rats. All animals were acclimated for one week before use and then fasted overnight with free access to water prior to a single dose of 200 mg/kg by oral gavages. All animal studies were conducted according to protocols approved by the Review Committee of Animal Care and Use at the Shanghai Institute of Materia Medica (Shanghai, China).

2.3.2. In vivo samples

Urine and feces during the period of 0–48 h post-dose were collected and combined separately. Blood samples via angular vein at 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h and 11 h, organs (heart, liver, kidneys, spleen, lung) and contents of intestinal tract at 2 h, 4 h, 7 h and 10 h were withdrawn from rats after administration of mangiferin. Plasma was separated from blood placed in heparinized Eppendorf tubes after centrifuging at 4000 × *g* for 10 min. 200 μl plasma obtained from each time point were pooled as drug containing plasma. Organs were weighed and homogenized with 0.1 M PBS buffer (pH = 7.4) in the ratio of 1 g:5 ml and the supernatants were separated out after centrifugation at 10,000 × *g* for 20 min. Contents of intestinal tract at each time point were diluted with saline solution to the volume of 10 ml. All samples were kept at −80 °C.

Urine, tissue homogenate, homogenized intestinal contents were pretreated according to the procedures as follows: 400 μl sample was loaded onto a SPE column that was preconditioned with 3 ml methanol and 3 ml water subsequently. The cartridge was then washed with 3 ml water and the analytes were eluted with 2 ml methanol. 50 mg 0–48 h feces sample was grounded and extracted by ultrasonication three times with 1 ml methanol for 30 min each time. Drug containing plasma was precipitated with 3 volumes of acetonitrile: acetic acid (9:1). The supernatant was separated after votexing and centrifuging. Extracting solutions with different pretreatment methods described above were all dried under nitrogen gas over a water bath of 37 °C. The residues were reconstituted in 100 μl methanol and centrifuged twice at 10,000 × *g* for 10 min prior to analysis. All the blank samples as control were prepared with the same method as drug containing samples.

2.3.3. In vitro samples

Anaerobic cultural solutions were prepared as literature described [16]. Fresh intestinal contents obtained from three SD rats in the control group were homogenized with anaerobic cultural solution in the ratio of 1 g:4 ml under anaerobic environment immediately and then filtrated with gauze. Mangiferin was added into 5 ml the above intestinal flora cultural solution to a final concentration of 100 μg/ml and cultured in anaerobic incubation bags over a shaking water-bath at 37 °C. After 2 h, 6 h, 10 h and 24 h, the transformations were terminated by cooling down to 4 °C. The sample preparation was in accordance with the procedures described for urine.

2.4. Analytical condition

Separations of the metabolites were achieved on a reversed-phase Inertsil ODS-3 column (4.6 mm × 250 mm, 5 μm, GL Sciences, Tokyo, Japan) connected to a Diamonsil C_{18} guard column (2.1 mm × 12.5 mm, 5 μm, Dikma Technologies, Beijing, China). The column temperature was set at 25 °C and the flow rate maintained at 0.8 ml/min. Mobile phase consisting of acetonitrile (A) and water containing 0.5% formic acid (B) used a linearly gradient program as follows: 10–23% A at 0–15 min, hold for 5 min, linearly gradient to 45% A in 25 min, then linearly gradient to 61% A at 60 min. An aliquot of 20 μl of the purified sample was injected automatically into the HPLC system for LC–MS^n analysis.

MS^n analyses were conducted in negative ion mode and the operating parameters were optimized as follows: collision gas, ultrahigh-purity helium (He); nebulizing gas, high-purity nitrogen (N_2); capillary voltage, 3.5 kV; end plate offset, 500 V; nebulizer, 30 psi.; drying gas flow rate, 10 l/min; drying gas temperature, 350 °C. For full scan MS analysis, the spectra were recorded in the range of 100–1000 Da.

3. Results and discussion

Mangiferin together with its two metabolites (northyriol and 1,7-dihydroxyxanthone) served for reference in the present work to elucidate the structures of other potential metabolites. Based on the direct comparison of samples from drug-treated and corresponding control animals, a total of thirty-three metabolites were detected and identified. Extracted ion chromatogram of mangiferin and its metabolites are presented in Fig. 1. The location, MS^n data along with the retention time of mangiferin and its metabolites are summarized in Table 1.

3.1. Characterization of mangiferin and its metabolites

Mangiferin (M0) and mangiferin glucuronide (M1)

Three characteristic product ions of mangiferin at *m*/*z* 403 ($[M-H-18]^-$), *m*/*z* 331 ($[M-H-90]^-$) and *m*/*z* 301 ($[M-H-120]^-$) were the most important information for identification of its metabolites. $[M-H]^-$ at *m*/*z* 597 given by M1 produced ion at *m*/*z* 421 with neural loss of 176 Da in the MS^2 spectral, which sequentially fragmented in the same pathway with the parent compound. So M1 might be monoglucuronidated conjugate of mangiferin.

Monomethyl mangiferin (M4, M8, M9, M10), dimethyl mangiferin (M11, M12) and monomethyl mangiferin glucuronide (M2, M3)

According to the literature [9,12,13], M4, M8, M9, M10, M11 and M12 were proposed to be methylated products of mangiferin while M2 and M3 being monomethyl mangiferin glucuronide. Moreover, at least one monomethylated and one dimethylated mangiferin were first reported as metabolites of mangiferin.

Norathyriol (M26)

By comparison with the standard and referring to the literature [13], M26 was unambiguously identified as norathyriol which has been described to possess various pharmacological activities [17,18].

Monomethyl norathyriol (M30, M31, M32)

Three metabolites coexisted in the extracted ion chromatogram (EIC) of *m*/*z* 273 showed an increase of 14 Da in molecular weight than that of norathyriol. Moreover, their product ion at *m*/*z* 258 via loss of CH_3 in the MS^2 spectral underwent similar sequential fragmentation with the parent ion of norathyriol. Thus M30,

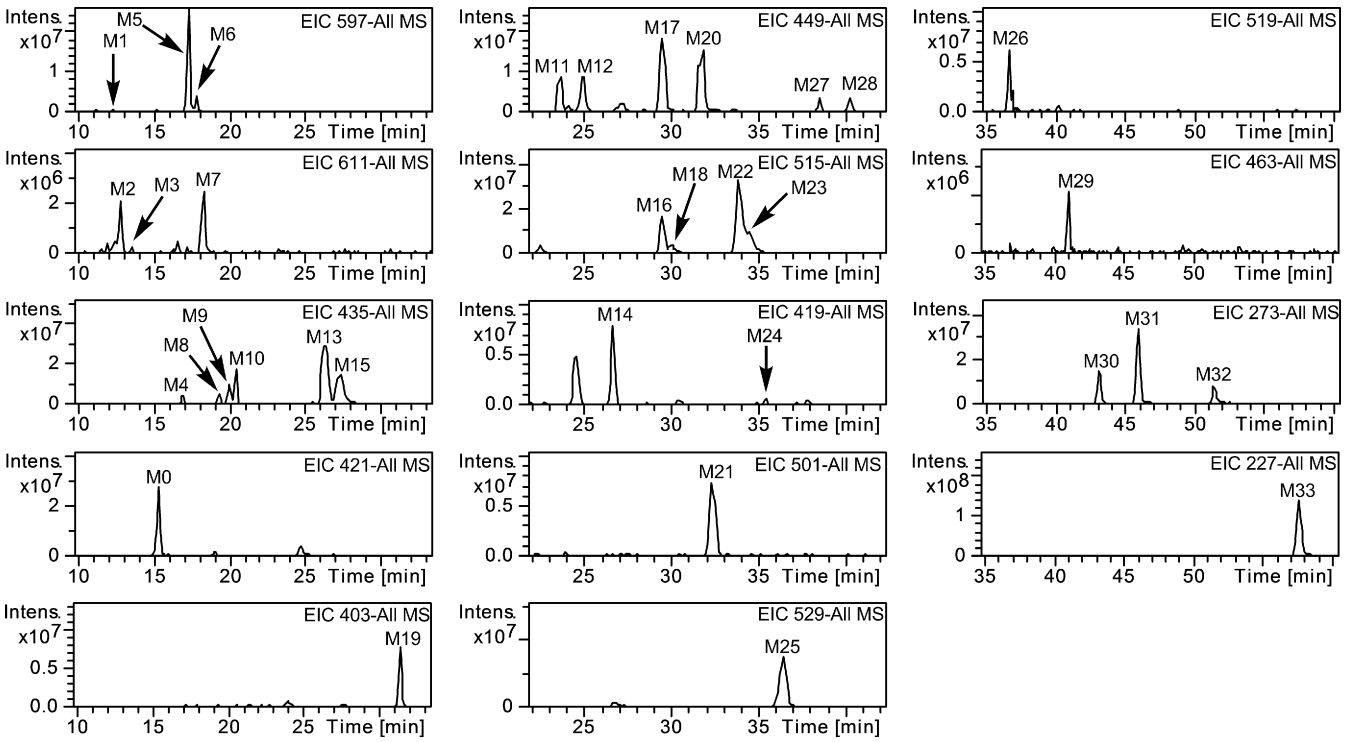

Fig. 1. Extracted ion chromatograms of mangiferin and its metabolites.

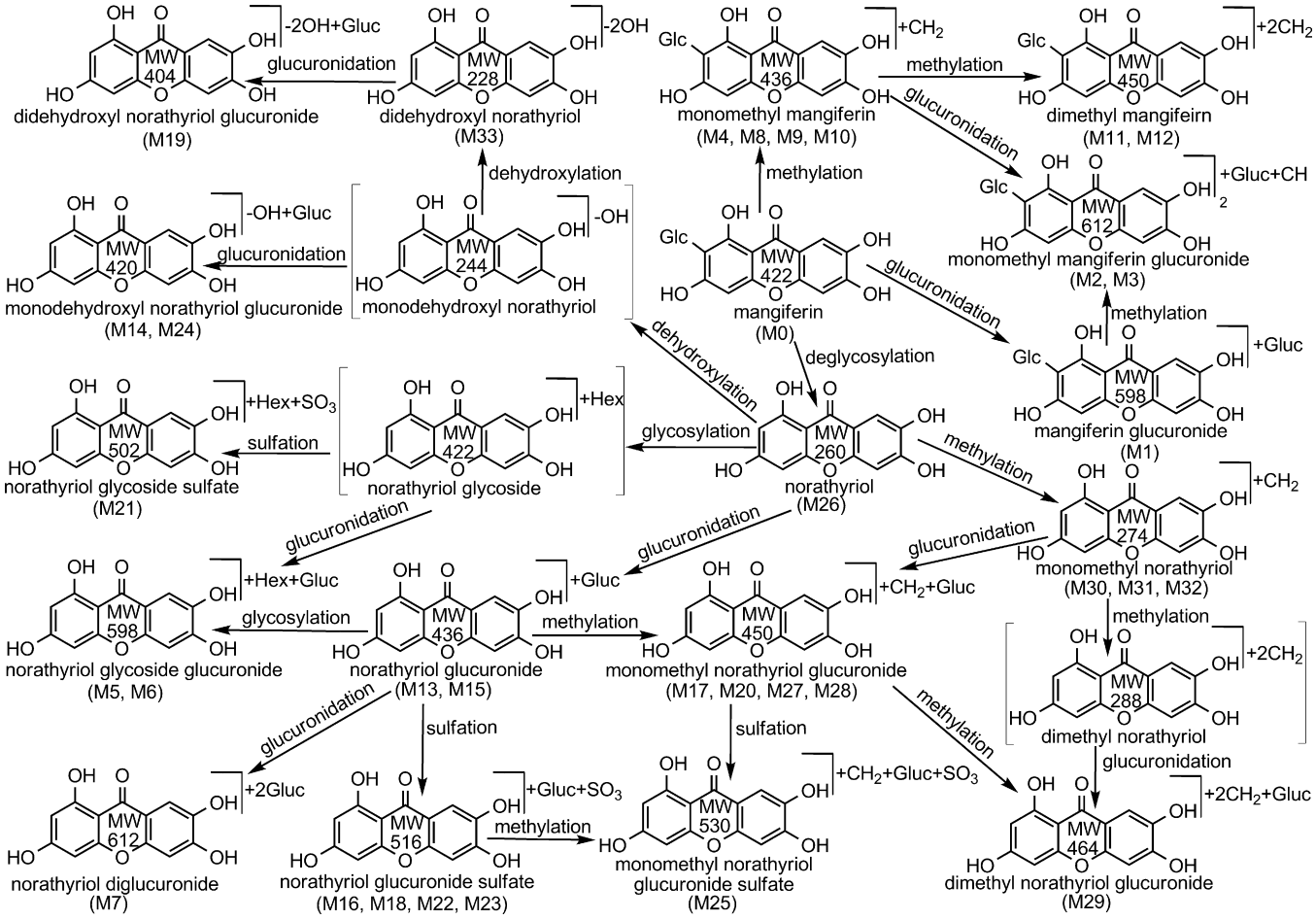

Fig. 2. The proposed metabolic pathways of mangiferin in rats.

Table 1
Chromatographic retention time, location, MS^n data and relative abundance of mangiferin and its metabolites.

No.	R_t (min)	Location										Precursor ion (m/z)	Data-dependent MS^n data(% base peak) (m/z)
		1	2	3	4	5	6	7	8	9	10		
M0	15.4	+	+	+	+	+	+	–	+	–	+	421[M−H]$^-$	MS^2 [421]: 403(15), 331(83), 301(100), 259(5)
M1	12.1	+	–	–	+	–	–	–	–	–	–	597[M−H]$^-$	MS^2 [597]: 579(37), 507(58), 477(54), 421(100), 403(13), 331(67), 301(47) MS^3 [421]: 403(11), 331(89), 301(100), 259(6)
M2	12.8	+	–	–	+	–	–	–	–	–	–	611[M−H]$^-$	MS^2 [611]: 593(13), 521(6), 491(18), 435(100) MS^3 [435]: 345(43), 315(100), 272(7)
M3	13.4	+	–	–	+	–	–	–	–	–	–	611[M−H]$^-$	MS^2 [611]: 435(100), 345(8), 315(8) MS^3 [435]: 417(16), 345(49), 315(100), 272(3)
M4	17.0	+	+	–	+	–	+	–	–	–	+	435[M−H]$^-$	MS^2 [435]: 420(5), 345(2), 315(100), 287(43), 272(19), 259(3) MS^3 [315]: 315(100), 272(1.2)
M5	17.4	+	–	–	+	–	–	–	–	+	–	597[M−H]$^-$	MS^2 [597]: 435(100), 417(77), 421(5), 259(15) MS^3 [435]: 259(100)
M6	17.9	+	–	–	+	–	–	–	–	–	–	597[M−H]$^-$	MS^2 [597]: 421(100), 435(6), 259(4) MS^3 [421]: 259(100), 421(6)
M7	18.3	+	–	–	–	–	+	–	–	–	–	611[M−H]$^-$	MS^2 [611]: 435(100), 259(6) MS^3 [435]: 259(100)
M8	19.4	+	+	+	+	–	+	–	+	+	–	435[M−H]$^-$	MS^2 [435]: 345(18), 315(100), 272(6), 259(41) MS^3 [315]: 315(100), 272(5)

Table 1 (*Continued*)

No.	R_t (min)	Location										Precursor ion (m/z)	Data-dependent MS^n data(% base peak) (m/z)
		1	2	3	4	5	6	7	8	9	10		
M9	20.0	+	–	–	–	–	–	–	–	–	–	435[M−H]$^-$	MS^2 [435]: 345(26), 315(100), 259(86) MS^3 [315]: 315(100), 272(5)
M10	20.4	+	+	–	+	–	–	–	–	–	–	435[M−H]$^-$	MS^2 [435]: 345(25), 315(100), 259(81) MS^3 [315]: 315(100), 272(5)
M11	23.8	+	–	–	–	–	–	–	–	–	–	449[M−H]$^-$	MS^2 [449]: 345(8), 315(15), 273(100), 258(12) MS^3 [273]: 273(100), 258(22) MS^4 [258]: 258(100), 230(12), 202(9)
M12	25.0	+	–	–	–	–	–	–	–	–	–	449[M−H]$^-$	MS^2 [449]: 345(7), 315(12), 273(100), 258(6) MS^3 [273]: 273(100), 258(8)
M13	26.1	+	–	+	–	–	–	–	–	–	–	435[M−H]$^-$	MS^2 [435]: 259(100)
M14	26.7	+	–	–	–	–	–	–	–	–	–	419[M−H]$^-$	MS^2 [419]: 243(100) MS^3 [243]: 243(100), 212(3), 143(4)
M15	27.2	+	–	+	–	–	+	–	–	+	–	435[M−H]$^-$	MS^2 [435]: 259(100) MS^3 [259]: 259(100), 229(24), 163(6)
M16	29.5	+	–	+	–	–	–	–	–	–	–	515[M−H]$^-$	MS^2 [515]: 435(100), 259(7) MS^3 [435]: 435(6), 259(100)
M17	29.5	+	–	+	–	–	+	–	–	+	–	449[M−H]$^-$	MS^2 [449]: 273(100) MS^3 [273]: 273(100), 258(11)
M18	30.0	+	–	–	–	–	–	–	–	–	–	515[M−H]$^-$	MS^2 [515]: 435(100), 259(67) MS^3 [435]: 259(100)
M20	31.8	+	–	–	–	–	–	–	–	+	–	449[M−H]$^-$	MS^2 [449]: 273(100) MS^3 [273]: 273(100), 258(30.3) MS^4 [258]: 258(100), 230(11)

Table 1 (*Continued*)

No.	R_t (min)	Location										Precursor ion (m/z)	Data-dependent MS^n data(% base peak) (m/z)
		1	2	3	4	5	6	7	8	9	10		
M21	32.3	+	–	–	–	–	–	–	–	–	–	501[M−H]$^-$	MS^2 [501]: 421(85), 259(100) MS^3 [421]: 259(100) MS^4 [258]: 258(100), 230(11)
M22	33.8	+	–	–	–	–	–	–	–	–	–	515[M−H]$^-$	MS^2 [515]: 435(100), 339(22) MS^3 [435]: 259(100)
M23	34.4	+	–	–	–	–	–	–	–	–	–	515[M−H]$^-$	MS^2 [515]: 435(31), 339(100), 259(43) MS^3 [339]: 259(100)
M24	35.3	+	–	–	–	–	–	–	–	–	–	419[M−H]$^-$	MS^2 [419]: 243(100)
M25	36.4	+	–	–	–	–	–	–	–	–	–	529[M−H]$^-$	MS^2 [529]: 449(100), 273(77) MS^3 [273]: 273(100), 258(18) MS^4 [258]: 230(4)
M26	36.8	+	+	–	+	–	+	+	+	–	+	519[2M−H]$^-$	MS^2 [519]: 259(100) MS^3 [259]: 231(1)
M27	38.4	+	–	–	–	–	–	–	–	–	–	449[M−H]$^-$	MS^2 [449]: 273(100) MS^3 [273]: 273(100), 258(3)
M28	40.1	+	–	–	–	–	–	–	–	–	–	449[M−H]$^-$	MS^2 [449]: 273(100) MS^3 [273]: 273(100), 258(3)
M29	41.1	+	–	–	–	–	–	–	–	–	–	463[M−H]$^-$	MS^2 [463]: 287(100) MS^3 [287]: 287(100), 272(14) MS^4 [272]: 272(100), 257(33)
M30	43.2	+	+	–	–	–	–	–	–	–	–	273[M−H]$^-$	MS^2 [273]: 258(100) MS^3 [258]: 230(1)
M31	45.9	+	+	–	–	–	–	–	–	–	–	273[M−H]$^-$	MS^2 [273]: 258(100) MS^3 [258]: 230(4)
M32	51.2	+	+	–	–	–	–	–	–	–	–	273[M−H]$^-$	MS^2 [273]: 258(100) MS^3 [258]: 230(2)
M33	57.3	–	+	–	–	–	–	–	–	–	–	455[2M−H]$^-$	MS^2 [455]: 227(100) MS^3 [227]: 183(5)

1, urine; 2, feces; 3, plasma; 4, intestinal contents; 5, heart; 6, liver; 7, spleen; 8, lung; 9, kidneys; 10, intestinal bacteria incubation; +, found; –, not found.

M31 and M32 were deduced to be monomethyl norathyriol, at least two of which were novel metabolites of mangiferin [11].

Norathyriol glucuronide (M13, M15) and norathyriol diglucuronide (M7)

According to the literature [13], M13 and M15 were proposed to be monoglucuronide conjugates of norathyriol. Furthermore, at least one of them was first reported as metabolite of mangiferin.

$[M-H]^-$ at *m/z* 611 given by M7 yielded no other product ions than *m/z* 435 in the MS^2 spectral and *m/z* 259 in the MS^3 spectral by successive loss of two glucuronide moieties, indicating that two glucuronyl units are attached on the norathyriol skeleton to form diglucuronide conjugate of aglycone of mangiferin.

Monomethyl norathyriol glucuronide (M17, M20, M27, M28) and dimethyl norathyriol glucuronide (M29)

$[M-H]^-$ at *m/z* 449 given by M17, M20, M27 and M28 and its characteristic product ions at *m/z* 273 were all increased by 14 Da compared with those of M13 and M15, suggesting that these analytes were monomethyl norathyriol glucuronide. Likewise, M29 might be dimethylated and monoglucuronidated metabolite of norathyriol.

Norathyriol glucuronide sulfate (M16, M18, M22, M23)

Four peaks with different retention times appeared in the extracted ion chromatogram (EIC) of *m/z* 515. All successive MS/MS fragmentations showed decrease of 176 Da and 80 Da in molecular weight ascribed to loss of a glucuronide and sulfate unit whereas the characteristic ions of C-glycoside xanthone were absent. Thus we concluded that M16, M18, M22 and M23 could be monoglucuronide and monosulfate conjugates of norathyriol.

Norathyriol glycoside glucuronide (M5, M6) and norathyriol glycoside sulfate (M21)

Metabolites eluted at 17.4 min and 17.9 min displayed $[M-H]^-$ at *m/z* 597. Product ions in their MS^2 and MS^3 spectral demonstrated the existence of a hexose ($597 \rightarrow 435$ and $421 \rightarrow 259$, respectively) and a glucuronide unit ($597 \rightarrow 421$ and $435 \rightarrow 259$, respectively). Nevertheless, no characteristic ions of C-glycoside xanthone appeared, indicating that mangiferin was first transformed into norathyriol via deglycosylation and further subjected to reglycosylation and glucuronidation. Similarly, we inferred that M21 was norathyriol glycoside sulfate.

Glycosylation as a rare metabolic pathway in animals has been reported previously in dietary flavonoids such as genistein [19] and EGCG ((−)-epigallocatechin-3-gallate) [20]. This is the first report of glycosylation pathway involved in the metabolism of mangiferin. What is worth mentioning here is that O-glycoside took place of the original C-glycoside.

1,7-dihydroxyxanthone (M33)

Compared with the standard, M33 was ascertained to be 1,7-dihydroxyxanthone, deglycosylated and didehydroxylated metabolite of mangiferin to which diverse interesting bioactivities such as anti-malarial [21], anti-cancer [22], antioxidant and vasodilatated functions [23] have been ascribed.

Monodehydroxyl norathyriol glucuronide (M14, M24) and didehydroxyl norathyriol glucuronide (M19)

$[M-H]^-$ at *m/z* 419 and *m/z* 403 showed neutral loss of 176 Da to generate product ions of *m/z* 243 and *m/z* 227, respectively. Previous studies have confirmed the existence of monodehydroxylated and didehydroxylated metabolites of norathyriol in rat urine post-dose of mangiferin [8,11]. It is proposed that these dehydroxylated products underwent glucuronidation prior to excreting into urine, leading to their glucuronide conjugates instead of the phase I metabolites detected in the present study. M14 and M24 were postulated to be monodehydroxylated and glucuronidated metabolites of norathyriol while M19 being its didehydroxylated and glucuronidated product.

3.2. Location of mangiferin and its metabolites

Location of mangiferin and its metabolites shown in Table 1 indicated that all compounds excluding M33 appeared in other samples could be detected in urine (M0–M32).

Four phenolic acids determined by Bock et al. [14] were not included in the compounds present in feces (M4, M8, M10, M26, M30, M31, M32, M33) in our research. It is presumably resulting from matrix suppression in LC/MS and its inferiority in detection of trace amount of phenolic acids to GC/MS.

Most of compounds detected in intestinal contents (M0, M1, M2, M3, M4, M5, M6, M8, M10, M26) appeared in the sample collected at 4 h post-dose. *In vitro* incubation further suggested intestinal bacteria involved in the formation of methylated and deglycosylated metabolites (M4, M26).

Polyphenols are reported to possess a significant binding affinity to proteins, leading to protein–polyphenol complexes that are believed to be the transport forms of polyphenols *in vivo* [24,25]. Thus it is not surprising that metabolites detected in plasma (M0, M8, M13, M15, M16, M17) are much fewer than those present in the urine.

After administrating mangiferin to rats, tissue distribution of the parent drug and its metabolites showed great differences. Only one compound was detected in heart (M0) and spleen (M26). M0 and its two metabolites (M8, M26) were observed in lung. Six metabolites (M4, M7, M8, M15, M17, M26) besides mangiferin were identified in liver. Five metabolites (M5, M8, M15, M17, M20) appeared in kidneys whereas the parent drug was absent.

4. Conclusion

In vivo and *in vitro* metabolism of mangiferin was extensively and comparatively investigated in the present study. Not only mangiferin itself underwent phase II metabolism, but its aglycone by intestinal flora degradation involved in extensive metabolism *in vivo*. Two phase I metabolites were unambiguously identified and structures of thirty-one phase II metabolites were tentatively elucidated. Besides sixteen fixed metabolites (M1, M5, M6, M7, M16, M17, M18, M20, M21, M22, M23, M24, M27, M28, M29), at least one monomethylated, one dimethylated mangiferin, two monomethylated and one glucuronidated norathyriol were first reported as metabolites of mangiferin. Metabolic pathways of mangiferin were supposed as deglycosylated, dehydroxylated, methylated, glycosylated, glucuronidated and sulfated transformations (shown in Fig. 2). Our efforts for structural confirmation of the metabolites and investigation of pharmacokinetics of the active metabolites are underway.

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