SUPPLEMENTARY MATERIAL

UPLC-Q-Orbitrap-MS² analysis of *Moringa oleifera* leaf extract and its antioxidant, antibacterial and anti-inflammatory activities

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Abstract: *Moringa oleifera* leaf acetone extract (MLE) was prepared. Phytochemicals of MLE and their antioxidant, antibacterial, and anti-inflammatory activities were evaluated. Results showed that MLE contained total phenolic content of 20.16 mg gallic acid equivalents/g dry weight. A total of 39 compounds were identified by mass spectrometry. The contents of acetyl-glucomoringin, caffeoylquinic acid, feruloylquinic acid, and coumarylquinic acid were high. MLE had high DPPH⁻ and ABTS⁺⁺ scavenging activities and reducing powder. In addition, MLE could effectively inhibit *S. aureus* and *B. subtilis*, but little effect on *E. coli* was found. The anti-inflammatory effect of MLE was evaluated using a lipopolysaccharide (LPS) -induced RAW 264.7 cell model. MLE significantly inhibited nitric oxide (NO) production and inducible NO synthase (iNOS) mRNA levels in LPS-induced RAW 264.7 cells. The inhibitory activity increased in a dose-dependent manner. The bioactivities of MLE were related to its phenolic content and phenolic profiles.

Keywords: *Moringa oleifera* leaf; phytochemicals; antioxidant; antibacterial; anti-inflammatory
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Experimental

Chemicals and reagents

The *Moringa oleifera* leaf was provided from Yunnan Tianyou Technology Development Co, Ltd (Yunnan, China), and identified by Dr. Xudong Sun of Institute of Botany, The Chinese Academy of Science. A voucher specimen (No. Sun20170308) has been deposited at the Yunnan Institute of Food Safety, Kunming University of Science and Technology. Acetonitrile and formic acid of HPLC were obtained from Merck Co. Inc (Darmstadt, Germany). 2,2-Diphenyl-2-picryl-hydrayl (DPPH), 2,2′-azino-bis (3-ethylbenothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tri-2-pyridinyl-1,3,5-triazine (TPTZ), lipopolysaccharide (LPS), and trolox were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Kanamycin, vancomycin and the standards used for quantitative and qualitative evaluations of the phytochemical compounds were purchased from Shanghai Yuanye Bio-Technology Co. (Shanghai, China).

Preparation of MLE

The extraction procedure was performed according to the study by Sandhu & Gu (2010) with some modifications. A mass of 20 g ML was extracted with 100 mL of acetone: water: acetic acid=70:29.7:0.3 (v/v), successively assisted by vortex for 30s, ultrasonic for 5min, stand at temperature for 5min, and ultrasonic for another 5 min. Then, the mixture was centrifuged for 15 min at 5000 rpm and the supernatant was collected. The extraction process was repeated two more times with the same solvent (100mL×2). The supernatants were combined and concentrated by a rotary evaporator under vacuum at 40 °C and the concentrated MLE was lyophilized for use.

Determination of total phenolic content

Total phenolic content was analyzed by the Folin-Ciocalteu colorimetric method (Zhuang et al. 2017). Briefly, 0.5 mL MLE was mixed with 2.5 mL 10% Folin-Ciocalteu reagent and shaken for 30 s. The mixture was kept for 5 min, and 2 mL 7.5% sodium carbonate aqueous solution was successively added. The mixture was kept for 60 min in the dark at room temperature and its absorbance was read at 765 nm using a TU-1901 spectrophotometer (Persee Inc., Beijing, China) with gallic acid as the standard. The result was expressed as milligrams gallic acid equivalents (GAE)/g dry weight (DW) of ML.

Phytochemical composition profile
Ultra-performance liquid chromatography (UPLC) analysis was performed using an Ultimate 3000 Series UPLC systems (Thermo Scientific, Waltham, USA) equipped with a vacuum degasser, binary pump, auto-sampler, and thermostated column compartment. Chromatographic separation was performed using a reversed-phase Hypersil Gold C18 column (100 mm × 2.1 mm i.d., 1.9 μm, Thermo Fisher, Runcorn, United Kingdom). Water containing 0.1% formic acid (A) + acetonitrile (B) were used as the mobile phase in the gradient mode: 0–1 min, 5% B; 1–5 min, 15% B; 5–10 min, 38% B; 10–12 min, 65% B; 12–18 min, 80% B; 18–20 min, 100% B; 20–25 min, 100% B. The flow rate was 0.2 mL/min and the injection volume was 5 μL.

A Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany) equipped with an electrospray ionization source was used in negative ionization mode for accurate molecular mass and fragment information. The mass spectrometer was operated with the following parameters: capillary temperature, 320 °C; spray voltage, 3.5 kV; AGC target value, 1e6; sheath gas (N2) flow rate, 32 arbitrary units (a.u.); auxiliary gas (N2) flow rate, 10 a.u.; mass range was 100–1000 m/z. The resolution was set at 70,000 for accurate mass measurement and at 35,000 for MS2 measurement. Mass data was acquired in profile mode. Data analysis was performed using XCaliburTM software (Thermo Fisher, Bremen, Germany).

Quantities were calculated from peak areas in the UPLC profile, using external calibration curves established with standards (Shanghai Yuanye Bio-Technology, Shanghai, China) by qualitative analysis and semi-quantitative analysis (Zhuang et al. 2017). The content of each identified compound was expressed as μg/g DW.

**Determination of antioxidant activities**

**DPPH--scavenging assay**

DPPH--scavenging and ABTS•+-scavenging assay was performed by the previous method (Oegen et al. 2006). IC_{50} was expressed as the concentration of the test sample at 50% of ABTS•+-scavenging activity. The ferric-reducing antioxidant power (FRAP) assay was performed by the previous method (Zhuang et al 2017), and IC_{50} value was defined as the concentration of the test sample in the reaction mixture that gave 0.500 absorbance units.

**Antibacterial activity and minimum inhibitory concentration (MIC) assay**

Antibacterial activity was tested in *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC29213 and *Bacillus subtilis* ATCC6633 obtained from
E. coli, S. aureus, and B. subtilis were grown overnight in 5 mL Luria-Bertani medium (containing 0.5% yeast extract, 1% peptone, and 1% sodium chloride) at 37 °C. The antibacterial activity of the test sample was performed using the method of Tang et al. (2013). Briefly, the mid-logarithmic phase of bacteria was centrifuged (10,000 r/min, 4 °C, 30 s) and adjusted to 10^6 colony forming units per mL (CFU/mL) by physiological saline solution. Aliquots of 100 µL test sample (0–1000.00 µg/mL) and 100 µL bacterial suspension were added to 96-well microplates and incubated for 24 h at 37 °C. MIC was defined as the lowest sample concentration that showed no increase in the optical density (OD 600) read on a Multiskan MK3 microplate reader (Thermo, Waltham, USA).

**Determination of anti-inflammatory activity**

**Assay of RAW 264.7 cell viability**

Murine RAW 264.7 cells were purchased from Kunming Cell Bank, Chinese Academy of Sciences (Yunnan, China). RAW 264.7 cells were stimulated with LPS using the previous method (Ryu et al. 2017). The cytotoxicity assay of MLE on LPS-stimulated RAW 264.7 cells was performed by MTT method (Sun et al. 2015). The MLE sample (0–200 μg/mL) showed no significant toxicity (p>0.05) to the RAW 264.7 cells.

**Measurement of nitric oxide (NO) and iNOS gene expression**

RAW 264.7 cells were stimulated with 1.5 µg/mL LPS for 12 h and seeded onto a 96-well plate at 200 µL (4×104) per well for 24 h. The cells were then incubated with MLE solutions (40, 60 and 80 μg/mL) for 12 h. The nitrite concentration was detected using the NO determination kit (SpectraMax M5, Molecular Device), following the manufacturer’s instructions. The iNOS gene expression of RAW 264.7 cells were determined by previous method (Sun et al. 2015).

**Statistical Analyses**

Experimental data were presented as the mean ± standard deviation. Date was analyzed using one-way analysis of variance (ANOVA). Statistical analyses were indicated using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Means were accepted as significantly difference at 95% confidence interval (p <0.05).

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Fig S1. Total ion chromatogram in negative mode scan obtained by UPLC-Q-Orbitrap-MS² of MLE
| N o. | Compound                          | $t_R$ (min) | [M-H]$^-$ (m/z) | Error (ppm) | MS$^2$ | Content (µg/g DW) |
|-----|-----------------------------------|------------|-----------------|-------------|--------|------------------|
|     | **Organic acids**                 |            |                 |             |        |                  |
| 1   | Citric acid$^a$                    | 1.33       | 191.0187        | 0.319       | 87-111 | 199.71±3.24      |
| 2   | Malic acid$^a$                     | 1.40       | 133.0129        | -2.028      | 71-73  | 658.86±4.21      |
| 17  | Benzoic acid$^b$                   | 8.86       | 121.0281        | -2.776      | 95-108-121 | 16.82±1.00    |
| 33  | Trihydroxyocta-decahydroquinic acid$^b$ | 13.32       | 327.2171        | 1.618       | 211-229 | 54.62±1.21      |
| 35  | Hydroperoxyocta-decahydroquinic acid$^b$ | 13.99       | 309.2067        | 2.245       | 171-71  | 5.73±0.25       |
| 37  | 9-Hydroxyoctadeca-10,12,15-trienoic acid$^b$ | 15.35       | 293.2118        | 2.383       | 171-121 | 85.94±0.41      |
| 38  | Linolenic acid$^a$                 | 18.71      | 277.2167        | 1.924       | 135-71  | 506.46±6.32      |
| 39  | Linoleic acid$^a$                  | 20.45      | 279.2325        | 2.125       | 93-146-118-214-2 | 192.30±6.18  |
|     | **total**                          |            |                 |             |        | 1720.44          |
|     | **Amino acid**                     |            |                 |             |        |                  |
| 6   | L-Phe$^a$                          | 2.79       | 164.0705        | -0.105      | 72-148 | 182.46±4.21      |
| 8   | L-Try$^a$                          | 4.81       | 203.0816        | 0.146       | 116-142 | 143.99±2.67      |
|     | **total**                          |            |                 |             |        | 326.45           |
|     | **Hydroxybenzoic acid derivatives**|            |                 |             |        |                  |
| 4   | Protocatechuic acid hexoside$^c$   | 2.48       | 315.0719        | 2.671       | 108-152-109-153 | 55.50±1.28  |
| 5   | Salicylic acid O-glycoside$^d$     | 2.58       | 299.0769        | -1.944      | 137    | 11.50±0.96       |
|     | **total**                          |            |                 |             |        | 67.00            |
|     | **Hydroxycinamic acid derivatives**|            |                 |             |        |                  |
| 7   | 3-Caffeoylquinic acid$^e$          | 4.54       | 353.0873        | 1.704       | 135-191-179 | 2218.13±20.76 |
| 9   | Coumaric acid$^e$                  | 5.18       | 163.0388        | -0.924      | 119    | 42.40±2.11       |
| 10  | Coumaroylquinic acid$^e$           | 6.19       | 337.0925        | 2.184       | 119-191-163-173 | 878.30±12.94 |
| 11  | Feruloylquinic acid$^f$            | 6.95       | 367.1028        | 1.311       | 134-193 | 900.98±10.67     |
| 12  | Coumaryl-hexoside$^c$              | 7.05       | 325.0924        | 2.080       | 119-163 | 35.90±3.61       |
| 31  | Dicaffeoylquinic acid$^f$          | 12.17      | 515.1187        | 0.597       | 179-191-353 | 33.49±2.83     |
|     | **total**                          |            |                 |             |        | 4109.20          |
|     | **Flavonols**                      |            |                 |             |        |                  |
| 13  | Quercetin diglycoside              | 7.10       | 625.1404        | 0.823       | 463-301 | 4.60±0.26        |
| 15  | Quercetin rutinoside$^e$           | 7.86       | 609.1456        | 1.016       | 285    | 8.99±0.37        |
| 19  | Quercetin-3-O-glucoside$^f$        | 9.88       | 463.0875        | 0.837       | 300-301 | 162.23±7.11      |
| 20  | Myricetin-3-O-glucoside$^f$        | 9.88       | 479.0821        | 0.215       | 316-317 | 2.10±0.14        |
| 21  | Quercetin-acetyl-glycoside$^f$     | 10.05      | 505.0979        | 0.461       | 300-301 | 399.89±14.81     |
| 22  | Quercetin malonyl hexose$^g$       | 10.05      | 549.0876        | 0.171       | 300-301-505 | 126.96±3.49   |
| 23  | Quercetin-hydroxy-methyl glutaroyl glycoside$^g$ | 10.19       | 607.1297        | 0.558       | 300-301 | 114.54±2.65      |
| 24  | Kaempferol 3-O-glucoside$^h$       | 10.45      | 447.0926        | 0.833       | 284-285 | 150.46±9.31      |
|    | Compound Description                  | Rf  | M/z       | m/z 10000  | m/z 1000  | m/z 100  | m/z 10  | m/z ± 10  |
|----|---------------------------------------|-----|-----------|------------|-----------|---------|---------|-----------|
| 25 | Isorhamnetin 3-O-glucoside\(^b\)       | 10.57 | 477.1032  | 0.959      | 314-315   | 21.97 ± 1.11 |
| 26 | Kaempferol\(^a\)                      | 10.72 | 285.0401  | 2.580      | 229-257-151 | 5.70 ± 0.21 |
| 27 | Kaempferol malonyl hexose\(^a\)       | 10.72 | 533.0928  | 2.580      | 284-285   | 114.43 ± 7.23 |
| 28 | Kaempferol glycoside–hydroxyl-methylglutarate\(^b\) | 10.81 | 591.1348  | 0.649      | 285-284   | 82.51 ± 11.63 |
| 29 | Isorhamnetin acetyl hexose\(^b\)      | 10.86 | 519.1136  | 0.526      | 314-315   | 59.50 ± 2.19 |
| 30 | Kaempferol acetyl glucoside\(^b\)     | 11.36 | 489.1033  | 1.181      | 284-285   | 57.50 ± 1.96 |
| total |                                   |     |           |            |           | 1311.38 |

### Flavones

|    | Compound Description                  | Rf  | M/z       | m/z 10000  | m/z 1000  | m/z 100  | m/z 10  | m/z ± 10  |
|----|---------------------------------------|-----|-----------|------------|-----------|---------|---------|-----------|
| 16 | Multiflorin B isomer\(^g\)            | 8.22 | 593.1505  | 0.596      | 353-383-473-593 | 86.91 ± 1.47 |
| 18 | (Vitexin) Apigenin glucoside\(^g\)    | 9.69 | 431.0977  | 0.897      | 283-311-341 | 24.58 ± 0.92 |
| total |                                   |     |           |            |           | 111.49 |

### Thioglycosides

|    | Compound Description                  | Rf  | M/z       | m/z 10000  | m/z 1000  | m/z 100  | m/z 10  | m/z ± 10  |
|----|---------------------------------------|-----|-----------|------------|-----------|---------|---------|-----------|
| 3  | Glucomoringin\(^i\)                   | 2.12 | 570.0950  | 1.013      | 97-259-328 | 242.42 ± 11.28 |
| 14 | Acetyl-glucomoringin\(^i\)            | 7.43 | 612.1053  | 0.348      | 97-259   | 2293.08 ± 38.58 |
| 32 | Isothiocyanate derivative\(^i\)       | 12.79 | 326.0699  | 12.79      | 58       | 35.93 ± 1.52 |
| 34 | Isothiocyanate derivative\(^i\)       | 13.38 | 370.0959  | 13.38      | 58       | 603.92 ± 16.31 |
| 36 | Isothiocyanate derivative\(^i\)       | 14.32 | 412.1064  | 0.506      | 58       | 512.96 ± 11.42 |
| total |                                   |     |           |            |           | 3688.31 |

The concentrations of compounds were quantified by standards (a) and semi-quantified by Linolenic acid (b), protocatechuic acids (c), salicylic acids (d), coumaric acids (e), ferulic acids(f), quercetin (g), kaempferol (h), and isothiocyanate (i).
Table S2 The MIC values of antibacterial activities of MLE (μg/mL)

| Bacterial   | *Escherichia coli* | *Staphylococcus aureus* | *Bacillus subtilis* |
|-------------|--------------------|-------------------------|---------------------|
| MLE         | >1000              | 62.50                   | 250.00              |
| Kanamycin   | 1.56               | 0.78                    | 15.63               |
| Vancomycin  | 250.00             | 7.81                    | 500.00              |
Fig S2. Inhibitory effects of MLE on NO production (A) and relative iNOS mRNA levels (B) in LPS-stimulated RAW 264.7 macrophage cells. Bar values (means) of the sample-treated groups with different letters are significantly different (p<0.05)