Determination of oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin in mouse blood by UPLC-MS/MS and its application to pharmacokinetics

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Received: August 16, 2021 • Accepted: October 19, 2021

ABSTRACT

In this study, a UPLC-MS/MS method was developed to measure the concentrations of the flavonoids oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin in the blank mouse blood, and the method was then used in the measurement of the pharmacokinetics of the compounds in mice. Oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin were administered intravenously at a dose of 5 mg kg⁻¹, and the mouse blood (20 µL) was withdrawn from the caudal vein 0.0833, 0.25, 0.5, 1, 2, 4, 6, 8, and 10 h after administration. The mobile phase used for chromatographic separation by gradient elution was composed of acetonitrile and water (0.1% formic acid). The analytes were detected by operating in electrospray ionization (ESI) positive-ion mode using multiple reactions monitoring (MRM). The intra-day and inter-day accuracy ranged from 86.2 to 109.3%, the intra-day precision was less than 14%, and the inter-day precision was less than 15%. The matrix effect ranged from 85.3 to 111.3%, and the recovery of the analytes after protein precipitation were all above 78.2%. This method had the advantages of high sensitivity, accuracy, and recovery, and it had excellent selectivity, which enabled it to be applied to measuring the pharmacokinetics of the analytes in mice.

KEYWORDS

oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, mouse, pharmacokinetics

INTRODUCTION

Oroxylum indicum (L.) Vent is a species of small flowering trees belonging to the family of Bignoniaceae. It is native to India, Nepal, and southern China and often grows on hillsides, along streams, and in shrubs up to 1,000 m above sea level. O. indicum (L.) Vent is shaped like a wide, flaky butterfly wing, whose flower extends from the base to the outside, 5–200 px long and 3.5–112.5 px wide. In recent years, different parts of O. indicum (L.) Vent have been used in traditional medicine because they exhibit various biological activities, such as anti-inflammatory [1–3], antibacterial [4–7], anticancer [2, 8–10], and anti-atherosclerosis [11]. In addition, O. indicum (L.) Vent has been used as an expectorant, for protecting liver function [12–14], treating hypoglycemia, preventing and treating cataracts and liver cancer [15–18], and protecting nervous system function [19–21]. The chemical constituents of
**EXPERIMENTAL**

**Chemicals and animals**

Oroxin A, oxorin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, guaijaverin, and cirsimarin (internal standard, IS) (purity >98%, Fig. 1) were purchased from Chengdu Mansite Bio-Technology Co., Ltd. (Chengdu, China). HPLC-grade methanol and formic acid were purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water was prepared by a Millipore Milli-Q water system (Bedford, MA, USA). Mice (male, body weight: 20–22 g) were obtained from the Animal Experimental Center of Wenzhou Medical University.

**Instruments and conditions**

An ACQUITY H-Class UPLC and a XEVO TQS-micro triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA) equipped with a UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) was used in this study for quantitative analysis. The column temperature was maintained at 40°C during chromatographic separation. The mobile phase consisted of a gradient elution of acetonitrile and water (0.1% formic acid) with a flow rate of 0.4 mL min⁻¹ and a run time of 3 min. The method comprised the following sequence: 0–0.2 min, 10% (isocratic) acetonitrile; 0.2–1.5 min, 10–80% acetonitrile; 1.5–2.0 min, 80% (isocratic) acetonitrile; 2.0–2.2 min, 80–10% acetonitrile; 2.2–3.0 min, 10% (isocratic) acetonitrile. Nitrogen was used as the desolvation gas (900 L h⁻¹) and the nebulizing gas (50 L h⁻¹). The capillary voltage was set to 2.5 kV, the ion source temperature was 150°C, and the desolvation temperature was 450°C. ESI operating in positive-ion mode using MRM was used to monitor the transitions of m/z 433.1 → 271.1 (cone voltage: 25 V; collision voltage: 22 V) for oxorin A, m/z 595.2 → 271.1 (cone voltage: 8 V; collision voltage: 18 V) for oxorin B, m/z 285.1 → 270.1 (cone voltage: 54 V; collision voltage: 20 V) for oroxylin A, m/z 461.1 → 285.1 (cone voltage: 10 V; collision voltage: 12 V) for oroxyloside, m/z 255.1 → 152.9 (cone voltage: 70 V; collision voltage: 24 V) for chrysin, m/z 579.2 → 255.1 (cone voltage: 10 V; collision voltage: 26 V) for chrysin 7-O-beta-gentiobioside, m/z 435.1 → 303.1 (cone voltage: 18 V; collision voltage: 10 V) for guaijaverin, and m/z 477.2 → 315.1 (cone voltage: 30 V; collision voltage: 22 V) for cirsimarin (IS).

**Standard curve preparation**

Stock solutions (100 μg mL⁻¹) of oxorin A, oxorin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, guaijaverin, and cirsimarin were prepared in a 1:1 (v/v) mixture of methanol:water. Then, a series of working solutions (20, 50, 200, 500, 2,000, 5,000, and 10,000 ng mL⁻¹) of these compounds were prepared by diluting the stock solutions with methanol. All solutions were refrigerated at 4°C until further use. An aliquot of each of the standard working solutions was added to a sample of blank mouse blood to prepare a series of solutions with concentrations of 2, 5, 20,
50, 200, 500, and 1,000 ng mL\(^{-1}\). These solutions were injected into the UPLC-MS/MS instrument, and the peak areas were plotted against their respective concentrations to generate the standard curves of oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin.

**Blood sample pretreatment**

A 20 µL aliquot of mouse blood was added to a 1.5-mL centrifuge tube containing 100 µL of cirsimarin (50 ng mL\(^{-1}\)) in methanol, and the mixture was mixed vortexed for 1 min and then centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant (80 µL) was transferred to a liner pipe in vial.

**Method validation**

The UPLC-MS/MS method was validated in accordance with international standards by measuring the selectivity, matrix effects, linearity, precision, accuracy, recovery, and stability of the method.

To investigate whether endogenous substances present in the mouse blood interfered with the determination of the substance to be measured and internal standard, the UPLC-MS/MS results of six blank mouse blood samples from different sources were compared to the results of the LLOQ samples prepared with the corresponding blank mouse blood. The LLOQ is the lowest concentration of analyte in a sample which can be quantified reliably. For pharmacokinetic studies the LLOQ adapted to lower than 5% of the \(C_{\text{max}}\).

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*Fig. 1. Chemical structures of oroxin A (A), oroxin B (B), oroxylin A (C), oroxyloside (D), chrysin (E), chrysin 7-O-beta-gentiobioside (F), guaijaverin (G), and cirsimarin (IS) (H)*
Seven different concentrations of each analyte in blank mouse blood over the concentration range of 2–1000 ng mL\(^{-1}\) were respectively operated under "blood sample pretreatment.” The concentration of each was plotted against the peak area ratio of the analyte (in addition to the IS), and the weighted (\(W = 1/X^2\)) least square method was utilized for linear regression analysis, the equation of which was interpolated to determine the concentration of the analytes in the mouse blood.

Three concentrations (2, 4, 180, and 900 ng mL\(^{-1}\)) of each of the analytes in blank mouse blood were prepared for quality control, and six samples of each of the three concentrations were analyzed and tested within 3 days according to the "blood sample pretreatment” method. The QC samples are analyzed against the calibration curve, and the obtained concentrations are compared with the nominal value to get the value of accuracy. Precision was demonstrated for the LLOQ, low, medium and high QC samples, within a single run and between different runs, i.e. using the same runs and data as for the demonstration of accuracy.

Aliquots (20 \(\mu\)L) of blood from six different mice from different sources were added to 100 \(\mu\)L of methanol, and after vortexing for 1 min followed by centrifugation at 13,000 rpm for 10 min at 4 °C, the supernatants were removed and added to a fresh tube. The quality control samples of low and high concentrations were then added to the tubes containing the supernatants. After vortexing for 1 min, a 2 \(\mu\)L aliquot of the mixture was injected into the UPLC-MS/MS for analysis, and the corresponding peak area (A) was measured. The 20 \(\mu\)L of blood that was removed was replaced with 20 \(\mu\)L of deionized water, and the corresponding peak area (B) was measured using the same method. The matrix effect was then calculated by comparing the peak area ratio of the two treatments at each concentration using the formula: \(A/B \times 100\%\).

Samples of low, medium, and high concentrations (2, 4, 180, and 900 ng mL\(^{-1}\)) of the analytes in blank mouse blood were prepared, and 6 samples of each concentration were analyzed according to the operation described under the “blood sample pretreatment” section. Meanwhile, an aliquot (20 \(\mu\)L) of mouse blank blood was added to 100 \(\mu\)L of methanol, and the mixture was vortexed for 1 min and centrifuged for 15 min at 13,000 rpm. The supernatant was removed and added to another centrifuge tube, and the control samples with concentrations of 2, 4, 180, and 900 ng mL\(^{-1}\) were added to these tubes. After vortexing for 1 min, the supernatants were removed, and 2.00 \(\mu\)L of the supernatant was analyzed by UPLC-MS/MS to determine the peak area of the concentrations (\(n = 3\)). The extraction recovery was then calculated by comparing the ratio of the chromatographic peak area after extraction to the chromatographic peak area of the samples without extraction.

The stabilities of the analytes were investigated by comparing the accuracy of the quantitation of the three concentrations (4, 180, and 900 ng mL\(^{-1}\)) of oroxin A, oroxin B, oroxin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin after repeated freeze-thaw cycles, after sitting in the autosampler for 2 h, and after storing at −20 °C for 30 days. These results were compared to freshly prepare blank blood samples.

**Pharmacokinetics**

A total of 42 mice were randomly divided into 7 groups. Doses (5 mg kg\(^{-1}\)) of oroxin A, oroxin B, oroxin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin were administered to the mice intravenously (i.v.). The blood (20 \(\mu\)L) was withdrawn from the caudal vein at 0.0833, 0.25, 0.5, 1, 2, 4, 6, 8, and 10 h after administration and collected into 1.5-mL centrifuge tubes. After treatment of the blood, the plasma was injected into the UPLC-MS/MS for analysis. The Masslynx 4.1 software package (Waters Corp.) was used for data acquisition and processing, and the DAS 2.0 software package was used to analyze the pharmacokinetic parameters of the analytes.

**RESULTS**

**Selectivity**

Figures 2 and 3 display the UPLC-MS/MS chromatograms of the blank mouse blood samples supplemented with oroxin A, oroxin B, oroxin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin. The results showed that, not only did the gradient elution enable the effective separation of the analytes, but also the endogenous substances in the mouse blood samples had little effect on the measurement of the samples.

**Standard curve**

The correlation coefficients (R\(^2\)) of the standard curves of oroxin A, oroxin B, oroxin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin in mouse blood that were acquired over the range of 2–2000 ng mL\(^{-1}\) were all higher than 0.992. In the equations of the standard curves, x represented the concentration of compound in mouse blood, and y represented the peak area ratio of drugs, as shown in Table 1.

**Accuracy, precision, recovery, and matrix effect**

As shown in Table 2, the intra-day and inter-day accuracy of all of the analytes were 86.2–109.3%. The intra-day precision was less than 14%, and the inter-day precision was less than 15%. The matrix effect ranged from 85.3 to 111.3%. In addition, the recovery was all above 78.2%.

**Stability**

Based on the results of stability tests of the blood samples stored in the autosampler at ambient temperature for 2 h, stored at −20 °C for 30 days, and subjected to three freeze-thaw cycles, the UPLC-MS/MS analysis method demonstrated excellent stability, accuracy, and precision (Table 3), which indicated that the method met the requirements for the detection of analytes in biological samples.
Pharmacokinetics

Blood concentration-time curves were generated by plotting the administration time against the drug concentration (Fig. 4). The obtained pharmacokinetics data were all processed by the DAS2.0 software, and the pharmacokinetic parameters were calculated using the non-atrioventricular model (Table 4).

DISCUSSIONS

The selection of the electrospray ionization mode in mass spectrometry is often used in methodological research [29–31]. In this study, we compared the sensitivities of the detection of x in both positive and negative mode and found that ESI positive-ion mode was more sensitive than negative-ion mode, so we chose ESI positive-ion mode for detection of the analytes. To ensure the best liquid chromatography conditions, endogenous interfering substances should be separated as much as possible from both the analytes of interest and the internal standard. Because oroxin A, oroxin B, orxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, guaijaverin, and cirsimarin (IS) in blank blood.

**Fig. 2.** UPLC-MS/MS chromatograms of oroxin A, oroxin B, orxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, guaijaverin, and cirsimarin (IS) in blank blood
protein precipitation using methanol were the best compared to the other two solvents, so methanol was used for precipitation.

UPLC-MS/MS was used for the quantitative determination of the flavonoid analytes in mouse blood [32, 33]. Compared to traditional HPLC analysis, the time of UPLC was significantly faster [34–38], with the sample analysis being completed in 3 min. In this study, a UPLC-MS/MS method was developed to determine the concentrations of oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, guaijaverin, and cirsimarin (IS).

In this study, we developed a UPLC-MS/MS method to measure the concentrations of seven kinds of flavonoid compounds in mouse blood and then used this method to study the pharmacokinetics after intravenous administration. Compared with the previously study on determination

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**Table 1. Parameters of the standard curves of oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin in mouse blood**

| Compound                        | Regression Equation       | Correlation Coefficient ($R^2$) | Linear Range (ng mL$^{-1}$) | LLOQ (ng mL$^{-1}$) |
|---------------------------------|---------------------------|---------------------------------|----------------------------|---------------------|
| Oroxin A                        | $y = 0.0002x - 0.0001$    | 0.9965                          | 2–2000                     | 2                   |
| Oroxin B                        | $y = 0.0005x - 0.0004$    | 0.9956                          | 2–2000                     | 2                   |
| Oorxylin A                      | $y = 0.0011x - 0.0004$    | 0.9991                          | 2–2000                     | 2                   |
| Oroxyloside                     | $y = 0.0005x - 0.0004$    | 0.9974                          | 2–2000                     | 2                   |
| Chrysin                         | $y = 0.0009x - 0.0005$    | 0.9978                          | 2–2000                     | 2                   |
| Chrysin 7-O-beta-gentiobioside  | $y = 0.0032x + 0.0028$    | 0.9994                          | 2–2000                     | 2                   |
| Guaijaverin                     | $y = 0.0007x - 0.0004$    | 0.9998                          | 2–2000                     | 2                   |

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**Fig. 3. UPLC-MS/MS chromatograms of blank mouse blood spiked with oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, guaijaverin, and cirsimarin (IS)**

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### Table 2. Accuracy, precision, matrix effect, and recovery of oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin in mouse blood (n = 6)

| Compound     | Concentration (ng mL⁻¹) | Precision (% RSD) | Accuracy (%) | Matrix effect (%) | Recovery (%) |
|--------------|-------------------------|------------------|--------------|------------------|--------------|
|              |                         | Intraday         | Inter-day    | Intraday         | Inter-day    |
| Oroxin A     | 2                       | 109.2            | 92.8         | 11.6             | 14.2         | 101.1        | 82.3         |
|              | 4                       | 89.5             | 92.5         | 11.4             | 11.4         | 103.2        | 80.6         |
|              | 180                     | 91.7             | 95.4         | 5.1              | 5.1          | 107.7        | 84.7         |
|              | 900                     | 100.5            | 98.9         | 8.5              | 8.5          | 101.9        | 82.1         |
|              | 2                       | 86.2             | 108.5        | 11.9             | 12.6         | 104.8        | 78.2         |
| Oroxin B     | 4                       | 108.5            | 102.5        | 9.5              | 9.2          | 100.8        | 90.9         |
|              | 180                     | 103.1            | 102.3        | 3.5              | 6.5          | 108.5        | 81.2         |
|              | 900                     | 99.9             | 104.4        | 10.3             | 4.5          | 104.4        | 87.4         |
|              | 2                       | 88.2             | 94.4         | 10.6             | 10.2         | 89.8         | 85.5         |
| Oronylin A   | 2                       | 86.6             | 100.7        | 8.3              | 7.9          | 88.7         | 90.6         |
|              | 4                       | 104.2            | 100.6        | 6.9              | 8.1          | 86.3         | 92.2         |
|              | 180                     | 95.6             | 109.3        | 5.6              | 3.7          | 85.3         | 89.4         |
|              | 900                     | 93.9             | 93.3         | 6.2              | 5.6          | 110.1        | 86.8         |
| Oroxyloside  | 4                       | 99.1             | 101.5        | 6.5              | 7.5          | 107.4        | 89.1         |
|              | 180                     | 98.4             | 100.4        | 9.6              | 9.1          | 111.3        | 85.5         |
|              | 900                     | 97.9             | 100.2        | 7.9              | 6.3          | 104.1        | 81.9         |
| Chrysin      | 2                       | 95.1             | 93.9         | 8.4              | 11.8         | 109.4        | 82.5         |
|              | 4                       | 99.6             | 97.3         | 9.6              | 5.7          | 110.4        | 83.1         |
|              | 180                     | 95.6             | 97.3         | 7.1              | 6.9          | 100.8        | 81.4         |
|              | 900                     | 101.3            | 102.7        | 8.1              | 8.3          | 106.6        | 83.5         |
| Chrysin 7-O-beta-gentiobioside 4 | 107.7 | 94.8 | 6.8 | 7.2 | 100.5 | 88.9 |
|              | 180                     | 95.2             | 97.1         | 8.7              | 8.2          | 100.8        | 85.7         |
|              | 900                     | 107.7            | 97.4         | 4.3              | 9.3          | 101.1        | 84.5         |
|              | 2                       | 94.2             | 107.6        | 13.2             | 12.3         | 100.5        | 95.5         |
| Guaijaverin  | 4                       | 107.3            | 106.7        | 6.3              | 7.1          | 103.3        | 85.2         |
|              | 180                     | 109.3            | 94.5         | 6.3              | 4.4          | 103.7        | 84.5         |
|              | 900                     | 104.5            | 97.9         | 8.3              | 4.4          | 105.6        | 80.3         |

### Table 3. Stability of oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin in mouse blood

| Compound     | Concentration (ng mL⁻¹) | Autosampler ambient | Ambient 2h | −20 °C 30d | Freeze-thaw |
|--------------|-------------------------|---------------------|------------|------------|-------------|
| Oroxin A     | 4                       | 107.5               | 90.8       | 103.2      | 105.1       | 9.7         |
|              | 180                     | 99.9                | 98.3       | 98.4       | 104.2       | 8.2         |
|              | 900                     | 97.6                | 102.5      | 95.3       | 99.8        | 10.8        |
| Oroxin B     | 4                       | 103                 | 98.3       | 106.1      | 103.5       | 9.3         |
|              | 180                     | 99.7                | 97.7       | 103.5      | 96.4        | 8.7         |
|              | 900                     | 98.2                | 102.5      | 93.1       | 89.4        | 9.1         |
| Oronylin A   | 4                       | 97.8                | 106.8      | 101.3      | 90.1        | 9.2         |
|              | 180                     | 91.9                | 97.3       | 94.8       | 89.7        | 9.6         |
|              | 900                     | 104.5               | 99.7       | 93.9       | 106.1       | 7.8         |
| Oroxyloside  | 4                       | 101.8               | 98.4       | 96         | 103.5       | 7.9         |
|              | 180                     | 98.7                | 95.8       | 102        | 108.9       | 4.5         |
|              | 900                     | 93.5                | 100.8      | 108.3      | 110.4       | 7.3         |
| Chrysin      | 4                       | 102.9               | 103.3      | 107.8      | 108.5       | 10.5        |
|              | 180                     | 101.2               | 102.7      | 108.7      | 108.7       | 8.3         |
| Chrysin 7-O-beta-gentiobioside 4 | 98.3 | 97.4 | 104.3 | 93.6 | 8.7 |
| Guaijaverin  | 4                       | 102.3               | 94.8       | 104.7      | 102.6       | 9.4         |
Fig. 4. Blood concentration-time curves of oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin after intravenous administration to mice

Table 4. The main pharmacokinetic parameters of oroxin A, oroxin B, oroxylin A, oroxyloside, chrysin, chrysin 7-O-beta-gentiobioside, and guaijaverin in mouse blood (n = 6)

| Parameters                      | \( \text{AUC}_{0-1\text{h}} \) (ng mL\(^{-1}\)·h) | \( \text{AUC}_{0-\infty} \) (ng mL\(^{-1}\)·h) | \( t_{1/2\alpha} \) (h) | \( \text{CL}_{z/F} \) (L h\(^{-1}\) kg\(^{-1}\)) | \( V_{z/F} \) (L kg\(^{-1}\)) | \( C_{max} \) (ng mL\(^{-1}\)) |
|---------------------------------|---------------------------------|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Oroxin A                        | 32.9 ± 5.0                      | 33.5 ± 4.9                      | 0.8 ± 0.1       | 151.4 ± 20.6    | 183.6 ± 38.4    | 75.5 ± 8.4      |
| Oroxin B                        | 215.1 ± 46.6                    | 217.4 ± 47.5                    | 0.6 ± 0.2       | 24.0 ± 5.7      | 18.9 ± 5.8      | 455.2 ± 104.2   |
| Oroxylin A                      | 184.4 ± 17.1                    | 208.6 ± 19.6                    | 5.2 ± 4.0       | 24.1 ± 2.2      | 172.6 ± 117.2   | 333.7 ± 62.2    |
| Oronxyloside                    | 632.8 ± 113.3                   | 643.9 ± 108.7                   | 2.8 ± 1.1       | 7.9 ± 1.2       | 32.5 ± 14.6     | 1,702.2 ± 367.7 |
| Chrysin                         | 749.8 ± 164.5                   | 763.6 ± 174.9                   | 1.9 ± 0.9       | 6.8 ± 1.6       | 17.2 ± 5.0      | 1,494.9 ± 246.4 |
| Chrysin 7-O-beta-gentiobioside  | 521.7 ± 70.7                    | 580.5 ± 62.0                    | 4.3 ± 1.8       | 8.7 ± 1.1       | 54.0 ± 23.8     | 878.9 ± 166.9   |
| Guaijaverin                     | 1,108.0 ± 215.6                 | 1,208.4 ± 226.7                 | 4.3 ± 0.6       | 4.2 ± 0.7       | 25.9 ± 6.1      | 1,017.4 ± 259.1 |
of investigated flavonoids and pharmacokinetic study, the pharmacokinetic of different flavonoids compounds in O. indicum (L.) Vent was first reported in mice.

ACKNOWLEDGMENTS

This work was supported Beijing Kangmeng Charity Foundation (H20013B), People’s Livelihood Fund Project of Rui’an Science and Technology Bureau (MS2018019).

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