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Phytochemical characterization, antioxidant potential, and health risk assessment of Radix Oryzae Glutinosae

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Abstract

Radix Oryzae Glutinosae (ROG) has been used as a traditional anhidrotic agent in China. Ten samples were systematically assessed based on four aspects of their chemical profiles and antioxidant activity. The former was achieved using ultraviolet-visible spectra (UV), infrared absorption spectra (IR), proton nuclear magnetic resonance spectroscopy (1H NMR), and inductively coupled plasma-mass spectrometry (ICP-MS) fingerprints, and antioxidant activity was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay, and the ferric reducing antioxidant power (FRAP) assay. The presence of amino acids, flavonoids, organic acids, and sugars was indicated in all samples. A hierarchical cluster analysis and a canonical discriminant analysis were introduced to interpret the results of the multi-pattern fingerprint. The results of the correlations between the individual elements and between each element with the different types of metabolites displayed many interesting patterns. In vitro studies revealed that all samples displayed antioxidant activities. A pollution status examination and heavy metal evaluation of the ROG samples were performed. The human health risk assessment associated with the intake of potentially harmful elements in herbs was calculated in terms of the estimated daily intake, the target hazard quotient, and the lifetime cancer risk. The Nemerow multi-factor pollution index results suggested that all samples belonged to the serious pollution level. The results of the noncarcinogenic risk assessment study indicated that the pattern of consumption of this herb in China seems to suggest an excessive health hazard associated with some toxic elements studied. The carcinogenic risk results suggested that there is a potential risk due to As, Cr, Cd, and Ni for consumers, and these may contribute to the population cancer burden through ROG ingestion. This study provides insights into the organic medicinal constituents and the trace elements of the herb. The results will enable this herb to be used more effectively and safely.

Keywords: Antioxidant activity, Chemical profiles, Human health risk assessment, Pollution index, Radix Oryzae Glutinosae

1. Introduction

Traditional Chinese medicines (TCMs) are the fundamental source of healthcare in China due to their historical and cultural background [1,2]. However, many issues regarding TCMs, such as their quality, safety, and efficacy, remain problematic because a few compounds that are the so-called biomarkers do not adequately describe the complex composition of these herbal medicines. Radix Oryzae Glutinosae (ROG) is the root of Oryza sativa L. var. glutinosa Matsum and, is a crop that belongs to the Gramineae family. A decoction of ROG has typically been prescribed to treat spontaneous sweating and night sweating, thirsty throat, hepatitis, and filariasis in TCM [3,4]. ROG is used as an...
important folk medicine and a functional food in China, and it grows in rice production areas throughout China. The large consumption of this herb indicates an urgent need for comprehensive studies regarding its metabolite constituents for quality evaluation purposes because authenticity and high-quality coherence can guarantee expected remedial outcomes from the use of TCM. A comprehensive chemical analysis consists of the correlation between pharmacological activities and complex chemical mixtures. Chemical fingerprints can capture the complexity of a Chinese herb, and this is a result of its various chemical compositions and the diagnostically diverse abundance levels of its constituents. In the current study, a metabolomics approach is used to differentiate ROG samples that originated from 10 different rice production areas based on their ultraviolet-visible spectra (UV), infrared absorption spectra (IR), and proton nuclear magnetic resonance spectroscopy (1H NMR) fingerprints. To the best of our knowledge, there is no information regarding the antioxidant activity of ROG. Therefore, we also designed this study to investigate the antioxidant properties of the hydroalcoholic extract obtained from ROG.

Chinese herbs constitute an important link in the transfer of elements from soil to humans [5]. Oral exposure to herbs polluted by heavy metals could cause a series of adverse health effects. However, a pollution status examination and health risk assessment of the consumption of the combined elements in ROG have not been conducted. In this study, the mineral elements in ROG samples are determined using the inductively coupled plasma-mass spectrometry (ICP-MS) method. The single-factor pollution index method (PI) and the Nem-erow multi-factor pollution index method (PN) are used to analyze the heavy metal pollution degree in the ROG samples under the combined activity of single heavy metal elements and assorted metal elements. Indices for the health risk evaluation are then reported as the estimated daily intake (EDI), the target hazard quotient (THQ), and the lifetime cancer risk (CR).

2. Materials and methods

2.1. Chemicals and samples

The ethanol (analytical grade) was purchased from Shanghai Suyi Chemical Reagent Co. Ltd. (Shanghai, China). Potassium bromide (KBr), tetramethylsilane (TMS), deuterium oxide (D2O), nitric acid (HNO3), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), anhydrous potassium persulfate (K2S2O8), aqueous iron sulfate (FeSO4·7H2O), and L-ascorbic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Deionized water was obtained from a Millipore water purification system (EMD Millipore Corporation, Burlington, MA, USA).

A total of 10 ROG samples were collected from the Chinese herb pharmacy and Chinese medicinal material markets all over China. The samples were thoroughly washed using distilled water and then dried at 50 °C in a drying cabinet until constant weights were achieved. The dried samples were milled and then passed through a 60-mesh sieve. Finally, they were stored in plastic bags at 4 °C prior to analysis.

2.2. UV-vis spectroscopic analysis

A total of 0.150 g of dried powder of ROG dissolved in 20.0 mL of 50% ethanol (ethanol: water, 1:1, v/v) was extracted ultrasonically for 30 min. The obtained extract was filtered through quantitative filter paper (Whatman, Maidstone, Germany), and the filtrate was collected for the UV-vis analysis. The UV-vis absorbance spectra were collected using a UV-1801 spectrophotometer (Beijing Beifen-Ruili Analytical Instrument Co., Ltd., Beijing, China) in a 1.00 cm pathlength quartz cell against a blank of 50% ethanol. The scanning range was 190–700 nm at 1.0 nm intervals. The UV-vis fingerprint profile of water, chloroform, 50% ethanol, and ethanolic extracts of ROG were selected in a wavelength range of 200–400 nm due to the proper baseline and sharpness of the peaks. The spectrum of each sample was collected in duplicate to obtain an average result. In the resultant data matrix, 10 samples using 201 absorption intensities were finally used for the statistical analysis.

2.3. FT-IR spectroscopic analysis

An accurately weighed quantity (1.05 g) of the herbal powder from each sample was immersed in 140 mL of 50% ethanol in a conical flask. The flasks were shaken to mix the herbal powder with the solvent before the mixtures were ultra-sonicated for 30 min at 23 °C. The extracts were then filtered, and the filtrate was concentrated to complete the drying process under reduced pressure. The IR spectra of the sample extracts were conducted on a Nicolet is50 IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with OMNIC software. The sample extract was pressed into a 1 mm
tub for analysis. The 1H NMR spectra of the herbal extracts were accumulated in the 40 KBr pellet for the FT-IR measurement. Ten scans were averaged over the 0.00–0.02 ppm width forming a region at 20 °C. Every spectrum was acquired using the NOESYPRESAT pulse sequence to suppress the large signal peak from the residual water. For each analysis, 64 transients were collected as 32 K data points with a spectral width of 9615.4 Hz using a relaxation delay of 2.0 s and an acquisition time of 1.70 s. TMS was used as an internal reference for the chemical shift (δ) of 0.0 ppm of the NMR signals. The free induction decay signals were zero filled and exponentially multiplied by a 0.3 Hz line-broadening factor prior to the Fourier transformation. These measurements were conducted using the Chenomx NMR suite version 7.7 software (Chenomx Inc., Edmonton, Canada).

All of the 1H NMR spectra were imported into MestreNova version 6.1.1 software (Mestrelab Research, Santiago de Compostela, Spain), and phasing and baseline corrections of the spectra were manually executed. A spectral alignment was conducted due to the tiny chemical shift difference among the different spectra. The acquired spectral intensities were scaled to TMS and binned into spectral regions of a 0.02 ppm width forming a region of 0.00–8.52 ppm to provide 233 total integrated chemical shift regions per NMR spectrum. The signals at δ 0.86–0.90 ppm, δ 3.32–3.36 ppm (residual ethanol), and δ 4.72–4.96 ppm (water) were excluded. Each spectrum was then normalized using the total integral area and converted to an ASCII file with identical parameters.

2.4. 1H NMR analysis

A total of 5 mg of the dried herbal extract was dissolved in D2O and transferred to a 5-mm NMR tube for analysis. The 1H NMR spectra of the herbal extracts were recorded using a 14.1 T Agilent DD2 600 MHz NMR spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 5 mm triple resonance cold probe, operating at 599.793 MHz and maintained at 20 °C. Every spectrum was acquired using the NOESYPRESAT pulse sequence to suppress the large signal peak from the residual water. For each analysis, 64 transients were collected as 32 K data points with a spectral width of 9615.4 Hz using a relaxation delay of 2.0 s and an acquisition time of 1.70 s. TMS was used as an internal reference for the chemical shift (δ) of 0.0 ppm of the NMR signals. The free induction decay signals were zero filled and exponentially multiplied by a 0.3 Hz line-broadening factor prior to the Fourier transformation. These measurements were conducted using the Chenomx NMR suite version 7.7 software (Chenomx Inc., Edmonton, Canada).

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2.5. Antioxidant assay in vitro

2.5.1. Extract preparation

Each accurately weighed sample powder (6.00 g) was extracted three times using 50 mL of a 19:1 (v/v) ethanol-water solution for 2 h at 85 °C, and the respective extract solutions were combined. The final extract was evaporated to dryness using rotary evaporation under reduced pressure (60 Pa) at 40 ± 2 °C. The obtained crude extract of each sample was collected for the antioxidant activities assay.

2.5.2. DPPH radical scavenging assay

DPPH powder (19.7 mg) was dissolved in 250 mL of ethanol to make the 0.20 mmol/L DPPH radical standard solution. The prepared solution was stored in the refrigerator (4 °C) and kept in the dark. The radical scavenging activity of the ROG extracts against the DPPH radical using spectrophotometry was conducted as described by Wang et al. (2017) [6] with minor modifications. The DPPH cation solution (2.0 mL) was mixed with 2.0 mL of the sample extract and incubated for 20 min in the dark at room temperature. Reduction at an absorbance at 514 nm was monitored by the UV-vis spectrophotometer. The percentage of inhibitory activity was calculated as: \[ \frac{(A_{\text{negative control}} - A_{\text{sample}})}{A_{\text{negative control}}} \times 100\% \]
where \(A_{\text{sample}}\) denotes the absorbance of the sample solution (2.0 mL) with the DPPH solution (2.0 mL), and \(A_{\text{negative control}}\) denotes the absorbance of ethanol (2.0 mL) with the DPPH solution (2.0 mL). The results were expressed as the IC50 (half maximal inhibitory concentration) value (mg/mL), where the IC50 indicates the 50% scavenging effect concentration of the extract. L-ascorbic acid was used as the positive control. Testing of each sample was performed in triplicate.

2.5.3. ABTS radical scavenging assay

A phosphate-buffered saline (PBS) powder was dissolved in distilled water to prepare a 0.01 mol/L PBS solution. The cation-radical ABTS+ solution was generated one day prior to the analysis by mixing stock solutions of ABTS (7.0 mmol/L) and potassium persulfate (140.0 mmol/L). Then the resulting solution was homogenized and incubated at ambient temperature for 12–16 h in darkness. The ABTS solution was diluted with the phosphate buffer until an absorbance value of 0.70 ± 0.02 AU at 734 nm was achieved.

The ABTS assay was evaluated using the method reported by Kuo et al. (2021) [7] with slight modifications. Aliquots of 0.5 mL of each sample solution with various concentrations were mixed thoroughly with 4.5 mL of the ABTS working solution. The resulting mixture was allowed to stand at ambient temperature for 10 min, and the absorbance was read at 734 nm against a reference solution using the UV-vis spectrophotometer. The inhibition percentage was calculated using the following equation: \[ \frac{(A_{\text{negative control}} - A_{\text{sample}})}{A_{\text{negative control}}} \times 100\% \]
where \(A_{\text{sample}}\) denotes the absorbance of the sample solution (0.5 mL) with the ABTS solution (4.5 mL), and \(A_{\text{negative control}}\) denotes the
absorbance of ethanol (0.5 mL) with the ABTS solution (4.5 mL). The results are expressed as the IC<sub>50</sub> value (mg/mL), where IC<sub>50</sub> indicates the 50% scavenging effect concentration of the extract. L-ascorbic acid was used as the positive control. The testing for each sample was performed in triplicate.

2.5.4. Ferric reducing antioxidant power assay

The Ferric reducing antioxidant power (FRAP) working solution was freshly produced by mixing the 300 mmol/L acetate buffer (pH 3.6), the 20 mmol/L ferric chloride solution, and the 10 mmol/L TPTZ in the 40 mmol/L HCl solution at a ratio of 10:1:1 (v/v/v). The FRAP reagent was warmed at 37 °C prior to use.

The FRAP assay was estimated as described by Giri et al. (2017) and modified for this study [8]. Briefly, 3.0 mL of the FRAP reagent was added to 100 μL of the sample solution and incubated at 37 °C for 15 min. The absorbance was read at 596 nm against a reference solution using a UV-vis spectrophotometer. The calibration curve was established with the FeSO<sub>4</sub>·7H<sub>2</sub>O solutions with nine different concentrations (0.10, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, and 2.00 mmol/L). The results are expressed as millimole (mmol) of Fe<sup>2+</sup> equivalents per gram of dry weight.

2.6. Multielement analysis

The purchased herb samples were pulverized using an XQM-0.4 planetary ball mill (Tianchuang Corp., Changsha, China). A total of 0.2 g of each sample was accurately weighed directly into the Poly tetra fluoroethylene digestion vessel (in triplicate). Then, 5 mL of concentrated HNO<sub>3</sub> (65%, v/v) was added and digested using an XT-9916 Microwave Digestion System (Xintuo Corp., Shanghai, China). One randomly selected vessel was filled with nitric acid only and taken through the entire procedure as a blank. After cooling, the digest was filtered through a 0.22 μm pore size membrane filter and then diluted with ultra-pure water to 20 mL in a volumetric flask for further analysis. A Standard Reference Material (GBW10020 Citrus Leaves from China National Institute of Standards and Technology, Beijing, China) was used for validation of the analytical method. The calibration standards were prepared by dilution of the stock standard solutions (1000 μg/mL) of the corresponding elements using deionized water. The concentrations of Co, Be, Ba, Sb, Fe, Cr, Mn, Ni, Zn, As, Cd, Pb, Cu, and Bi were measured using the Agilent 7500cx ICP-MS (Agilent Technologies, Santa Clara, CA, USA).

2.7. Pollution assessment

To evaluate the herb contamination degrees, the single-factor pollution index (PI) and the Nemerow multi-factor pollution index (PN) were calculated. The PI calculation equation is defined as follows:

\[
PI = \frac{C_i}{S_i}
\]

where \(PI\) represents the pollution index of the ith pollutant in the herb, and \(C_i\) and \(S_i\) are the measured concentrations of a heavy metal (i) in the herb and the evaluation criteria, respectively [9].

According to the PI, the contamination statuses of the heavy metals were divided into the following five classes: no pollution (PI ≤ 1.0), mild pollution (1.0 < PI ≤ 2.0), moderate pollution (2.0 < PI ≤ 3.0), severe pollution (3.0 < PI ≤ 5.0), and very severe pollution (PI > 5.0).

The PN considers all of the individual assessment factors and can reflect the general herb pollution. The comprehensive pollution index was calculated using the following equation:

\[
PN = \sqrt{\frac{1}{2} \left( \frac{(C_i/S_i)_{ave}}{S_i} + \frac{(C_i/S_i)_{max}}{S_i} \right)}
\]

where \(PN\) is the comprehensive pollution index, and \((C_i/S_i)_{ave}\) and \((C_i/S_i)_{max}\) are the average and maximum values of the single-factor contaminant index, respectively [10].

The PN was classified into the following five levels: safe level (PN ≤ 0.7), warning level (0.7 < PN ≤ 1.0), light pollution level (1.0 < PN ≤ 2.0), medium pollution level (2.0 < PN ≤ 3.0), and serious pollution level (PN > 3.0).

2.8. Dietary intake approximations and the health risk assessment

The consumption of each herb (g/day) was multiplied by the median value of each element (mg/kg dry weight) in that type of herb. For the estimation of the consumption of each element (μg/kg body weight/day), both low-dose herb consumers and high-dose herb consumers were considered, and the estimations were performed for adults (60 kg/bw).

2.8.1. Estimated daily intake

The estimated daily intake (EDI) of heavy metals via the ingestion route was calculated using the following equation:
where \( C \) is the concentration of contaminants in the herb (mg/kg); \( IR \) is the ingestion rate (g/person/day); and \( BW \) is the body weight of the residents (adult, 60 kg) [11].

The EDI values of the elements for low-dose and high-dose consumers were compared with the reference values to evaluate the risk-benefit ratio. The values of the acceptable daily intake (ADI) were reported by the World Health Organization/Food and Agriculture Organization of the United Nations (WHO/FAO). According to the WHO/FAO standard, the ADI is the equivalent of the reference Dose for Chronic Oral Exposure (RfD) in the United States. No official acceptable daily exposure value has been established for Bi, so this element was excluded from the risk-benefit ratio analysis in this study.

2.8.2. Target hazard quotient

The target hazard quotient (THQ) is the ratio of the exposure dose to the reference dose. It is employed for the noncarcinogenic health risk assessment of pollutants in herbs due to the consumption of the herbs. The desired value of the THQ is expected to be \(<1\) for the maximum safe limit; however, a THQ \(>1\) suggests the possibility of ensuing systemic toxic effects. The THQ was calculated using the following equation:

\[
\text{THQ} = \frac{EFr \times ED \times IR \times C}{AT \times BW \times RfD},
\]

where \( EFr \) is the exposure frequency (from 365 days per year for people who consume an herb seven times a week and 12 days per year for people who consume an herb once a month); \( ED \) is the exposure duration (70 years); \( IR \) is the ingestion rate (kg/day); \( C \) is the concentration of the element in herbs (mg/kg); \( AT \) is the averaging time for non-carcinogens; \( BW \) is the average body weight of residents (adult, 60 kg); and the \( RfD \) values (mg/kg/day) provided by WHO/FAO (2021) for the different elements are 0.0003 for As, 0.8 for Fe, 0.3 for Zn, 0.003 for Pb, 0.02 for Ni, 0.04 for Cu, and 0.001 for Cd [12].

2.8.3. Carcinogenic risk

The carcinogenic risk (CR) was employed for cancer health risk assessment. The CR values of As, Pb, Cr, and Ni from dietary exposure were calculated using the following equation:

\[
\text{CR} = \text{EDI} \times \text{SF},
\]

where CR is the carcinogenic risk of a single element, and SF is the ingestion slope factor for a particular element. CR is categorized as the cancer risks belonging to very low (\( CR \leq 1 \times 10^{-6} \)), low (\( 1 \times 10^{-6} < CR \leq 1 \times 10^{-4} \)), moderate (\( 1 \times 10^{-4} < CR \leq 1 \times 10^{-3} \)), high (\( 1 \times 10^{-3} < CR \leq 0.1 \)), or very high (\( CR > 0.1 \)). The desired CR value is expected to be \(<1 \times 10^{-4}\), which is the safe limit [13].

2.9. Statistical analysis

All tests were performed in triplicate. The data are all presented as mean \( \pm \) standard deviation (SD). Descriptive analyses were conducted on all of the observed variables. The arithmetic mean, SD, range, and median were computed for the continuous variables. The analyses were conducted using SPSS software, version 20.0 (IBM., Corp., Armonk, NY, USA). The correlation analysis and the hierarchical cluster analysis were performed using R, version 3.6.3 (Free Software Foundation, R Development Core Team, Auckland, New Zealand).

3. Results and discussion

3.1. UV fingerprints of the ROG extracts

The extraction solvents and periods were optimized. Various solvents were tested (Fig. S1; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional), including ethanol, 50% ethanol, chloroform, and water. Among the four different extracts, the 50% ethanolic extract demonstrated the presence of the maximum absorbance and peak numbers.

Ten ROG samples were extracted using 50% ethanol and analyzed using UV-vis spectroscopy to achieve insight into the major chromophores found in them. Fig. 1a depicts the UV spectral fingerprint (200–400 nm) of the different ROG samples. Fig. 1b is a representative fingerprint that was obtained from the average vector at the same wavelength of the 10 UV spectra. The UV-vis spectra show that the characteristic UV range was principally between 200 and 360 nm. The spectrograms show three absorption maxima near 225, 285, and 320 nm. The strongest absorption at 215–235 nm corresponded to the \( \pi-\pi^* \) transition of the unsaturated carboxyl and carbonyl groups in ROG [14,15]. The stronger absorption at 275–295 nm belonged to the \( \pi-\pi^* \) transition and indicated that there was a phenolic constitute or nucleic acid in the samples [16]. The stronger absorption at 310–330 nm can be assigned to the n-\( \pi^* \) transition.
or the electron delocalization due to the C–O–C coupling [17].

3.2. Acquisition of the IR fingerprints of the ROG extracts

Fig. 1c shows the FT-IR spectra of the structural features of 10 batches of the sample extracts. All of these 10 spectrograms represent 11 typical characteristic peaks that had similar peak times (RSD <2%). Fig. 1d is the referential fingerprint from the average vector at the same wavenumber of the 10 IR fingerprints.

The mid-infrared wavenumbers of the absorption peaks and preliminary assignments are summarized in Table S1. According to the referential one, the FT-IR spectrum showed an intense band centered at 3424.70 cm⁻¹ that was attributed to the O–H and N–H stretching vibrations [18]. The band occurring at 2926.68 cm⁻¹ corresponded to the asymmetric C–H stretching vibrations of the CH₂ and CH₃ groups [19]. The band at 1630.04 cm⁻¹ was assigned to either the stretching vibration of the hydrogen-bonded or conjugated C=O or the stretching vibration of C=C [20]. Both assignments contributed together to this band. The band at 1603.32 cm⁻¹ was
assigned to the stretching vibration of C=O in an aromatic ring or the stretching vibration of conjugated C=O or C=N [21,22]. The absorption band at 1513.48 cm⁻¹ belonged to the C=O stretching vibration of the aromatic ring. The band appearing at 1420.19 cm⁻¹ was ascribed to either the C−H bending vibrations of the CH₃ and CH₂ groups [23] or the configuration of the amide (amide III) C−N [24]. The sharp band at 1384.47 cm⁻¹ appeared to be due to the δ C−H vibration of CH₃. The band appearing at 1278.39 cm⁻¹ was possibly associated with the non-symmetrical stretching vibration of C−O−C or the stretching vibration of C−O of the aryl esters [20]. The bands at 1119.77 cm⁻¹ and 1080.19 cm⁻¹ were primarily contributed by the C−O−C or C−O−H stretching vibrations in esters, ethers, polysaccharides, phenols, and alcohols [25]. The band at 618.39 cm⁻¹ was assigned to the out-of-plane O−H bending vibration or the out-of-plane C=O bending vibration of amide [26].

3.3. ¹H NMR spectroscopic analysis

3.3.1. ¹H NMR spectra of the ROG extracts

The representative ¹H NMR spectrum of the ROG is shown in Fig. 1e. The ¹H NMR spectra of the aqueous ethanol extracts indicated the presence of amino acid, carbohydrate, and phenolic compounds according to the visual inspection. Carbohydrate signals primarily in the range of 3.0–5.3 ppm were more prominent compared with the other spectral regions (the amino acids in the chemical shift region of 0–3.0 ppm and the phenolics in the region of 5.3–8.3 ppm). The aromatic region signals were smaller than those in the carbohydrate or amino acid region. Visual inspection of the chemical shift region from 0–8.5 ppm showed a similar trend regarding the overall metabolites from the different origins, although some metabolite resonances displayed a higher intensity.

This study regarding the nonvolatile extract of ROG revealed the presence of a broad spectrum of chemical compounds such as amino acids, flavonoids, carbohydrates, and terpenoids. A total of 25 compounds were identified in ROG, as shown in Table S2; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional. The amino acids included L-phenylalanine, histidine, leucine, glycine, hydroxyproline, proline, arginine, and lysine. The terpenoids included cycloartenol, p-cymene, and thymol. The flavonoid only included kaempferol. The organic acids included citric acid, taurine, and 2-ketoisovaleric acid. The carbohydrates included fructose, maltose, sucrose, α-glucose, and β-glucose. The signal at 8.25 ppm may have been due to the adenosine that was observed [27]. The signals attributable to L-phenylalanine at 7.30, 7.15, 3.93, and 3.25 ppm were also present [27]. These findings were consistent with the truth that organic acids, amino acids, triterpenoids, and flavonoid glycosides are soluble in aqueous ethanol. The assignment of the primary metabolites was based on a comparison with the relevant literature [27–29] and the Chemomx database.

3.3.2. ROG correlation analysis results by applying ¹H NMR

The correlation coefficients of the ¹H NMR spectral data among the 10 samples were calculated (Table S3; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional). The results indicated that the compositional profile of the samples from different geographical regions was correlated, especially for the carbohydrate portion. The primary differences were detected in the amino acid region, where a wide range of coefficients was observed (0.086–0.997). In a comparison with the aromatic substance contents (coefficient range: 0.620–0.986), the carbohydrate metabolites were quite similar (coefficient range: 0.797–0.993).

All of the metabolites data in the ROG samples from the different regions are also presented as heatmaps for a visual image of how the metabolite levels differed among them. Three heat maps (Fig. 2a–c) were generated to display the varying amino acid/organic acid, sugar, and aromatic substance metabolites based on the ¹H NMR chemical shift (horizontal) along the ROG (vertical) from 10 different provinces. This perspective of the metabolite differences in the ROG samples was based on the results of the semi-quantitative analysis obtained from the ¹H NMR normalized ratios. The hierarchical cluster analysis of the acquired ¹H NMR spectral data (Fig. 2) showed that the samples from Wuhu (S1) and Huaihua (S6) were metabolically similar. In addition, the samples from Meizhou (S2), Yulin (S3), Suqian (S7), and Quzhou (S10) were grouped closely together in the three subsets. The close metabolic relationship of the samples might have been due to the similarity in the intrinsic (such as genetic make-up) or extrinsic (such as soil conditions and climate) factors, and this, was consistent with the literature [30]. From the overview of the total metabolites, we discovered that the amino acid/organic acid compounds displayed more intensive spectral signals than the other compounds. The semi-quantitative analysis of the amino acid/organic acid compounds by a heatmap.
is displayed in Fig. 2a. The amino acids levels (0.0–3.4 ppm) in the ROG samples from Huaihua City (S6), and the sample from Dujiangyan City (S8) were significantly ($p < 0.05$) higher than that of the other samples. The ROG samples from Wuhu City (S1) contained the highest contents of the sugar compounds corresponding to 3.4–5.5 ppm (Fig. 2b). For the aromatic substance dataset (Fig. 2c), the ROG sample from Huanggang City (S5) contained the highest content of aromatic substances. The sample from Xinyang City (S4) contained the second highest, and the ROG sample from Dujiangyan City (S8) contained the lowest.

### 3.4. Antioxidant activity

This work aimed to investigate whether ROG exhibits antioxidant activity. The antioxidant capacities of the 10 ROG extracts were evaluated using a DPPH assay, an ABTS assay, the FRAP assay, and the results are shown in Figs. 3a, b, and 3c, respectively. The $IC_{50}$ (Fig. 3a) ranged from 0.113 mg/mL for S6 (from Huaihua City) to 0.253 mg/mL for S7 (from Suqian City), reflecting a 2.2-fold difference in the scavenging DPPH radical. The antioxidant activity order of the individual samples was $S7 > S5 > S10 > S1 > S4 > S2 > S8 > S3 > S9 > S6$. The $IC_{50}$ (Fig. 3b) ranged from 0.09 mg/mL for S1 (from Wuhu City) to 0.017 mg/mL for S4 (from Xinyang City) in the scavenging ABTS radical. The FRAP value (Fig. 3c) was from 0.504 mmol/g for S5 (from Huanggang City) to 1.224 mmol/g for S2 (from Meizhou City). The results of three in vitro assays among the ten samples with reference revealed that high antioxidant potential activities were exhibited by all of the ROG samples, but they were still considerably less effective DPPH and ABTS radical scavengers than in L-ascorbic acid. This information provides references for the rational use of ROG in China.
Fig. 4. Trace elements in the ROG samples. (a) Box-and-whisker diagram of concentrations (log10 transformed) of each of the 14 measured elements. (b) Correlation between the analyzed elements regarding their accumulation in the ROG samples (Heatmap) with a demonstration of a hierarchical cluster tree plot to display the groups of similarly accumulated elements. (c)–(e) Correlation between the $^1$H NMR signal intensity of metabolites and the content of mineral elements in the ROG samples. (f) Results of the single factor pollution index (PI) and the Nemerow's multifactor index (PN) in the ROG samples.
3.5. Multielemental analysis in the ROG samples

3.5.1. Concentrations and descriptive statistics of the 14 elements

An analysis of the results of ROG indicated that Co, Be, Ba, Sb, Fe, Cr, Mn, Ni, Zn, As, Cu, Cd, and Pb were detectable in all samples except for Bi (Table S4; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional). Bi was below the detectable level in some samples. Moreover, the highest content of Bi was found in the sample from Wuhu City (1.691 mg/kg). Among the tested elements, Fe showed the highest concentration levels, whereas Sb was found to be the lowest. Microelements showed a mean concentration order of: Fe > Mn > Zn > Ba > Cu > As > Pb > Ni > Cr > Co > Cd > Bi > Be > Sb. The abundance of Fe, Mn, Ba, Zn, As, and Cu in the present study might indicate that these elements are commonly found in ROG. In Fig. 4a, a box-whisker plot displays for the statistical distribution of the concentrations of each of the 14 measured elements in the ROG samples. A log transform (log10) was used for the data. Visible differences in the elemental contents were discovered among the samples studied. The box and whisker diagram shows the concentrations of the 14 elements that contributed to the differentiation among the 10 sites.

3.5.2. Correlation analysis between the elements

To investigate the interrelationships among the analyzed elements and evaluate the potential for profiling in the 10 ROG samples, we applied a correlation analysis using a Pearson test. A heatmap analysis with a dendrogram tree based on a hierarchical cluster analysis was used to display the correlations between the particular element contents in the studied herb species. By using the complete hierarchical clustering and correlation distances, dendrograms with cluster groupings were acquired. According to the heatmap analysis, the correlation between the analyzed elements regarding the accumulation in ROG is presented below (Fig. 4b). As can be seen in Fig. 4b, the tested elements were categorized into three variable groups in ROG: (1) Pb, Cd, Zn, As, Bi, and Sb; (2) Ni, Co, Mn, Cr, Be, Fe, and Ba; and (3) Cu.

Table S5; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional summarizes the correlation pathways of the elemental contents in ROG. The correlations among the elements were confirmed by considering those with a significant correlation coefficient value (>0.80) only. The significance level for described correlation was lower than 0.05 (p < 0.05). Because both of the values of r were greater than 0.8 and a significant correlation existed at a p level of 0.05, this proved that strong correlations existed between Ni and Co (0.87), Ni and Ba (0.87), Ni and Fe (0.80), Ni and Cr (0.86), Co and Cr (0.82), Fe and Cr (0.92), Pb and Cd (0.95), Be and Co (0.88), Be and Fe (0.91), Be and Cr (0.97), and Be and Ni (0.91) in ROG. These correlations may have been due to the chemical features of the determined elements that are prone to follow similar metabolic pathways in the herbs.

3.5.3. Canonical correlation analysis

The R-function (psych and pheatmap packages) was used to calculate the correlation between the 1H NMR signal intensity of metabolites and the content of mineral elements in the ROG samples. The variables of each system were divided primarily into three sections: organic and amino acid, sugar, and aromatic substance regions. Fig. 4c–e shows the correlation between the 1H NMR signal intensity of the metabolites and the content of the mineral elements in the ROG samples. As can be seen in Fig. 4c, positive correlations existed between the contents of the analyzed elements and the signal intensity of the organic and amino acids (δ 0.0–1.8 ppm). The heatmap displays the positive correlations between the signal intensity of sugars (δ 3.7–4.8 ppm) and the contents of Co, Mn, Cu, and Bi and, the negative correlations between the As, Sb, Cd, and Pb contents (Fig. 4d). Large negative correlations between the As and Sb contents and the signal intensity of each metabolite, except for some organic and amino acids (δ 0.0–3.4 ppm), and the positive correlations between Bi and the metabolites were observed in the ROG samples (Fig. 4c–e). The combination of the 1H NMR profiling and the elemental concentration with the correlation analysis provided a new method for elucidating the role of mineral elements in the metabolomics of ROG. The difference in the correlation could represent a reflection of the dissimilarity in the biosynthesis of metabolites.

3.6. Pollution status

The average values of the single-factor pollution index for the four heavy metals in ROG followed the order of As > Cd > Cu > Pb. The mean values of PI of Pb were less than one in ROG, but the concentrations of Pb in some samples were still above the maximum permitted value. The PI values of As were
greater than one in all of the samples (Fig. 4f), indicating that this element was enriched in the ROG herbs of the study area. This was primarily due to the great bioaccumulation ability of As in ROG [31]. In the ROG samples, approximately 100% were polluted by As, 50% were polluted by Cd, 30% were polluted by Pb, and 90% were polluted by Cu. As, Cd, Cu, and Pb are the common “pollution elements”. This can be attributed to discharges from different anthropogenic sources, such as the mining and smelting industries, agricultural activities, vehicle exhaust emissions, and landfills [9]. Higher PI values in the samples from Wuhu, Meizhou, Yulin, Huanggang, and Dujiangyan were observed, and this indicated that the occurrences of these four heavy metals in the soil of these cities were significantly high. The PN results suggested that all of the ROG samples belonged to the serious pollution level, indicating that the ROG samples had serious levels of heavy metals that required attention prior to use. Therefore, an effective heavy metal elution method is urgently needed for ROG to exclude metals, especially As.

3.7. Health risk assessment

3.7.1. Estimated daily intake of elements

The results of the EDI appraisal of elements in ROG are presented in Table 1. As can be seen in Table 1, the highest EDIs were those of Fe, Mn, Zn, and Ba, followed at a distance by Cu and As. Of the toxic elements studied, As exceeded the ADI level by 18–37 times. The present study results indicated that restriction needs to be imposed on ROG consumption.

3.7.2. Target hazard quotient of elements

The THQs of the investigated elements due to the consumption of the ROG herbs were determined, which are presented in Table 1. The determined health risk due to digestion exposure showed that Sb had the minimum THQ value for ROG. The rank order of elements based on their THQ values was As > Fe > Mn > Cr > Cu > Pb > Cd > Ba > Zn > Ni > Co > Be > Sb. Individual elements posed no health risk based on their THQ values, except for As and Bi (the elements of which the THQ values were not available in this study). However, the calculated THQ values of As in ROG for both low dose and high dose consumers exceeded the safe level.
3.7.3. Carcinogenic risk of the potentially harmful elements

Table 2 lists the calculated carcinogenic risks for the toxic trace elements in ROG. In this study, our investigated carcinogenic risks were due to As, Cr, Pb, Cd, and Ni, and nearly all of the values exceeded the threshold value of $10^{-4}$ except for Pb. Specifically speaking, low-dose and high-dose As were at high-level risks, and Cr, Cd, and Ni were at moderate-level risks. The results indicated that the potential cancer risks from human oral exposure to As, Cr, Cd, and Ni in the ROG that was sampled were not acceptable. In summary, evaluated health risk indices suggested that ROG consumption, even once per month, may cause potential health risks due to metal toxicity. Therefore, great care has to be taken with regular ROG consumption.

4. Conclusions

In this study, we used the UV, IR, and $^1$H NMR methods for an investigation of the same ROG samples in combination with the utility of specific analytical techniques and databases. Three efficient and low-cost analytical methods were used to investigate the antioxidant activities of the ROG samples. A heavy metal contamination assessment and human health risk assessment were performed to investigate whether the Chinese population is confronted with a potential health risk due to ROG exposure. The obtained results showed the presence of amino acids, flavonoids, organic acids, and sugars in all of the ROG samples and also demonstrated for the first time that ROG possessed the antioxidant capacity corresponding to the content of phenol. Unfortunately, the PN results suggested that all of the ROG samples belonged to the serious pollution level, indicating that the ROG samples had serious levels of heavy metals and required attention prior to use. The evaluated health risk indices suggested that ROG consumption once per month may cause a potential health risk due to metal toxicity. Therefore, great care needs to be taken during regular ROG consumption. The present investigation provides insights into the organic medicinal constituents and the inorganic trace elements of ROG for the first time, and the results will enable this herb to be used more effectively and safely.

Conflict of interest

The authors declare no conflict of interest to disclose.

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Supporting information.

MIR wavenumbers (cm$^{-1}$) and preliminary assignments in the range of 400–4000 cm$^{-1}$ for ROG samples (Table S1; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional), assignment of $^1$H NMR spectra peaks of ROG (Table S2; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional), correlation analysis results of the ROG samples by applying $^1$H NMR (Table S3; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional), results of determination of the selected elements (mg/kg dry weight) in the ROG samples from ten regions using the ICP-MS method (Table S4; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional), correlation matrix for the elemental concentration in the ROG samples (n = 10) (Table S5; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional), UV-vis diagrams of the different extraction solvents (Fig. S1; https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3426&context=journal&type=additional).

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