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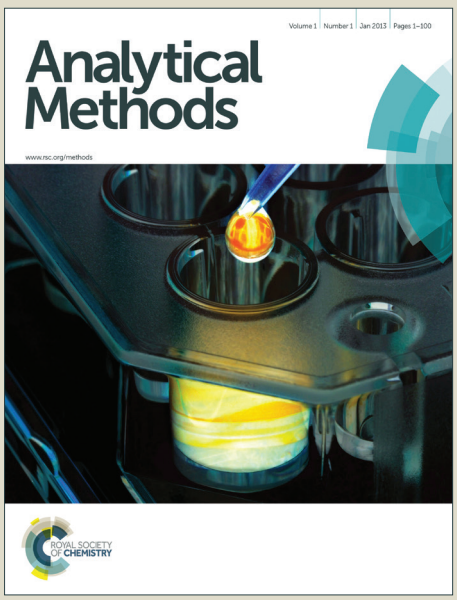

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Identification of *in vivo* components in rats after oral administration of lotus leaf flavonoids using ultra fast liquid chromatography with tandem mass spectrometry

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Abstract

The herb of lotus (*Nelumbo nucifera*) leaves is the traditional Chinese medicine He Ye, which is commonly used to treat sunstroke, assuage thirst, and cure both diarrhea and fever in China. Lotus leaves are rich in flavonoids, which exhibit various biological activities. However, the *in vivo* components, including parent compounds and their metabolites after consumption of the leaves have not been investigated extensively. In the present study, a method based on ultra fast liquid chromatography with tandem mass spectrometry (UFLC-MS/MS) was established to identify the *in vivo* components in rats after oral administration of a lotus leaf flavonoid extract. Plasma and urine samples were collected before and after dosing and treated by liquid-liquid extraction with ethyl acetate, and followed by UFLC-MS/MS assay. Q1 (first quadrupole) full scan combined with multiple reaction monitoring (MRM) survey scan were used for the detection of parent flavonoids and their metabolites. MRM-information dependent acquisition (IDA) of enhanced product ions (MRM-IDA-EPI) was used for the structural identification of detected components. A total of thirty-seven components were identified, including quercetin-3-O-glucuronide, quercetin-3-O-glucoside, quercetin-3-O-galacoside, quercetin and kaempferol, as well as their methylation, glucuronidation, and sulfonation metabolites. The result may help better understand the pharmacological activities of the traditional Chinese medicine He Ye.

Keywords: *Nelumbo nucifera*; Lotus leaves; flavonoids; *in vivo*; metabolites; UFLC-MS/MS

1. Introduction

Lotus (*Nelumbo nucifera*) is utilized not only as an ornamental plant and a dietary staple, but also as a traditional Chinese medicine in China and consumed around the world. All parts of lotus, including leaves, leaf stalks, flower stalks, flower petals, flowers, seeds and rhizomes can be used for medical purposes. The dried lotus leaves known as "He Ye" is officially listed in Chinese Pharmacopoeia and used to treat sunstroke, assuage thirst, and cure both diarrhea and fever. The leaves are rich in flavonoids with relative high content of 7.72 mg/g in fresh mature leaves, in comparison with other parts of lotus (Chen et al., 2012). Pharmacology studies indicated that lotus leaf flavonoids have a wide range of biological effects, including antioxidant, antibacterial, anti-HIV, anti-obesity, and antitumor activities (Kashiwada et al., 2005; Ono et al., 2006; Wu et al., 2012; Yang et al., 2011).

Although pharmacological activities of lotus leaf flavonoids have been extensively studied, the investigation on their *in vivo* components has not yet been documented so far. It is considered that a crude herb contains lots of components and only those being absorbed into the blood can display their bioactivities (Wang et al., 2005). The identification the *in vivo* components of a crude herb extract, including the parents components and their metabolites, is a very important work for better understanding its pharmacological activities; providing scientific evidences for elevating the quality control approaches of the crude herbal medicine and their preparations, and searching active metabolites for new drug discovery (Cao et al., 2011). Therefore, the present study was carried out to create a new analytical method, the aim is to identify the *in vivo* components of lotus leaf flavonoids after oral administration, and further to better

understand the pharmacological actions of the Chinese herbal medicine He Ye.

2. Materials and Methods

2.1 Chemicals

Quercetin-3-O-glucuronide was purchased from Sigma-Aldrich (St Louis, MO, USA). Quercetin, quercetin-3-O-galactoside, quercetin-3-O-glucoside, kaempferol and kaempferol-3-O-glucoside were purchased from Weikeqi Biological Technology Co. Ltd (Chengdu, Sichuan province, China). HPLC-grade methanol and acetonitrile were obtained from Fisher Co. Ltd. (Waltham, MA, USA). Formic acid and other reagents were all of analytical grade and were purchased from Beijing Chemical Reagent Company (Beijing, China). Milli-Q (Millford, MA, USA) water was used throughout the study.

2.2 Preparation of lotus leaf flavonoid extract

Lotus leaves were purchased from Tong Ren Tang herb shop in Beijing and the plant species was identified as *Nelumbo nucifera* Gaertn by professor Ben-Gang Zhang from the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The leaves (500 g) were powdered and extracted three times by refluxing with 80% ethanol for 2 h. The extract solutions were pooled, filtered, and concentrated to remove ethanol under reduced pressure. Then, the residue was dissolved in adequate 0.1% HCl in water (2 L). After filtration, the filtrate was loaded onto a column packed with 600 g of D001 resin, which is strongly acidic cation exchange resin with styrene structure and particle size from 0.3 to 1.2 mm (Chemical Plant of NanKai University, Tianjin, China), and the column was

then washed with water. The passing filtrate and washing water were collected and then successively loaded onto another column packed with 600 g of polyamine (Merck, Germany). After finishing the loading, polyamine column was also washed with water. The washed D001 resin column was eluted with 10 L of 95% ethanol containing 1% ammonia, and the eluent was concentrated to dryness under reduced pressure to yield a dark brown powder, which is mainly composed of alkaloid compounds and named as alkaloid fraction (6.81 g). The polyamine column was eluted with 10 L of 95% ethanol, and the eluent was concentrated to dryness under reduced pressure to obtain a yellow powder (14.98 g), which is mainly composed of flavonoid compounds and named as lotus leaf flavonoid extract (LFE).

2.3 Identification of the components in LFE

The identification of the components existed in LFE was performed by using a Waters ultra performance liquid chromatography instrument connected with a photodiode array UV detector (UPLC-UV) (Milford, MA, USA). Separation was carried out on a Thermo Syncronis C_{18} column (50×2.1mm, 1.7 μm; San Jose, CA, USA) maintained at 40°C. The mobile Phase was consisted of 0.1% formic acid in acetonitrile (solvent A) and 0.5% formic acid in water (solvent B) with a linear gradient elution at a flow rate of 0.3 mL/min. The elution program was as follows: 5-25% A (0-18 min); 25-60% A (18-25 min); 60-5% A (25-26 min); 5-5% A (26-30 min). The eluent was monitored by the UV detector and its on-line UV spectra (210-400 nm) were collected continuously during the running. The injection volume was 5 μL (2 mg/ml in 80% methanol). The identification of detected components was carried out by comparing their retention times and on-line UV spectra with those of relevant reference compounds, and further confirmed by their

mass data of molecular ion ($[M-H]^-$) and fragment ions, that were obtained in Q1 (first quadrupole) full scan using the following ultra fast liquid chromatography with tandem mass spectrometry (UFLC-MS/MS) method.

The UFLC-MS/MS system was consisted of a Shimadzu Prominenece UFLC system (Kyoto, Japan) connected to an AB SCIEX 5500 Q-Trap mass spectrometer equipped with an electrospray ionization source (Foster City, CA, USA). The chromatographic conditions were the same as described for UPLC assay. The mass conditions were set as the followings, the TurboIonSpray interface was operated in the negative ion mode at -4500 V; curtain gas, 20 psi; ion source temperature, 500°C; collision gas, medium; ion source of Gas 1 and Gas 2 were set both at 60 psi; declustering potential, -100 V and entrance potential, -10 V. The mass data were acquired in the range of *m/z* 100–800.

2.4 Animals

Male Sprague-Dawley rats (200±20 g) were supplied by Vital River Experimental Animal Co. Ltd (Beijing, China). The animal experiment was approved by the Animal Ethics Committee at the Institute of Medicinal Plant Development of the Chinese Academy of Medical Sciences. The rats were housed under standard conditions of temperature, humidity and light, with free access to standard rodent diet and water before experiment. On the day before the experiment, the rats were subjected to a light surgery. A polyethylene catheter (0.50 mm ID, 1.00 mm OD, Portex Limited, Hythe, Kent, England) was cannulated into the right jugular vein under anesthesia with an intraperitoneal dose of 10% chloral hydrate at 3.50 mL/kg. After surgery, the rats were placed individually in metabolism cages to allow their recovery for at least 24 h. The rats were fasted over-night with free access to water prior to LFE administration.

2.5 Drug Administration and sample collection

The rats (n=6) received an oral dose of LFE at 45 mg/kg by gavage administration. Blood samples were collected into heparinized tubes through the catheter, before (blank) and at 0.5 and 1 h after dosing, and then centrifuged at 8000 rpm, 4°C for 5 min for plasma separation. Urine samples over a 12 h period before and post dosing were also collected by a urine reservoir, which contained 2 mL of 0.1% HCl to prevent the possible degradation of parent compounds and their metabolites. All of collected blank plasma, blank urine, dosing plasma and dosing urine from six rats were pooled together separately and stored at -20°C until assay.

2.6 Biological sample preparation

An 2 mL-aliquot of plasma or urine sample was mixed with 0.3 mL of HCl (1 mol/L) to adjust the sample pH value at 4-5, and then extracted with 10 mL of ethyl acetate by vortexing for 10 min. The clear ethyl acetate layer was transferred into a glass tube and concentrated to dryness by a gentle steam of nitrogen at 25°C. The residue was reconstituted in 100 μL of 80% methanol, and 5 μL of the resulting sample solution was injected into an UFLC-MS/MS system for the identification of *in vivo* components, including parent flavonoids and their metabolites.

2.7 Identification of components in plasma and urine

The assay for plasma and urine samples was performed by using the above UFLC-MS/MS method described in Section 2.3, under the same chromatographic and mass conditions. Q1 full scan and multiple reaction monitoring (MRM) survey scan was used for the detection of parent flavonoids and their metabolites. MRM-information

dependent acquisition (IDA) of enhanced product ions (MRM-IDA-EPI) was used for the structural identification of detected components. The MRM ion pairs were built by the biotransformation of the two basic flavonoid units, quercetin (*m/z* 301) and kaempferol (*m/z* 285) and generated by MetID software (AB SCIEX) using *m/z* 301 and 285 as "test articles". The biotransformation include the common phase I (oxidation and reduction) and Phase II (conjugation) metabolism reactions, such as $[M-H]^-$+16 (oxidation), $[M-H]^-$+14 (methylation), $[M-H]^-$+80 (sulfonation) and $[M-H]^-$+176 (glucuronidation), which are shown in Table 1. The collision energy was set at -40 V for the MRM survey scan and EPI mass spectral data were acquired from *m*/*z* 100 to *m*/*z* 800.

The transitions from molecule ions to fragment ions of parent flavonoids and their possible metabolites were used for MRM survey scans. The programs Analyst (version 1.6, Foster City, CA, USA) was used for data acquire and assay.

3. Results

3.1. Identification of the flavonoids in LFE

An UPLC-UV method by using a C_{18} column and a gradient solvent elution was established for the separation of the flavonoids in LFE. Fig. 1 shows the UPLC chromatogram of LFE, which is extracted at the wavelength of 360 nm. Nine peaks with the characteristics of flavonoid UV absorbance were found. In which, The components appeared at the peaks C, D, E, F, H and I were identified as quercetin-3-O-galactoside, quercetin-3-O-glucuronide, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, quercetin and kaempferol, respectively, by comparing their retention times and on-line UV spectra with those of authentic references, and further confirmed by their molecule ions and fragment ions in Q1 full scan using UFLC-MS/MS assay. Other three

compounds appeared at the peaks A, B and G (Fig. 1) were identified as quercetin-3-O-arabinopyranosyl-galactopyranoside, syringetin-3-O-glucoside and myricetin-3-O-glucoside, respectively, by comparing their mass spectra data (Table 2) with those described in previous publication (Chen et al., 2012). Fig. 2 shows the chemical structures of identified flavonoids, and Table 2 shows their chromatographic and mass data. These flavonoids are mainly the glycosylation and glucuronidation derivatives of quercetin and kaempferol.

3.2. Identification of the *in vivo* components of LFE in rats

The mass chromatograms from Q1 full scan and MRM-IDA-EPI survey scan of plasma and urine samples after oral administration of LFE were compared with those of relevant blank samples to identify the *in vivo* components, including the parent flavonoids and their possible metabolites. Fig. 3 shows the typical UFLC-MS/MS chromatograms of urine (A and C) and plasma (B and D) samples after oral administration of LFE. In which, A and B were detected by the 23 MRM transitions generated by quercetin (*m/z*, 301) as "test article", and C and D were detected by the 23 MRM transitions generated by kaempferol (*m/z*, 285) as "test article". A total of thirty-seven components were identified, including quercetin-3-O-glucuronide, quercetin-3-O- glucoside, quercetin-3-O-galacoside, quercetin and kaempferol, as well as their methylation, glucuronidation, and sulfonation metabolites. Table 3 shows the chromatographic and mass data of the identified components.

3.2.1. Quercetin and quercetin glycosides

Peaks 15, 17 and 33 were detected in both urine and plasma. The peak 33 had the $[M-H]^-$ ion at *m/z* 301, which yielded the fragment ions at *m/z* 179 and 151 (Mullen et al.,

2004). Peaks 15 and 17 had the $[M-H]^-$ ion at *m/z* 463 and fragment ion at *m/z* 301 ($[M-H]^-$–162, loss of one galactose or glucose unit). Meanwhile, the three peaks 33, 15 and 17 showed the same LC retention times with those of reference standards, therefore, they were identified as quercetin, quercetin-3-O-galactoside and quercetin-3-O-glucoside, respectively.

3.2.2. Quercetin glucuronides

Peaks 1, 3, 8, 9, 13 and 14, detected in both urine and plasma, had the $[M-H]^-$ ion at *m/z* 653 and fragment ions at *m/z* 477 ($[M-H]^-$–176, loss of one glucuronyl unit) and 301 ($[M-H]^-$–352, loss of two glucuronyl units). Therefore, they were tentatively identified as quercetin diglucuronides.

Peaks 16, 18 and 28, detected in both urine and plasma, had the $[M-H]^-$ ion at *m/z* 477 and fragment ion at *m/z* 301 ($[M-H]^-$–176, loss of one glucuronyl unit). Therefore, they were tentatively identified as quercetin monoglucuronides. In addition, peak 16 was identified as quercetin-3-O-glucuronide since it had the same LC retention time and mass spectra with that of reference standard.

3.2.3. Quercetin sulfates and quercetin glucuronide sulfates

Peaks 23 and 26, detected in both urine and plasma, had the $[M-H]^-$ ion at *m/z* 381 which yielded the fragment ion at *m/z* 301 ($[M-H]^-$–80, loss of SO_3). Therefore, they were tentatively identified as quercetin sulfates.

Peaks 4, 7, 11, and 12, detected in urine and/or plasma, had the $[M-H]^-$ ion at *m/z* 557 which yielded the fragment ions at *m/z* 477 ($[M-H]^-$–80, loss of SO_3), 381 ($[M-H]^-$–176, loss of one glucuronyl unit) and 301 ($[M-H]^-$–80–176, loss of one SO_3 and one

glucuronyl unit). Therefore, they were tentatively identified as quercetin glucuronide sulfates.

3.2.4. Methylquercetin

Peak 37 detected in urine had the $[M-H]^-$ ion at *m/z* 315 which yielded the fragment ion at *m/z* 301 ($[M-H]^-$–14, loss of one methyl unit). Therefore, it was tentatively identified as methylquercetin.

3.2.5. Kaempferol glucuronides

Peaks 2, 5 and 10, detected in urine and/or plasma, had the $[M-H]^-$ ion at *m/z* 637 which yielded the fragment ions at *m/z* 461 ($[M-H]^-$–176, loss of one glucuronyl unit) and 285 ($[M-H]^-$–352, loss of two glucuronyl units). Therefore, they were tentatively identified as kaempferol diglucuronides.

Peaks 20, 24, 25 and 30, detected in both urine and plasma, had the $[M-H]^-$ ion at *m/z* 461 which yielded the fragment ion at *m/z* 285 ($[M-H]^-$–176, loss of one glucuronyl unit). Therefore, they were tentatively identified as kaempferol monoglucuronides.

3.2.6. Kaempferol and kaempferol glucoside

Peak 36 was detected both in urine and in plasma and had the $[M-H]^-$ ion at *m/z* 285, which yielded the fragment ions at *m/z* 257 and 151. Meanwhile, the peak had the same LC retention time and mass data with that of reference standard, suggesting that the compound is kaempferol.

Peaks 19 and 22 had the $[M-H]^-$ ion at *m/z* 447 which yielded the fragment ion at *m/z* 285 ($[M-H]^-$–162, loss of one glucosyl unit). Therefore, they were identified as kaempferol glucosides. In addition, peak 22 had the same LC retention time and mass

spectra with that of reference standard of kaempfeerol-3-O-glucoside, suggesting that it is kaempfeerol-3-O-glucoside.

3.2.7. Kaempferol sulfate and kaempferol glucuronide sulfate

Peaks 21, 27 and 32, detected in both urine and plasma, had the $[M-H]^-$ ion at *m/z* 365 which yielded the fragment ion at *m/z* 285 ($[M-H]^-$–80, loss of SO_3). Therefore, they were tentatively identified as kaempferol sulfates.

Peak 6 detected in urine had the $[M-H]^-$ ion at *m/z* 541, which yielded the fragment ions at *m/z* 461 ($[M-H]^-$–80, loss of SO_3), 365 ($[M-H]^-$–176, loss of one glucuronyl unit) and 285 ($[M-H]^-$–80–176, loss of one SO_3 and one glucuronyl units). This suggests that the component might be kaempferol glucuronide sulfate.

3.2.8. Methylkaempferol glucoside

Peaks 34 and 35, detected in both urine and plasma, had the $[M-H]^-$ ion at *m/z* 461 which yielded the fragment ions at *m/z* 299 ($[M-H]^-$–162, loss of a glucosyl unit) and 285 ($[M-H]^-$–14–162, loss of one methyl unit and one glucosyl unit). Therefore, they were tentatively identified as methylkaempferol glucosides.

3.2.9. Methylquercetin glucoside and methylquercetin glucuronide

Peak 31, detected in urine, had the $[M-H]^-$ at *m/z* 477 which yielded the fragment ion at *m/z* 315 ($[M-H]^-$–162, loss of one glucosyl unit), suggests that the compound might be methylquercetin glucoside (Mullen et al., 2004).

Peak 29, detected in both urine and plasma, had the $[M-H]^-$ ion at *m/z* 491 which yielded the fragment ion at *m/z* 315 ($[M-H]^-$–176, loss of one glucuronyl unit). Therefore, it was tentatively identified as methylquercetin glucuronide (Mullen et al., 2004).

4. Discussion

LC-MS/MS technique is the most versatile tool for qualitative and quantitative analysis of herb constituents and drug metabolites, and is an integral part of pharmaceutical research due to its superb sensitivity and selectivity (Chen et al., 2012; Han et al., 2013; Zhang et al., 2009). The several detection methods, such as MRM scan, enhanced mass scan (i.e. Q1 full scan), neutral loss scan and precursor ion scan can be used as survey scans to trigger enhanced product ion (EPI) data acquisition for the detection and identification of *in vivo* components (Li et al., 2005; Mullen et al., 2007). In these methods, MRM-IDA-EPI is the most sensitive and specific, therefore, was used in the present study.

It is generally considered that only the compounds absorbed into the blood circulation have the chance to become active components after oral administration of crude herbal extracts or their prescriptions (Dong et al., 2012). However, not all of the components existed in herbs can be absorbed into the body. Exploring the *in vivo* components in the body after consumption of a crude herb extract may help to find out its bioavailable active components and to further better understand its pharmacological activities (Cao et al., 2011).

In the present study, we analyzed the components in LFE by using UPLC-UV and UFLC-MS/MS techniques. Nine flavonoid compounds were identified in LFE and are mainly glycoside and/or glucuronide derivates of quercetin and kaempferol. The compounds quercetin-3-O-galactoside, quercetin-3-O-glucuronide, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, quercetin and kaempferol were precisely identified by comparing the LC retention time and mass data with those of relevant standards. And

the other three compounds were tentatively identified and characterized due to the absence of standards. From the peak area response in the HPLC-UV chromatogram (Fig. 1), quercetin-3-O-glucronide was found the dominant compound, followed with quercetin-3-O-glucoside and quercetin-3-O-galactoside. Comparing with these glycosides, the two aglycones quercetin and kaempferol were found tenuity in LFE. These results suggest that the flavonoid compounds in lotus leaves are mainly existed as the form of glycosides.

To our knowledge, the present study is the first report on the *in vivo* components of LFE in rats by using UFLC-MS/MS with MRM-IDA-EPI method. Since quercetin and kaempferol are the two basic units of the flavonoid compounds identified in LFE, the two ions of *m/z* 301 (quercetin) and 285 (kaempferol) were used as the "test articles" in the biotransformation table in MetID software for building the MRM transitions in the detection of the components in rat plasma and urine. Finally, a total of thirty-seven components were identified, including quercetin-3-O-glucuronide, quercetin-3-O-glucoside, quercetin-3-O-galacoside, quercetin and kaempferol, as well as their glucuronidation, sulfonation, methylation metabolites. In which, quercetin glucuronides and kaempferol glucuronides were found to be dominant in the identified components. These results suggest that phase II biotransformation is the main metabolite pathway in the metabolism of flavonoid compounds. The similar results have been demonstrated in previous studies. The phase II metabolites, such as quercetin glucuronides and sulfates, quercetin glucuronide sulfates, and quercetin glucoside sulfates were found in human plasma after consumption of red onions, which are rich in flavonoids (Mullen et al., 2004). In another animal study, kaempferol was found to be quickly absorbed and

converted to its glucuronide and sulfate conjugates in rat gastrointestinal track (Zhang et al., 2010). Even though the flavonoid metabolites have been demonstrated in the previous studies, it is still very important to identify the *in vivo* components of LFE, due to their different chemical composition. The present study provides a detailed description on the chemical composition and the *in vivo* components of LEF, the main active components of lotus leaves. The results can help better understand the chemical materials of the commonly used Chinese herb medicine He Ye and its biological activities in the body.

5. Conclusion

The present study was performed to identify the *in vivo* components of LFE after oral administration to rats for the first time, by using UFLC-MS/MS methods with MRM-IDA-EPI survey scan and Q1 full-scan. A total of thirty-seven components were identified, including the parent flavonoids in LFE and their metabolites, which were mainly formed from quercetin, kaempferol and their glycosides through glucuronidation, sulfonation and methylation. The results can help better understand the chemical materials of the commonly used Chinese herb medicine He Ye and its biological activities in the body.

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Table1. Biotransformation for predicted common Phase I and Phase II metabolites

No	Gain/loss	Formula	Mass offset	Biotransformation type
1	1	O	16	Oxidation
2	-1	CH2	-14	Demethylation
3	1	CH_2	14	Methylation
4	2	CH_2	28	Di-methylation
5	1	SO_3	80	Sulfonation
6	1	$C_6O_6H_8$	176	Glucuronidation
7	2	$C_6O_6H_8$	352	Bis-glucuronidation
8	1	$C_6O_6H_8SO_3$	255	Glucuronidation and sulfonation
9	1	$C_6O_6H_8O$	192	Glucuronidation and oxidation
10	1	$C_{10}H_{15}O_6S$	305	GSH
11	1	$C_6O_6H_8O$	162	Glucosylation

Table 2. The chromatographic and mass data of identified flavonoids in LFE by UFLC-MS/MS

No	Rt (min)	$[M\text{-H}]^-$ (*m/z*)	Fragment ions (*m/z*)	Compounds
A	10.33	479	317 ($[M\text{-H}]^-$−Glc)	Myricetin-3-O-glucoside
B	11.10	595	433 ($[M\text{-H}]^-$−Gla), 301($[M\text{-H}]^-$−Gla−Ara)	Quercetin-3-O-arabinopyranosyl -galactopyranoside
C	12.41	463	301 ($[M\text{-H}]^-$−Gla)	Quercetin-3-O-galactoside
D	12.79	477	301($[M\text{-H}]^-$−Gln)	Quercetin-3-O-glucuronide
E	13.01	463	301($[M\text{-H}]^-$−Glc)	Quercetin-3-O-glucoside
F	14.97	447	285($[M\text{-H}]^-$−Glc)	Kaempferol-3-O-glucoside
G	17.81	507	345($[M\text{-H}]^-$−Glc), 329 ($[M\text{-2H}]^-$−Glc−Me), 315($[M\text{-2H}]^-$−Glc−2Me)	Syringetin-3-O-glucoside
H	19.64	301	179,151	Quercetin
I	21.92	285	257, 151	Kaempferol

Rt: Retention time; $[M–H]^-$: negatively charged molecular ion; Glc: glucosyl unit; Gal: galactosyl unit; Gln: glucuronyl unit; Me: methyl unit.

Table 3. The chromatographic and mass data of the *in vivo* components identified in rat plasma and urine after oral administration of LFE by UFLC–MS/MS

No	Rt (min)	Identified components	$[M-H]^-$ (*m/z*)	Fragment ions (*m/z*)	Location
1	7.05	Quercetin diglucuronide	653	477 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–2Gln)	U, P
2	8.57	Kaempferol diglucuronide	637	461 ($[M-H]^-$–Gln), 285 ($[M-H]^-$–2Gln)	U, P
3	8.72	Quercetin diglucuronide	653	477 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–2Gln)	U, P
4	9.10	Quercetin glucuronide sulfate	557	477 ($[M-H]^-$–SO_3), 301 ($[M-H]^-$–SO3-Gln)	U, P
5	9.39	Kaempferol diglucuronide	637	461 ($[M-H]^-$–Gln), 285 ($[M-H]^-$–2Gln)	U
6	9.46	Kaempferol glucuronide sulfate	541	461 ($[M-H]^-$–SO_3), 365 ($[M-H]^-$–Gln), 285 ($[M-H]^-$–SO_3–Gln)	U
7	9.50	Quercetin glucuronide sulfate	557	381 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–SO_3-Gln)	U, P
8	9.83	Quercetin diglucuronide	653	477 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–2Gln)	U, P
9	10.35	Quercetin diglucuronide	653	477 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–2Gln)	U, P
10	10.52	Kaempferol diglucuronide	637	461 ($[M-H]^-$–Gln), 285 ($[M-H]^-$–2Gln)	U, P
11	10.89	Quercetin glucuronide sulfate	557	381 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–SO_3–Gln)	U, P
12	11.28	Quercetin glucuronide sulfate	557	381 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–SO_3–Gln)	U, P
13	11.37	Quercetin diglucuronide	653	477 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–2Gln)	U, P
14	12.27	Quercetin diglucuronide	653	477 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–2Gln)	U, P
15	12.51	Quercetin-3-O- galactoside	463	301 ($[M-H]^-$–Gla)	U, P
16	12.53	Quercetin-3-O-glucuronide	477	301 ($[M-H]^-$–Gln)	U, P
17	12.87	Quercetin-3-O-glucoside	463	301 ($[M-H]^-$–Glc)	U, P
18	12.89	Quercetin glucuronide	477	301 ($[M-H]^-$–Gln)	U, P
19	13.90	Kaempferol glucoside	447	285 ($[M-H]^-$–Glc)	U
20	14.25	Kaempferol glucuronide	461	285 ($[M-H]^-$–Gln)	U, P
21	14.37	Kaempferol sulfate	365	285 ($[M-H]^-$–SO_3)	U, P
22	14.62	Kaempferol-3-O-glucoside	447	285 ($[M-H]^-$–Glc)	U, P
23	14.73	Quercetin sulfate	381	301 ($[M-H]^-$–SO_3)	U, P
24	14.81	Kaempferol glucuronide	461	285 ($[M-H]^-$–Gln)	U, P
25	15.14	Kaempferol glucuronide	461	285 ($[M-H]^-$–Gln)	U, P
26	15.21	Quercetin sulfate	381	301 ($[M-H]^-$–SO_3)	U, P
27	15.24	Kaempferol sulfate	365	286 ($[M-H]^-$–SO_3)	U, P
28	15. 42	Quercetin glucuronide	477	301 ($[M-H]^-$–Gln)	U, P
29	15.57	Methylquercetin glucuronide	491	315 ($[M-H]^-$–Gln), 301 ($[M-H]^-$–Gln–Me)	U
30	15.92	Kaempferol glucuronide	461	285 ($[M-H]^-$–Gln)	U, P
31	16.42	Methylquercetin glucoside	477	315 ($[M-H]^-$–Glc), 301 ($[M-H]^-$–Glc–Me)	U
32	17.36	Kaempferol sulfate	365	287 ($[M-H]^-$–SO_3)	U, P

33	19.35	Quercetin	301	179, 151	U, P
34	20.92	Methylkaempferol glucoside	461	299 ($[M–H]^-$–Glc), 285 ($[M–H]^-$–Glc–Me)	U
35	21.29	Methylkaempferol glucoside	461	300 ($[M–H]^-$–Glc), 285 ($[M–H]^-$–Glc–Me)	U
36	21.60	Kaempferol	285	257, 151	U, P
37	21.85	Methylquercetin	315	301 ($[M–H]^-$– Me), 151	U

Rt: Retention time; $[M–H]^-$: negatively charged molecular ion; Glc: glucosyl unit; Gal: galactosyl unit; Gln: glucuronyl unit; U: urine, P: plasma

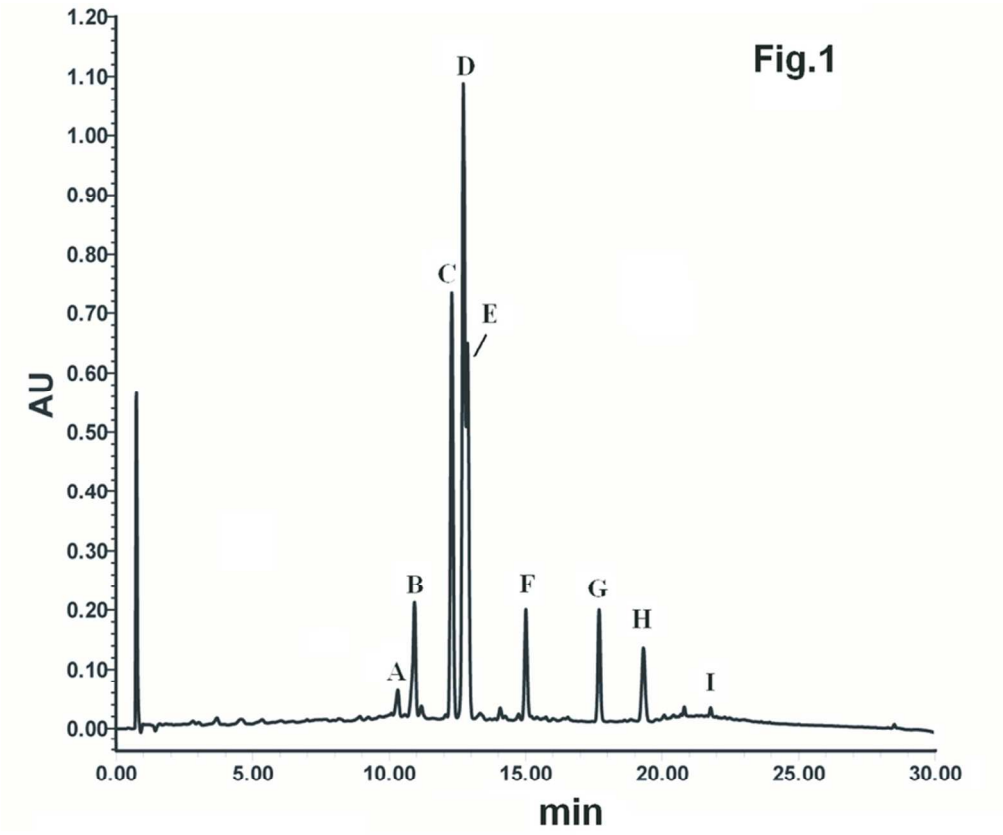


Fig. 1. UPLC-UV chromatogram (at 360 nm) of LFE.
40x34mm (600 x 600 DPI)

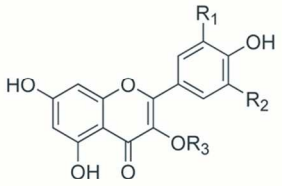


Fig.2

NO	Compound name	Substitution pattern		
		R1	R2	R3
A	Myricetin-3-O-glucoside	OH	OH	Glucose
B	Quercetin-3-O-arabinopyranosyl-galactopyranoside	OH	H	Arabinopose-galactose
C	Quercetin-3-O-galactoside	OH	H	Galactose
D	Quercetin-3-O-glucuronide	OH	H	Glucuronic acid
E	Quercetin-3-O-glucoside	OH	H	Glucose
F	Kaempferol-3-O-glucoside	H	H	Glucose
G	Syringetin-3-O-glucoside	OCH_3	OCH_3	Glucose
H	Quercetin	OH	H	H
I	Kaempferol	H	H	H

Fig. 2. Chemical structures of identified compounds in LFE.
49x39mm (600 x 600 DPI)

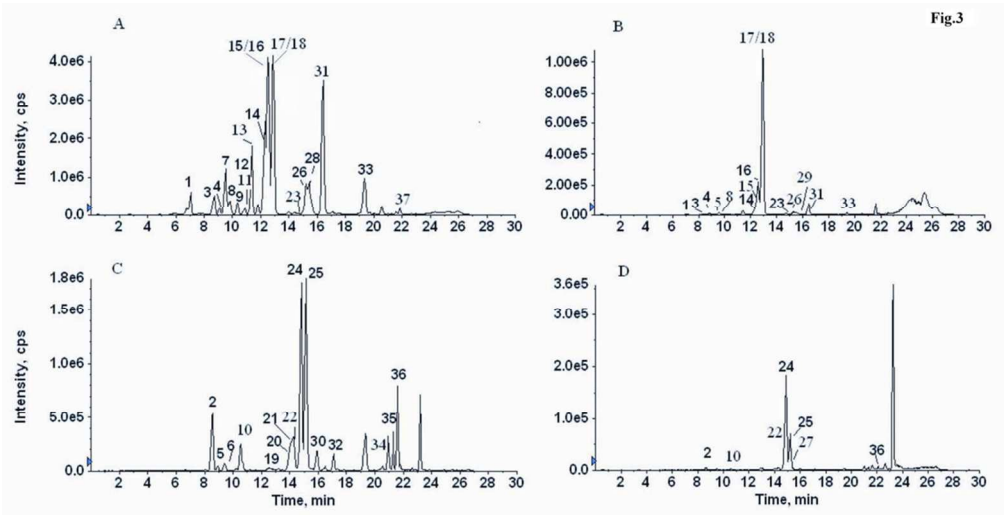
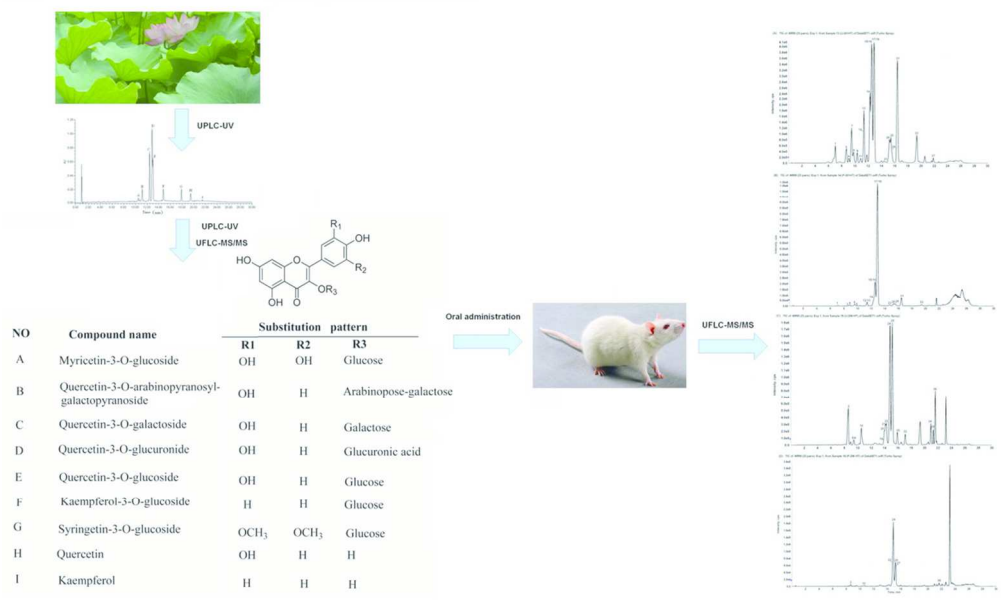


Fig. 3. The typical UFLC-MS/MS chromatograms of urine (A and C) and plasma (B and D) samples after oral administration of LFE. In which, A and B were detected by the 23 MRM transitions generated by quercetin (m/z, 301) as "test article", and C and D were detected by the 23 MRM transitions generated by kaempferol (m/z, 285) as "test article"
50x25mm (600 x 600 DPI)



NO	Compound name	Substitution pattern		
		R1	R2	R3
A	Myricetin-3-O-glucoside	OH	OH	Glucose
B	Quercetin-3-O-arabinopyranosyl-galactopyranoside	OH	H	Arabinopose-galactose
C	Quercetin-3-O-galactoside	OH	H	Galactose
D	Quercetin-3-O-glucuronide	OH	H	Glucuronic acid
E	Quercetin-3-O-glucoside	OH	H	Glucose
F	Kaempferol-3-O-glucoside	H	H	Glucose
G	Syringetin-3-O-glucoside	OCH_3	OCH_3	Glucose
H	Quercetin	OH	H	H
I	Kaempferol	H	H	H

Graphical Abstract
57x34mm (600 x 600 DPI)