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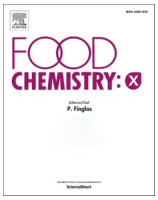

LC-MS/MS-based chemical profiling of water extracts of *Moringa oleifera* leaves and pharmacokinetics of their major constituents in rat plasma

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ABSTRACT

Moringa oleifera leaves (MOL) are native to India and have high biological activities. To better understand the basic pharmacodynamic materials, the chemical components in MOL and their pharmacokinetic properties were studied and quantitated using UPLC-Q-Exactive Orbitrap-MS. Forty-two compounds were identified, including phenolic acids and their derivatives, flavonoids, isothiocyanates, nucleosides, alkaloids, and other compounds. Two phenolic acids and six flavonoids were studied for their pharmacokinetic properties using UHPLC-MS/MS. Precision, accuracy, stability, matrix effects, and extraction recovery were verified. All substances that were measured reached their maximum within 0.5 h. Vicenin-2 had a high peak concentration and bioavailability. Kaempferol-3-O-rutinoside had a longer biological half-life than other components. The results from this study provide the data basis for subsequent comprehensive qualitative evaluation and potential MOL use in clinical applications.

1. Introduction

The *Moringa oleifera* Lam, commonly known as "Drumstick" (Pari & Kumar, 2002), is a naturalized species of the monogenetic family of the Moringaceae(Anwar et al., 2007). This plant is native to India but has been introduced to many other tropical regions over the years, and is widely distributed in Indonesia, the Philippines, and the Caribbean. The height of the Moringa tree is typically 5 to 10 m, sometimes even 15 m. It is a tree with a strong vitality and softwood. It has the ability to thrive in poor soil and remains largely unaffected by drought, showing rapid growth (Sánchez-Machado et al., 2006; Seshadri & Nambiar, 2003). The plant contains a variety of natural active ingredients and nutrients, including proteins, amino acids, minerals, vitamins, alkaloids, terpenes, flavonoids, phenolic acids, and isothiocyanate compounds, and has extremely high medicinal value. It is well known by the locals. Studies have been conducted to confirm some widely used medicinal properties (Cáceres et al., 1992; Leone et al., 2015). MO is also called the Miracle Tree, Tree of Life, Mother's Best Friend, God's Gift to Man, and Savior of the Poor by the locals due to its favorable properties (Mbikay, 2012).

Like other plants, *M. oleifera* has seeds, roots, flowers, leaves, fruits, and other useful parts, with different uses for each part. The tree's leaves are easy to harvest and are rich in chemical resources. They are the most commonly used part. They can be eaten directly along with the dried leaves or after extraction into an aqueous solution. In Nigeria, the MOL are eaten as a vegetable and can be used for stir-fry or soup and porridge. There has been no report of any side effects. In southern India, the leaves are also a popular food for babies and children. In many countries, desiccated MOL are widely recognized as a valuable source of protein, calcium, vitamins A, C, and E, β-carotene, amino acids, and various polyphenols. MOL are also used as growth supplements (Luqman et al., 2012; Singh et al., 2014). In addition to human consumption, MOL are also used in animal food. Because MOL are rich in natural antioxidants, they can improve the quality of meat, such as texture, color, flavor, and nutritional value (Moyo et al., 2012), and may increase food shelf life (Siddhuraju & Becker, 2003).

In addition to their edible value, a large number of scientific reports showed that MOL have antibacterial (Eilert et al., 1981), anti-cancer (Sreelatha et al., 2011), anti-inflammatory (Ezeamuzie et al., 1996),

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anti-ulcer, anti-oxidant (Diallo et al., 2001), anti-spasmodic, diuretic, anti-hyperglycemia (Ghasi et al., 2000), anti-hypertensive (Dangi et al., 2002), anti-hyperlipidemic, and other pharmacological effects. Almost all parts of this plant have been employed in the treatment of abdominal tumors, hysteria, scurvy, paralytic attacks, helminthic bladder issues, prostate complications, as well as sores, and addressing skin infections. MOL are used as an aphrodisiac and as a treatment for intestinal worms and asthma in Nigeria (Atawodi et al., 2010). They are used as an antipyretic and antidote in traditional Thai medicine (Sahakitpichan et al., 2011).

These extensive pharmacological effects of MOL are related to their abundant natural active substances (Hassan et al., 2021). Among them, flavonoids and phenolic acids are the most reported components, such as quercetin, kaempferol, and their glycoside derivatives, which have antioxidant and hypoglycemic properties, i.e., the lowering of blood lipid levels and anti-cancer effects. The various pretreatment procedures of MOL, such as extraction time and extraction temperature, may affect the effective content of these substances, which in turn affects their pharmacological activity.

In this study, the basic composition of MOL was examined, and the primary constituents were chosen for pharmacokinetic studies. We then developed a sensitive, efficient, and precise UHPLC-MS/MS method capable of simultaneously determining the analytes and internal standard (IS) in plasma samples of the MOL oral solutions, including chlorogenic and cryptochlorogenic acids, vicenin-2, vitexin, rutin, isoquercitrin, kaempferol-3-O-rutinoside and astragalin. After quantitation, the MOL extracts of interest were orally administered to lab test rats, as part of a pharmacokinetic study. The findings provide insight into evaluating MOL and exploring the underlying mechanisms of their pharmacological activities.

2. Experimental

2.1. Materials and reagents

The MOLs were obtained from the Yunnan Dayaoshan Trading Co., LTD (Yunnan, China). Chlorogenic acid (Purity >98%), cryptochlorogenic acid (Purity >98%), vicenin-2 (Purity >98%), vitexin (Purity >98%), rutin (Purity >98%), isoquercitrin (Purity >98%), kaempferol-3-O-rutinoside (Purity >98%), and astragalin (Purity >98%) were purchased from Dalian Meilun Biotechnology Co., LTD. LC/MS-grade acetonitrile was obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was obtained from Sigma-Aldrich (USA). HPLC-grade formic acid was obtained from the Damao Chemical Reagent Factory. Ultra-high-purity water was prepared with a water system purchased from Shenyang Jia he yi Technology Co., Ltd.

2.2. Plant material and extract preparation

Dried MOL were washed twice with ultra-pure water to remove mud and impurities and then thoroughly dried in the hood at room temperature for 3 days. The leaves were crushed and sieved (60 mesh sieve). 1 g of the crushed leaf powder was added to 10 mL ultrapure water, and reflux extracted twice at 100 °C. The water extract yield from *Moringa oleifera* leaves was 35.45%. The extracts were added to 100 mL of water for further quantification. The solutions were centrifuged at 12,000*g* for 10 min, then diluted tenfold, filtered through a 0.22 μm filter, and used for analytical quantitation.

The gastric lavage fluid for the pharmacokinetic study was prepared as follows:200.0 g of *Moringa oleifera* leaf powder was added to 10 times the weight of ultrapure water and reflux-extracted twice at 100 °C, each time for 1 h. The two filtrates were combined, evaporated, and concentrated at 45 °C to a crude drug amount of 3 g/mL.

2.3. Animals

Six male Sprague-Dawley rats (260–280 g) were procured from the Experimental Animal Center of Shenyang Pharmaceutical University. Prior to the study, these rats were accommodated in a controlled environment where the temperature was held at 20 ± 2 °C, relative humidity was maintained at 50 ± 20%, and a natural light-dark cycle was established for a week. Before the experiment, all rats underwent a 12-h fasting period during which they only had access to water for 12 h. The study was conducted conforming to the Guideline of Animal Experimentation of Shenyang Pharmaceutical University. The protocol was consented to by the Animal Ethics Committee of the university. The administered dose was 30 $g{\cdot}kg^{-1}$ (Raw medicinal·$weight^{-1}$) Certificate number: 210726211101403054. After the experiments, the rats were euthanized under deep anesthesia (intraperitoneal injection of sodium pentobarbital) for cervical dislocation. The animals were used for the pharmacokinetic research, which included three parts: determination of the content of active ingredients in actual plasma samples, validation of the methods used for content determination, determination of the drug concentration over time curves, and calculation of the pharmacokinetic parameters $T_{1/2}$, C_{max}, T_{max}, $AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$.

2.4. Component identification by UPLC-Q-Exactive Orbitrap-MS

A Vanquish Flex UPLC, comprising a quaternary solvent delivery system, and a column temperature controller were used for chromatographic separation. All samples were separated using an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.7 μm) at a temperature of 50 °C. Before the experiment, the effects of four different systems of the mobile phase, including methanol-water, methanol-0.1% formic acid, acetonitrile-water, and acetonitrile - 0.1% formic acid on the test substance, was investigated. We observed that the acetonitrile - 0.1% acid water system resulted in better component separation, allowing for improved compound identification. The mobile phase comprised water with 0.1% formic acid (eluent A) and acetonitrile (eluent B) using a gradient elution mode starting with10% B from 0 to 0.8 min, increasing to 10–20% B from 0.8 to 2.8 min, 20–70% B from 2.8 to 5.6 min, 70–90% B from 5.6 to 6.4 min, 90–100% B from 6.4 to 9 min, 100–100% B from 9 to 9.1 min, 100–2% B from9.1 to 10 min, and finally decreasing to 2–2% B. The flow rate was set to 0.3 mL/min, and the injection volume was configured as 5 μL.

The Orbitrap Exploris™ 120 (Thermo Scientific), a high-resolution tandem mass spectrometer, was utilized to detect the metabolites after chromatographic separation. The Q-Exactive spectrometer acquisition modes were set for positive and negative ion modes. The precursor ion spectra ranging from 70 to 1050 m/z were obtained at a resolution of 70,000 to reach an AGC target of $3e^6$. The maximum injection time applied was 100 ms. The fragment spectra were acquired at a 17,500 resolution with an AGC target of $1e^5$ and a maximum injection time of 50 ms. A quality control standard (Pooled sample) was obtained after every 10 samples to examine the stability of UPLC-Q-Exactive Orbitrap-MS.

2.5. Pharmacokinetic study of MOL

2.5.1. Analysis system

Quantitative analysis of the samples was conducted using an Agilent 1290 Series UHPLC system, a quaternary solvent delivery system, and a column temperature controller. Chromatographic separation was performed using an Agilent Zorbax SB-C18 RRHD column (2.1 × 100 mm , 1.8 μm). The mobile phase was composed of solvent A, aqueous phase containing 0.1% formic acid as buffer, and solvent B, acetonitrile, using a gradient elution process: 0–1 min, 5%–15% B; 1–2 min, 15–19% B; 2–6 min, 19–20% B, 6–6.5 min, 20–95% B; 6.5–7 min, 95–95% B; 7–8 min, constant at 95% B. The flow rate remained constant at 0.3 mL/min while the column temperature was set to 35 °C. The injection volume

was 2 μL.

Mass spectrometric detection and analysis were carried out in MS/MS mode. The flow rate of the drying gas (N_2) was 0.3 L/min. The temperature of the drying gas was set to 350 °C. A multi-response detection model was used for analysis. The Agilent MassHunter Workstation Acquisition Software Version B.05.01 and Qualitative Analysis Software Version B.07.00 were used for system control, data acquisition, and processing. Parameters for MRM component detection are shown in the **Supplementary Information, Supplementary Table 1.**

2.5.2. Preparation of standard solutions

The appropriate amounts of chlorogenic acid, cryptochlorogenic acid, vicenin-2, vitexin, rutin, isoquercitrin, kaemphenol-3-O-rutinosid, and astragalin were weighed. Stock solutions containing standard reference material were ultrasonicated in methanol to obtain solution concentrations at 83 μg·mL^{-1}, 86 μg·mL^{-1}, 56 μg·mL^{-1}, 692 μg·mL^{-1}, 104 μg·mL^{-1}, 102 μg·mL^{-1}, 90 μg·mL^{-1} and 67 μg·mL^{-1}. A proper amount of the stock solution of each reference substance was accurately mixed. The mixture was diluted with methanol to obtain 8 various standard concentrations that are listed in the **Supplementary Information, Supplementary Table 2.** A concentration of 100 μg·mL^{-1} of puerarin, after ultrasonication in methanol, was used as a reference and added as an internal standard to the stock solutions. The specific amounts of internal standard solution were diluted using methanol to achieve a concentration of 400 ng·mL^{-1}. The solutions were then stored in the refrigerator at a temperature of 4 °C for future use.

2.5.3. Sample preparation

The plasma contains numerous endogenous components that could significantly interfere with the target molecules. When comparing the effects of methanol and acetonitrile solvents on protein precipitation, we found that after methanol treatment, the response value of the tested substance was higher, and the matrix effect was small. Methanol was chosen as the pre-treatment solvent, and the effects of using methanol 4 times and 10 times on protein precipitation were investigated.

10 μL of methanol (volumes corresponding to the volumes of QC samples and the calibration curve) and 10 μL IS (400 ng·mL^{-1}) were introduced to 100 μL plasma samples. 400 μL of acetonitrile was then added to each sample and mixed using a vortex mixer for 3 min. Then, the mixture underwent centrifugation for 10 min at 14,000 rpm. The resulting supernatant was carefully transferred into a clean Eppendorf tube. The supernatant was further processed by evaporating it to dryness using a mild nitrogen gas flow. The residue obtained was then dissolved in 100 μL methanol, thoroughly mixed for 3 min using a vortex mixture, and centrifuged for an additional 10 min at 14,000 rpm. Then, 5 μL of the resulting supernatant was employed for further analysis using UHPLC-MS/MS.

2.5.4. LC-MS/MS method validation for quantifying MOL compounds in rat plasma

2.5.4.1. Specificity. Half an hour after the oral administration of the MOL extracts, the rat plasma samples were assessed by comparing them to spiked plasma samples, which contain working solutions and IS, and blank plasma samples obtained from six distinct rats. The evaluation aimed to determine the method's specificity and the identification of potential interferences from endogenous substances.

2.5.4.2. Calibration curves and the lowest limit of quantification (LLOQ). Blank rat plasma, individually spiked with the mixture of the standard solutions with varied concentrations and IS, was quantitatively monitored for three consecutive days, in repeated tests, to verify the calibration curve linearity. The calibration curves were derived by fitting the peak area ratios (y) of the analyte in relation to the IS against varying concentrations (x). The weighting factor was $1/x^2$. The LLOQ was estimated utilizing a signal-to-noise ratio of around 10 (S/N $\geq$ 10).

2.5.4.3. Precision and accuracy. Intra-day and inter-day precision and accuracy were examined by duplicate analysis of QC samples at varied concentrations: The concentrations of chlorogenic acid and cryptochlorogenic acid were 24, 600, and 4500 ng·mL^{-1}, respectively; The vicenin-2 concentrations were 22.4, 560, and 4200 ng·mL^{-1}. The vitexin concentrations were 19.2, 480, and 3600 ng·mL^{-1}. The rutin and isoquercitrin concentrations were 12.8, 320, and 2400 ng·mL^{-1}. The kaempferol-3-O- rutinoside concentrations were 3.2, 80, and 600 ng·mL^{-1}. The astragalin concentrations were 12, 300, and 2250 ng·mL^{-1}.

2.5.4.4. Recovery and matrix effects. The extracted plasma samples were spiked with the working solutions containing the IS; the post-extraction blank samples, which contain IS, were also spiked with the working solutions with three QC concentrations and were quantitated under the same analytical conditions. Recovery was determined by peak area differences of the peak areas between the corresponding extracted and post-extraction spiked samples. The matrix effects were determined from the peak area differences between the corresponding extracted samples and working solution mixtures. A total of six sample sets were analyzed in parallel.

2.5.4.5. Stability. Low, medium, and high concentrations of the QC samples were examined to investigate compound stability in rat plasma given the following test conditions: Storage of the sample in an autosampler at 4 °C for 12 h, storage at room temperature for 8 h, samples subjected to 3 freeze/thaw cycles, and storage of the sample at −80 °C for 30 days. Each of the aforementioned conditions was tested with three distinct QC concentrations.

2.5.4.6. Pharmacokinetic studies. The rats were acclimated to the facility for a week. Prior to the experiments, rats were fasting for 12 h, with the exception of readily available drinking water. Different doses of aqueous moringa leaf extract were orally administered to the rats in the early stages to ensure that the components remained within the detectable concentration range. At low dosages, astragalin and vitexin could not be detected continuously. This problem was resolved by increasing the dosage concentration to the maximum safe dose range. Finally, a dose of 30 g/kg of the MOL (in dry matter) was administered to the animals. Blood samples (100 μL each) were obtained from the rats' orbital venous plexus at specific time points: 0, 0.03, 0.08, 0.17, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h after administrating the extract. The samples underwent immediate centrifugation at 8000 rpm for 10 min, and then the supernatant was collected. The plasma samples were obtained and stored at −80 °C. "Phoenix Win Nonlin 6.0" software was utilized to determine the pharmacokinetic parameters.

3. Results

3.1. Identified compounds

A total of 43 compounds were identified by UPLC-Q-Exactive Orbitrap-MS, including 12 flavonoids, 18 phenolic acids, 8 alkaloids, 1 isothiocyanate, and 4 other types. (See Figure 1-3 and Table 1)

3.1.1. Flavonoids

The retention time of compound 29 was 3.78 min. In negative ion mode, the molecular ion $[M-H]^-$ of compound 29 was at *m/z* 609.1464. The molecular formula of compound 29 was predicted to be $C_{27}H_{30}O_{16}$ by the Xcalibur software. During collision-induced dissociation (CID), one molecule of rutin is dissociated to form secondary ion fragments $[M-H-Rha-Glu]^-$ with *m/z* 300.0276. Glycones can undergo loss of neutral fragments and produce characteristic fragment ions [M-H-Rha-Glu-H-

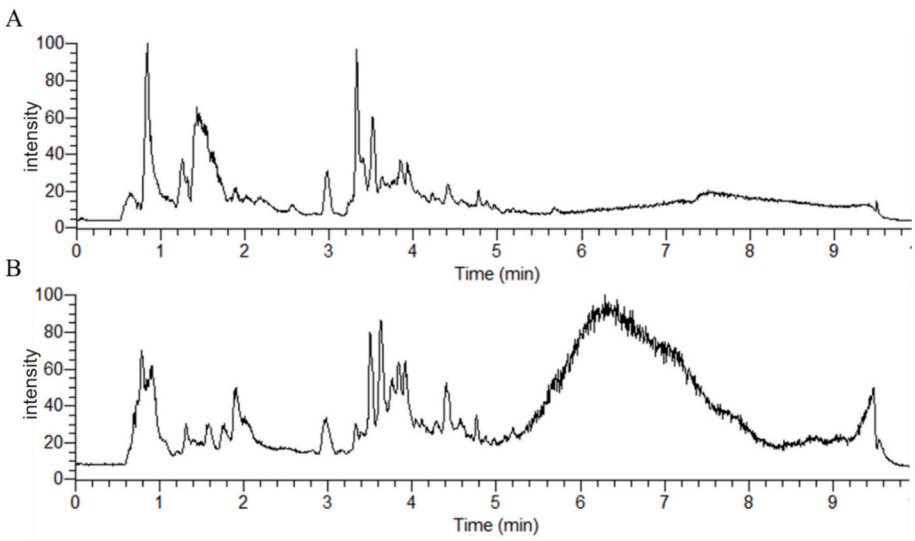

Fig. 1. The total ion chromatograms of *Moringa oleifera* leaf extract in (A) positive ion mode and (B) negative ion mode.

CO]$^-$ at m/z 271.0249, and undergo retro-Diels-Alder (RDA) cleavage to produce 151.0039 fragment ions. Compound 29 was identified as rutin through comparison with information available in the literature (Hui et al., 2023; Yi et al., 2016).

The retention time of compound 34 was 3.91 min. In negative ion mode, its molecular ion [M-H]$^-$ was at *m/z* 447.0931. Xcalibur software predicted that its molecular formula was $C_{21}H_{20}O_{11}$, with one glucose ring removed. The fragment ions [M-H-Glu]$^-$ m/z 284.0326, [M-H-OCH_3]$^-$ m/z 255.0299, and [M-H-CO]$^-$ m/z 227.0350 are obtained by loss of OCH_3 and CO fragments, respectively. A comparison with the literature confirms its identity as astragalin (Guo et al., 2019; Tatiane et al., 2019; Yi et al., 2016).

3.1.2. Phenolic acids

The retention time of compound 23 was 3.53 min. In negative ion mode, its molecular ion [M-H]$^-$ was at *m/z* 353.0880. Xcalibur software predicted that its molecular formula was $C_{16}H_{18}O_9$. The caffeic acid acyl group can be removed to form fragment ions [M-H-$C_9H_6O_3$]$^-$ at *m/z* 191.0562, and the molecular ions can also be fragmented to form a secondary ion [M-H-$C_7H_{10}O_5$]$^-$ with m/z 179.1350. The loss of one CO_2 fragment can produce fragment ions at *m/z* of 135.0452. By comparing the mass spectrum information reported in the literature, the compound was confirmed to be chlorogenic acid (Guo et al., 2019; Hui et al., 2023; Yi et al., 2016).

The retention time of compound 12 is 2.31 min. In negative ion mode, its molecular ion [M-H]$^-$ is at m/z 315.0724. Xcalibur software predicted that its molecular formula is $C_{13}H_{16}O_9$. After the loss of a glucose fragment [M-H-Glu]$^-$ with *m/z* 153.0194 is formed, and the molecular ion [M-H-CHO_2]$^-$ further fragments to give a m/z of 108.0217. By comparing the mass spectrum information reported in the literature, the compound was identified as protocatechuate-4-O-glucoside (Qiang et al., 2020).

3.1.3. Alkaloids

The retention time of compound 2 is 0.87 min. In positive ion mode, its molecular ion [M + H]$^+$ is at m/z 138.0551. Xcalibur software predicts that its molecular formula is $C_7H_7NO_2$. This compound generated a fragment ion [M + H-COOH]$^+$ at m/z 94.0652. By comparing the mass spectrum information reported in the literature, the compound was confirmed as trigonelline (Gilmar et al., 2022; Tatiane et al., 2019).

3.1.4. Isothiocyanate

The retention time of compound 18 was 3.34 min. In negative ion mode, its molecular ion [M-H] $^-$ was at m /z 570.0956, The Xcalibur software predicted its molecular formula to be $C_{20}H_{29}NO_{14}S_2$. After collusion-induced dissociation, rhamnoside and HSO_4 fragments were removed to obtain [M-H-Rha-HSO_3] $^-$ at m/z 328.0856, and [M-H-$C_{14}H_{17}NO_5S$] $^-$ at m/z 259.0135. By comparing the mass spectrum with information found in the literature, the compound was identified as glucomoringin (Guo et al., 2019).

3.2. Pharmacokinetic results

3.2.1. Validation of the bioanalysis method

3.2.1.1. Specificity. Method specificity was evaluated by comparing the analyte retention times and the presence of interference peaks of the respective chromatograms. Fig. 4 illustrates the chromatograms of (a) blank plasma samples, (b) blank plasma samples spiked with analytes and IS, and (c) plasma samples obtained subsequent to the oral administration of the MOL extract to the rats. Consistent retention time and the absence of any interference peaks were observed for all analytes among the samples.

3.2.1.2. Linearity and LLOQ. The concentrations of chlorogenic acid and cryptochlorogenic acid were in the range of 12–6000 ng·mL^{-1}. The concentrations of vicenin-2 were 11.2–5600 ng·mL^{-1}. The concentrations of vitexin were 9.6–4800 ng·mL^{-1}. The concentrations of rutin and isoquercitrin were 6.4–3200 ng·mL^{-1}. The concentrations of kaempferol-3-O-rutinoside were 1.6–800 ng·mL^{-1}. For the simulated plasma samples with a concentration of 6–3000 ng·mL^{-1}, the chromatograms were obtained using a Plasma sample pretreatment method, and each concentration was determined in both samples. Calibration parameters and LLOQs of eight compounds are shown in **Supplementary Information, Supplementary Table 3**.

3.2.1.3. Precision and accuracy. The precision and accuracy for both intra- and inter-day assessments were examined using six replicates of QC samples, containing analytes at low, medium, and high concentrations, as previously outlined. The results are presented in **Supplementary Information, Supplementary Table 4**. The accuracy (expressed as Relative Error, RE) varied from −7.68% to 13.15%, while the precision (expressed as Relative Standard Deviation, RSD) ranged from 1.37% to 12.2%, demonstrating a reliable method that has been developed.

3.2.1.4. Recovery and matrix effect. **Supplementary Table 5** in **the**

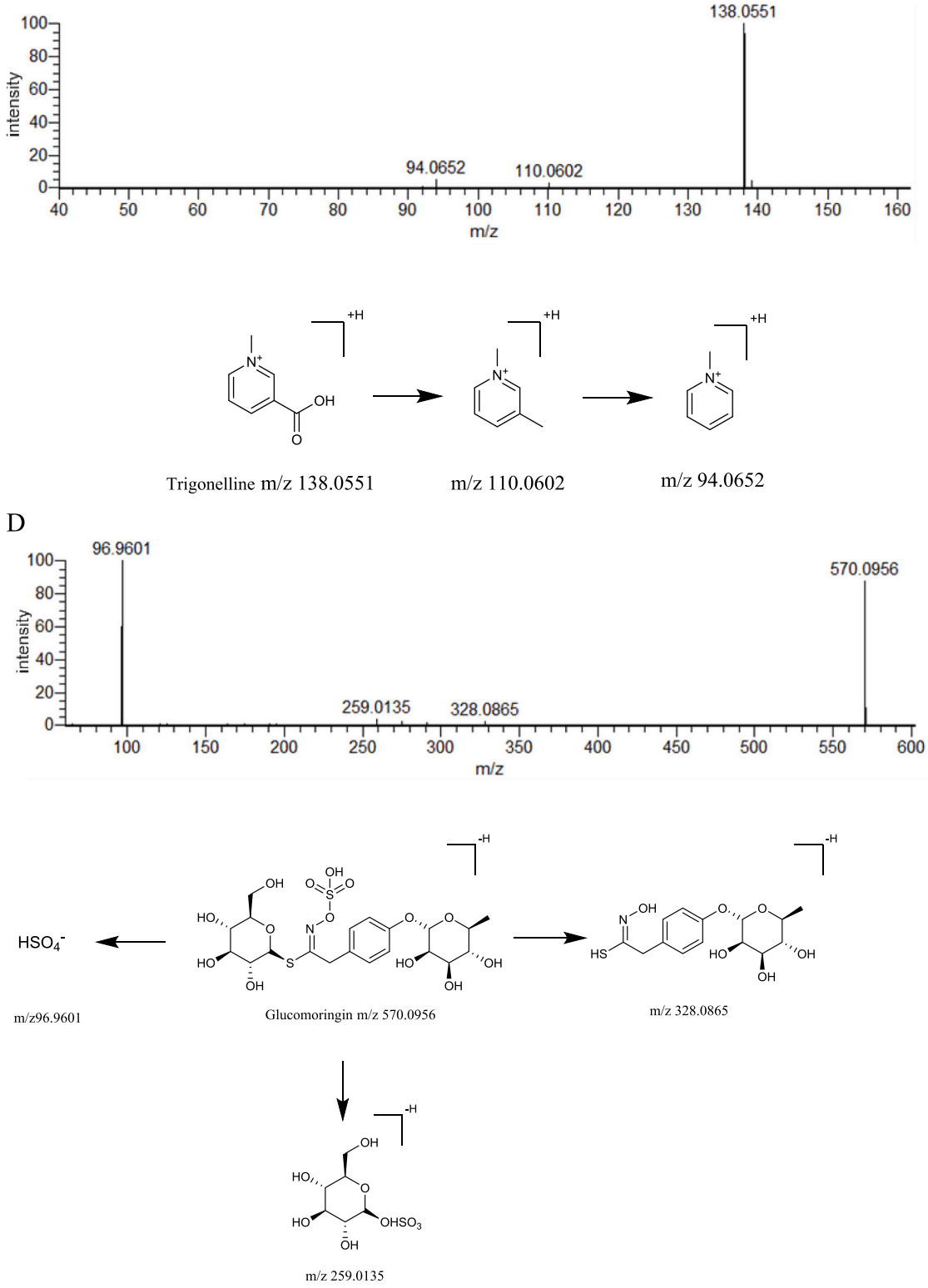

Fig. 2. The product ion spectrum and fragmentation pattern of rutin (A), chlorogenic acid (B), trigonelline (C), and glucomoringin (D).

Supplementary Information shows that the extraction recovery of all 8 analytes, across three varied concentrations, fell within the range of 73.52% to 98.75%. Moreover, the matrix effects on all analytes ranged between 81.66% and 113.68%. The results revealed that the matrix effect and extraction recovery are within satisfactory limits.

3.2.1.5. Stability. The stability of the 13 analytes during sample collection and processing was examined under varied storage conditions assessed with spiked plasma samples at three different QC concentrations. **Supplementary Table 6** in **the Supplementary Information** shows that the RSDs for all tested samples were consistently below 13.78%, indicating that all analytes remained stable at the four different test conditions.

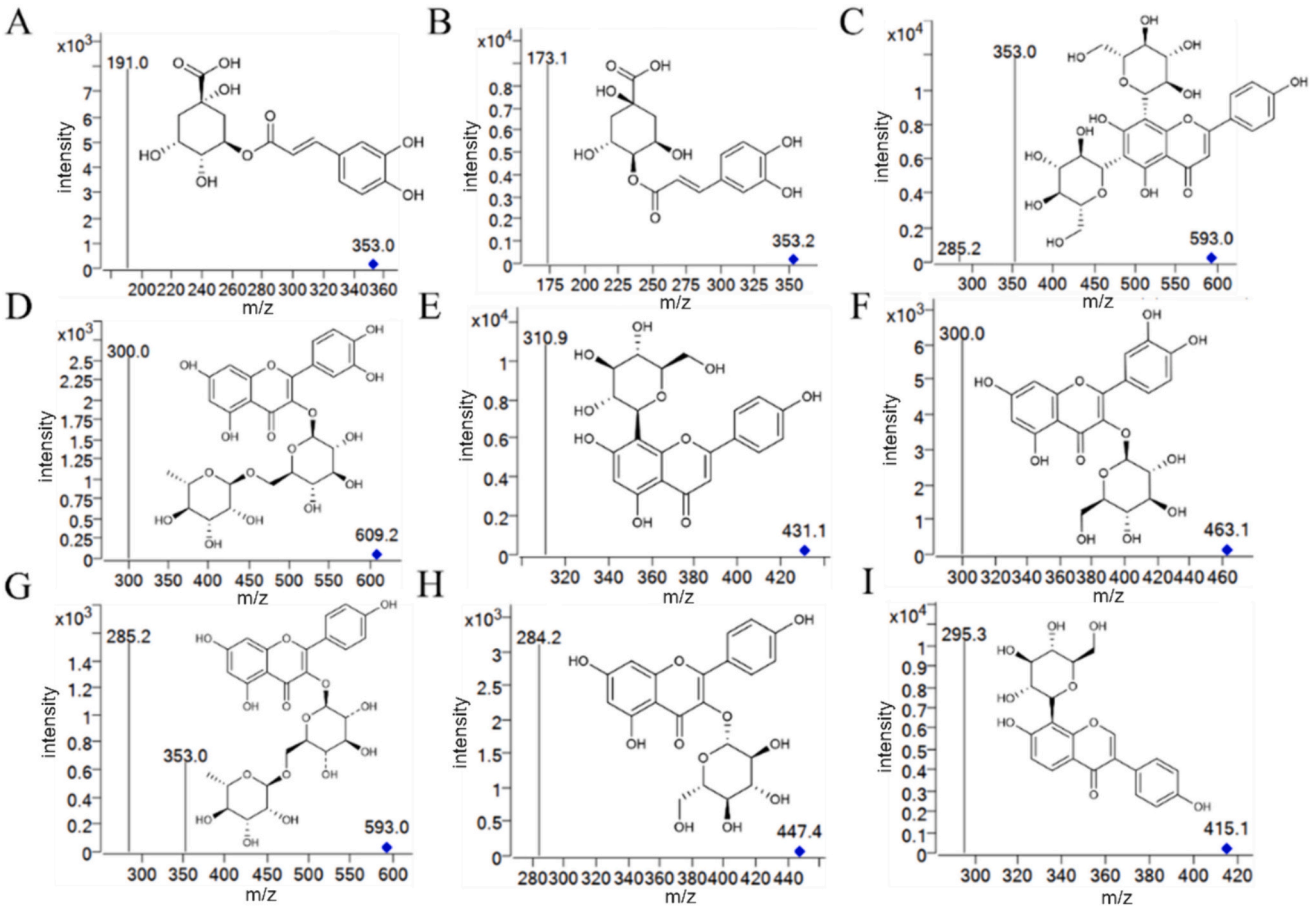

Fig. 3. Mass spectra of chlorogenic acid (A), cryptochlorogenic acid (B), vicenin-2 (C), rutin(D), vitexin (E), isoquercitrin (F), kaempferol-3-O-rutinoside (G), astragalin (H), puerain (I) in negative ion mode.

3.2.1.6. Pharmacokinetic study. Plasma samples obtained from rats that were fed the MOL extracts (30.0 g/kg) were analyzed using UHPLC-MS/MS. Fig. 5 **(A) to (H)** shows the average component concentration versus time plots for the plasma samples obtained from the rats following the oral administration of the MOL extract ($n = 6$). The components include chlorogenic acid, cryptochlorogenic acid, vicenin-2, vitexin, rutin, isoquercitrin, kaempferol-3-O-rutinoside, and astragalin. **Supplementary Table 7** in the **Supplementary Information** shows a summary of the main pharmacokinetic parameters of the 8 analytes.

4. Discussion

In this study, the pharmacokinetics of water extracts from *Moringa oleifera* leaves in rats were investigated, and the UHPLC-MS/MS method was developed to simultaneously obtain the concentrations of chlorogenic acid, cryptochlorogenic acid, vicenin-2, vitexin, rutin, isoquercitrin, kaempferol-3-*O*-rutinoside and astragalin in plasma. The method of analysis was confirmed to be sensitive, rapid, and reliable. The pharmacokinetic parameters showed that all components could be detected within 5 min; the peak concentrations of each substance after oral appeared within 0.5 h, indicating that all compounds were absorbed into the bloodstream quickly and distributed to other tissues, which agrees with previous reports (Abdou et al., 2019; Mumtaz et al., 2021). Flavonoid glycosides contain sugar groups and have good water solubility, showing high peak concentrations in vivo. The peak concentrations of vicenin-2 and cryptochlorogenic acid were 1367.34 ng·mL^{-1} and 1058.74 ng·mL^{-1}, respectively, higher than the concentration levels of other components, indicating that their concentrations in plasma were larger. The differences in the number and location of hydroxyl groups in the aglycones and the substitution and glycosylation sites may be critical factors affecting their absorption. The peak time of isoquercitrin was 0.24 h with a maximum blood concentration of 643.38 ng·mL^{-1}, which could be detected within 12 h, consistent with the results of previous studies (Caicedo-Lopez et al., 2021; Cuellar-Núñez et al., 2021). The elimination half-life of each substance under investigation was short, indicating that it could be rapidly eliminated in vivo. The half-life of chlorogenic acid was 5.06 h, which is different from the values reported in previous studies. The components of traditional Chinese medicine are complex, and multiple components may interact, and result in interferences of pharmacokinetic characteristics among the components (Chen et al., 2020; Jaiswal et al., 2009; Sosa-Gutiérrez et al., 2018). After the oral administration of a typical traditional Chinese medicine extract, a variety of components enter the body. They may interact with each other, resulting in a so-called bi-peak phenomenon. The reasons are complex and may lead to delayed gastric emptying, hepatoenteric circulation, etc., and the transformations of the compounds found in the traditional Chinese medicine components. In the intestinal environment, components containing the same parent nucleus can easily be transformed (Balusamy et al., 2019; Nayak et al., 2016; Xie et al., 2018). Since vicenin-2 and vitexin have the same parent nucleus, the bi-peak may be an indicator of the mutual transformation of these two compounds. It may also be caused by hepatoenteric circulation, and the specific reasons need to be further studied in vivo.

5. Conclusions

This study developed a sensitive, efficient, and precise UHPLC-MS/MS method for the simultaneous determination of both the

Table 1
Compounds identified in *Moringa oleifera* leaves by UPLC-Q-Exactive Orbitrap-MS.

No.	Compound	Formula	Ion adduction	t_R/ min	Measured m/z (Da)	Cal m/z (Da)	ppm	Fragment ions (m/z Da)
1	Malic acid	$C_4H_6O_5$	$[M-H]^-$	0.80	133.0142	133.0131	8.3	115.0036, 89.0244, 71.0138, 43.0189
2	Trigonelline	$C_7H_7NO_2$	$[M+H]^+$	0.87	138.0551	138.0550	0.7	110.0602, 94.0652
3	5-Hydroxymethylfurfural	$C_6H_6O_3$	$[M+H]^+$	0.95	127.0392	127.0390	1.6	109.0286, 99.0442, 81.0336, 69.0336
4	*L*-Valine	$C_5H_{11}NO_2$	$[M+H]^+$	1.06	118.0863	118.0863	0.0	72.0808, 55.0543
5	Cytosine	$C_4H_5N_3O$	$[M+H]^+$	1.26	112.0506	112.0505	0.9	95.0241, 69.0448
6	Benzaldehyde	C_7H_6O	$[M+H]^+$	1.43	107.0491	107.0491	0.0	95.0492, 79.0543
7	Cinnamic acid	$C_9H_8O_2$	$[M+H]^+$	1.45	149.0598	149.0597	0.7	131.0491, 121.0648, 103.0543, 79.0543
8	*p*-Coumaric acid	$C_9H_8O_3$	$[M+H]^+$	1.51	165.0545	165.0546	−0.6	147.0441, 123.0441, 119.0492, 95.0492
9	Citric Acid	$C_6H_8O_7$	$[M-H]^-$	1.63	191.0197	191.0186	5.8	111.0088, 87.0088, 85.0295, 57.0346
10	Cinnamaldehyde	C_9H_8O	$[M+H]^+$	1.67	133.0649	133.0648	0.8	115.0545, 105.0700, 86.0965
11	*L*-Leucine	$C_6H_{13}NO_2$	$[M+H]^+$	1.72	132.102	132.1019	0.8	86.0964, 44.0495
12	Protocatechuic acid-4-*O*-glucoside	$C_{13}H_{16}O_9$	$[M-H]^-$	2.31	315.0724	315.0711	4.1	153.0194, 108.0217
13	Salicylic acid	$C_7H_6O_3$	$[M-H]^-$	2.49	137.0244	137.0233	8.0	93.0346, 109.0295 ,
14	Nicotinamide	$C_6H_6N_2O$	$[M+H]^+$	2.57	123.0554	123.0553	0.8	96.0445, 80.0496
15	Guanosine	$C_{10}H_{13}N_5O_5$	$[M+H]^+$	2.96	284.0981	284.0989	−2.8	152.0569, 135.0303, 110.0349
16	*L*-Phenylalanine	$C_9H_{11}NO_2$	$[M+H]^+$	3.31	166.0863	166.0863	0.0	149.0596, 131.0492, 120.0807, 103.0543
17	Gallic acid 4-*O*-glucoside	$C_{13}H_{16}O_{10}$	$[M-H]^-$	3.34	331.0671	331.0660	3.3	313.0564, 168.0065, 149.9958, 125.0244
18	Glucomoringin	$C_{20}H_{29}NO_{14}S_2$	$[M-H]^-$	3.34	570.0956	570.0946	1.8	328.0865, 259.0135, 113.0249, 96.9601
19	Adenosine	$C_{10}H_{13}N_5O_4$	$[M+H]^+$	3.34	268.1042	268.1040	0.7	136.0618, 119.0356, 57.0335
20	Gallic acid	$C_7H_6O_5$	$[M-H]^-$	3.36	169.0143	169.0131	7.1	125.0245, 107.0502, 81.0346, 69.0347
21	Neochlorogenic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	3.38	353.0897	353.0867	8.5	191.0561, 179.0350, 173.0458, 135.0452, 109.0295, 93.0345
22	Protocatechuic acid	$C_7H_6O_4$	$[M-H]^-$	3.40	153.0194	153.0182	7.8	109.0295, 111.0454, 137.0244
23	Chlorogenic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	3.53	353.0880	353.0867	3.7	191.0562, 179.0350, 135.0452, 111.0450
24	Vicenin-2	$C_{27}H_{30}O_{15}$	$[M-H]^-$	3.61	593.1511	593.1501	1.7	353.0664, 473.1087, 297.0767
25	7-Hydroxycoumarin	$C_9H_6O_3$	$[M+H]^+$	3.62	163.0392	163.0390	1.2	145.0286, 135.0442, 117.0337, 89.0387
26	*p*-Coumaroylquinic acid	$C_{16}H_{18}O_8$	$[M-H]^-$	3.63	337.0934	337.0918	4.7	191.0560, 163.0401, 119.0502, 111.0452, 93.0345
27	Cryptochlorogenic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	3.64	353.0891	353.0867	6.8	191.0562, 173.0456, 135.0452, 111.0454, 93.0346
28	Rutin	$C_{27}H_{30}O_{16}$	$[M-H]^-$	3.78	609.1464	609.1450	2.3	300.0276, 271.0249, 255.0301, 151.0399
29	Protocatechualdehyde	$C_7H_6O_3$	$[M-H]^-$	3.79	137.0244	137.0233	8.0	93.0346, 119.0141, 108.0218
30	3-*O*-Feruloylquinic acid	$C_{17}H_{20}O_9$	$[M-H]^-$	3.81	367.1024	367.1024	0.0	191.0562, 173.0456, 134.0373, 111.0453, 93.0345
31	Vitexin	$C_{21}H_{20}O_{10}$	$[M-H]^-$	3.83	431.0982	431.0973	2.1	341.0668, 311.0562, 283.0613
32	Kaempferol-3-*O*-rutinoside	$C_{27}H_{30}O_{15}$	$[M-H]^-$	3.83	593.1514	593.1501	2.2	285.0403, 255.0300, 227.0352
33	Isoquercitrin	$C_{21}H_{20}O_{12}$	$[M-H]^-$	3.90	463.0883	463.0871	2.6	300.0274, 271.0247, 255.0301
34	Astragalin	$C_{21}H_{20}O_{11}$	$[M-H]^-$	3.91	447.0931	447.0922	2.0	284.0326, 255.0299, 227.0350
35	Quercetin-acetyl-glycoside	$C_{24}H_{22}O_{15}$	$[M-H]^-$	3.93	505.0989	505.0977	2.4	300.0276, 271.0249, 255.0299, 151.0036
36	Isorhamnetin-3-*O*-glucoside	$C_{22}H_{22}O_{12}$	$[M-H]^-$	3.93	477.1040	477.1028	2.5	314.0433, 285.0405, 271.0248, 243.0300
37	Kaempferol	$C_{15}H_{10}O_6$	$[M+H]^+$	3.93	287.0555	287.0550	1.7	258.0527, 241.0505, 213.0546, 165.0185
38	Quinic acid	$C_7H_{12}O_6$	$[M-H]^-$	3.94	191.0553	191.0550	1.6	147.0450, 87.0088, 85.0295
39	Kaempferol-acetyl-glycoside	$C_{23}H_{22}O_{12}$	$[M-H]^-$	3.95	489.1039	489.1028	2.2	284.0327, 255.0300, 227.0350
40	Quercetin	$C_{15}H_{10}O_7$	$[M+H]^+$	3.99	303.0501	303.0499	0.7	285.0394, 257.0450, 247.0580, 165.0180
41	Ferulic acid	$C_{10}H_{10}O_4$	$[M-H]^-$	4.38	193.0506	193.0495	5.7	178.0272, 148.0763, 136.0167, 74.0248, 108.0217
42	Isorhamnetin	$C_{16}H_{12}O_7$	$[M-H]^-$	4.51	315.0511	315.0499	3.8	300.0275, 283.0255, 164.0115, 151.0038, 107.0139
43	α-Linolenic acid	$C_{18}H_{30}O_2$	$[M+H]^+$	5.20	279.2317	279.2319	−0.7	261.2229, 243.2112, 95.0856, 81.0700

components and ISs of the MOL in plasma samples. The methodology used in this pharmacokinetic investigation of the analytes in rat plasma could successfully be used after oral administration. The evaluated and validated pharmacokinetic parameters serve as a foundational framework for potential clinical MOL applications.

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CRediT authorship contribution statement

Jiahong Wang: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Yiyang Du:** Validation, Methodology, Investigation, Funding acquisition. **Jiahe Li:** Methodology, Investigation. **Bing Yu:** Visualization, Software, Methodology, Formal analysis, Data curation. **Chuang Ren:** Methodology, Investigation. **Tingxu Yan:** Validation, Funding acquisition, Formal analysis. **Ying Jia:** Software, Funding acquisition. **Bosai He:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Yiyang Du reports financial support was provided by National Natural Science Foundation of China. Tingxu Yan reports financial support was provided by National Natural Science Foundation of China. Tingxu Yan reports financial support was provided by Scientific Research Fund of Liaoning Provincial Education Department. Ying Jia reports financial support was provided by Scientific Research Fund of Liaoning Provincial Education Department. Ying Jia reports financial support was provided by Key Laboratory of polysaccharide bioactivity evaluation of TCM of Liaoning Province-Liaoning Distinguished Professor Project. Ying Jia reports financial support was provided by High-level innovation and entrepreneurship team of Liaoning Province. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work

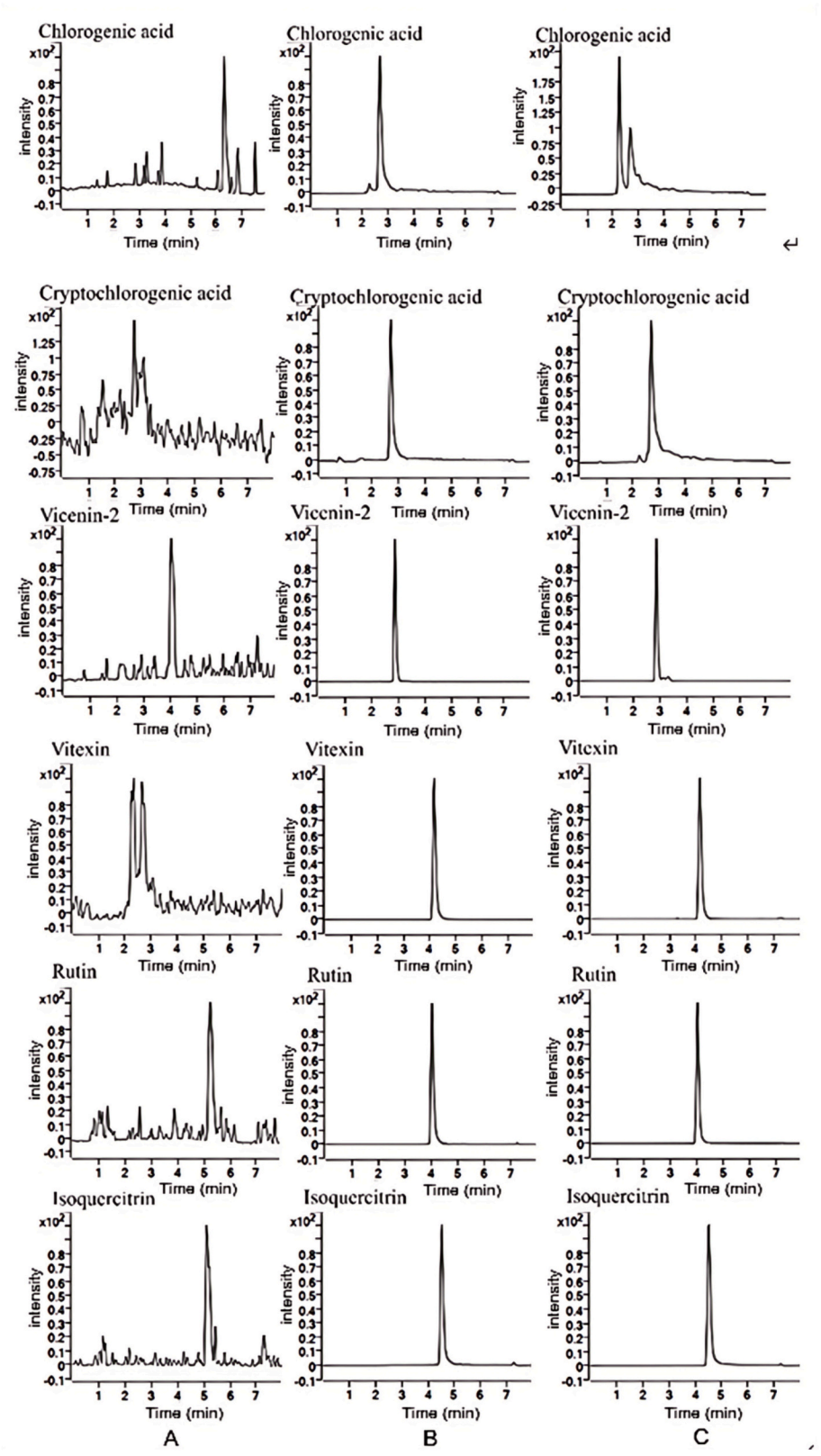

Fig. 4. Multiple Reaction Monitoring (MRM) chromatograms obtained from blank rat plasma (A), blank rat plasma spiked with analytes (LLOQ) and the IS (B), plasma sample obtained from a male rat 0.5 h after drug administration (C).

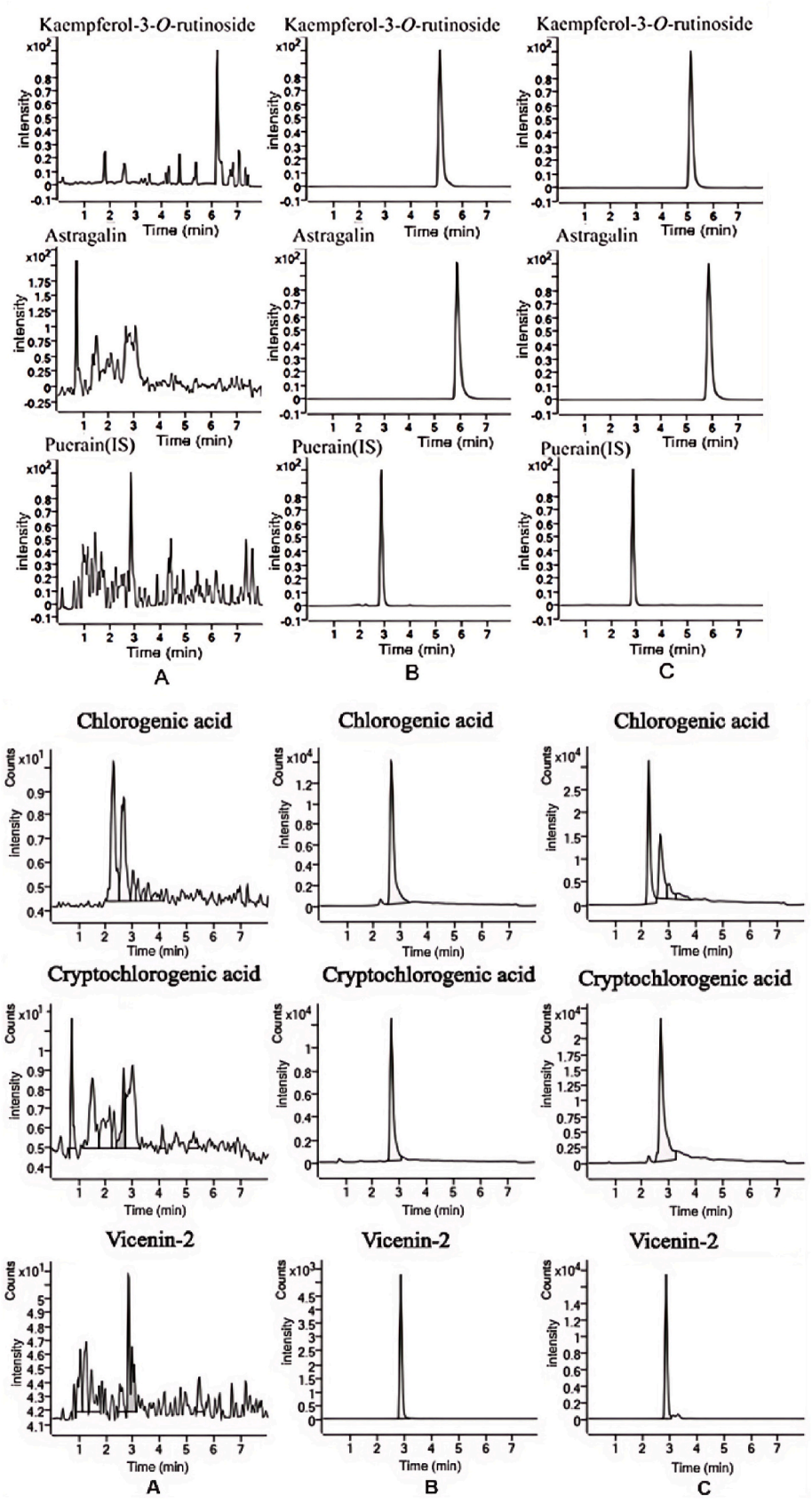

Fig. 4. (*continued*).

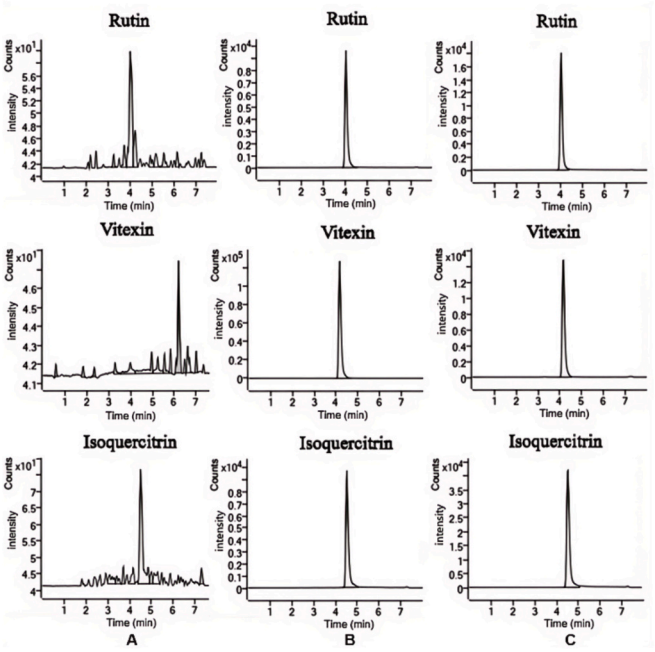

Fig. 4. (*continued*).

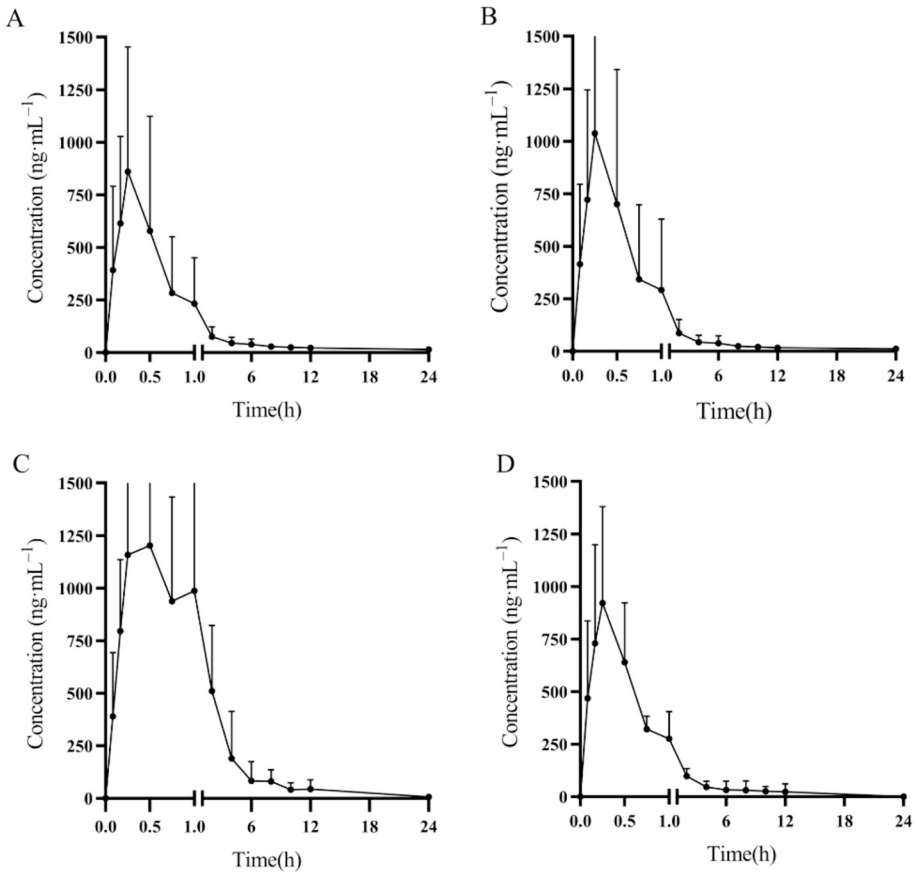

Fig. 5. The averaged concentration versus time plots for chlorogenic acid (A), cryptochlorogenic acid (B), vicenin-2 (C), vitexin (D), rutin (E), isoquercitrin (F), kaempferol-3-O-rutinoside (G), astragalin (H) in rat plasma following the oral administration of *Moringa oleifera* leaf extract ($n = 6$).

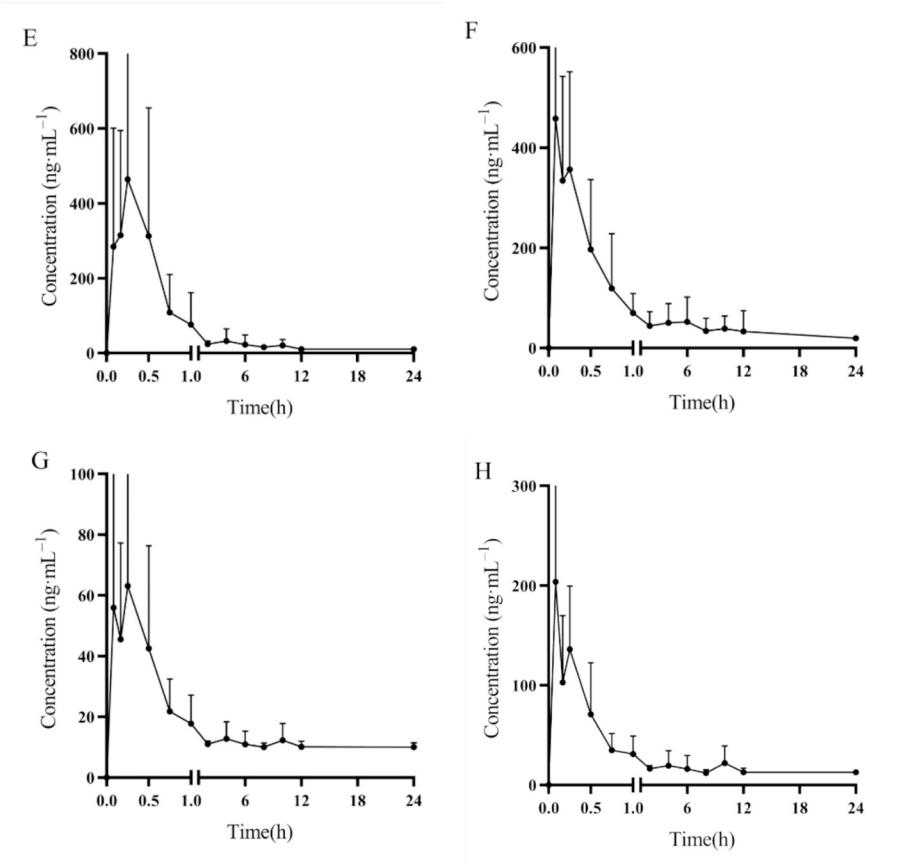

Fig. 5. (*continued*).

reported in this paper.

Data availability

The supporting data are available from the corresponding author(s) upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101585.

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