

SUPPLEMENTARY MATERIAL

Assessment of the Bioactive Compounds, Antioxidant and Antibacterial

Activities of *Isodon rubescens* as Affected by Drying Methods

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Abstract: The influence of natural drying (ND), hot-air drying (HD), vacuum drying (VD), infrared drying (ID) and freeze drying (FD) on bioactive compounds and bioactivities of *Isodon rubescens* (Hemsl.) were investigated in this study. The results showed that different drying methods resulted in the differences in bioactive compositions content, antioxidant and antibacterial activities of extracts from *I. rubescens*. FD sample possessed the highest content of total phenolics, total flavonoids, and several main phenolic compounds, as well as the stronger antioxidant and antibacterial activities, followed by ND, HD, and VD, the lowest for ID samples. For this reason, freeze drying would seem to be more advisable for the drying *I. rubescens*, and future studies could focus on the quality evaluation and optimizing various drying parameters.

Key words: *Isodon rubescens* (Hemsl.), drying, bioactive compound, antioxidant activity, antibacterial activity

Experimental

Plant materials

The fresh herb of *I. rubescens* was collected on August 15th 2015 in Lingchuan county, Jincheng City, Shanxi Province of China, and identified by Prof. Qing-Ping Hu, College of Life Sciences, Shanxi Normal University. The voucher specimen (HQP 201509) is deposited in the School of Food Science, Shanxi Normal University, China.

Chemicals/reagents

The 2, 4, 6-Tri (2-pyridyl)-s-triazine (TPTZ), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salts (ABTS), caffeic acid and rosmarinic acid were purchased from Sigma (USA). Chlorogenic acid, oridonin and ponacidin were purchased from Shanghai Pure-One Bio-tech Co. Ltd. (Shanghai, China). Nutrient agar (NA) and nutrient broth (NB) were from Beijing Aoboxing Bio-tech Co. Ltd. (Beijing, China).

Microorganisms and Culture

Two Gram-positive strains (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6051) and two Gram-negative bacteria (*Shigella dysenteriae* CMCC (B) 51252, *Escherichia coli* ATCC 25922) were provided by the College of Life Science, Shanxi Normal University, and cultured at 37 °C on NA or NB mediums.

Drying methods

Five drying methods were employed: (1) Natural drying (ND)- samples were dried for 3 days at 23–29 °C by sunshine and flowing air; (2) Freeze drying (FD)-samples were frozen at -40 °C and dried in a small lyophilizer with the condenser temperature and chamber vacuum for 12 h at 30 °C and 10-20 Pa respectively; (3) Hot-air drying (HD)- samples were dried in a cabinet dryer operated for 6 h at 60 °C; (4) Vacuum drying (VD)-samples were kept for 8 h in a vacuum oven set at 60°C and vacuum was maintained at 100 Pa; (5) Infrared drying (ID)- samples were dried for 5 h in an IR moisture analyzer at 60 °C. The dried *I. rubescens* were stored at 20-25 °C until analyzed. The drying conditions employed in each of these methods were selected after conducting trials to achieve a percentage moisture

content of < 5% (4.2% for ND, 2.8% for HD, 3.5% for VD, 3.3% for ID, 4.8% for FD). Moisture was measured by oven drying at 105 °C, and the dried samples were packaged in aluminum foil bags for further quality analysis.

Preparation of extracts

The extract was prepared according to the method as described by Feng and Xu (2014). Initially, the fifty grams of ground samples was blended with 500 mL ethanol and shaken at 150 rpm for 5 h at 25 °C, and then, the homogenates were centrifuged for 15 min at 4 °C and 5000 g. The supernatants were pooled and vacuum evaporated to dryness at 40 °C. Extracts were stored at – 40 °C for further analysis. The yield in each of the extracts was 2.21% for ND, 2.60% for HD, 2.05% for VD, 1.47% for ID, 2.54% for FD.

Determination of total phenolic and flavonoid content

Total phenolic content was determined using the Folin-Ciocalteu colorimetric method as described by Feng and Xu (2014). Briefly, an aliquot (0.5 mL) of the suitable diluted phenolic extracts, 2.5 mL of deionized water and 0.5 mL of 1.0 M Folin-Ciocalteu reagent were mixed within 10 mL volumetric flasks and vortexed. After 8 min, 1.5 mL of 7.5% sodium carbonate solution was added and mixed thoroughly. The absorbance of the reaction mixtures was measured at 765 nm wavelength after incubation for 2 h at room temperature. Gallic acid was used for calibration of the standard curve and total phenolic content was expressed as milligram gallic acid equivalent per gram dried weight (mg GAE/g DW).

The level of flavonoid was measured as described by Feng and Xu (2014). Briefly, an aliquot (1.0 mL) of diluted extracts and 0.3 mL of 5% NaNO₂ solution were mixed for 6 min. Then 0.3 mL 10% Al(NO₃)₃ was added and incubated for 6 min. Next, 4 mL of 4% NaOH was added. The final volume was adjusted to 10 mL with distilled water and mixed thoroughly. After 15 min, absorbance of the mixture was determined at 510 nm against the same mixture, without the sample, as a blank. Rutin was used for calibration of the standard curve and the content of flavonoids was expressed as milligram rutin equivalent per gram dried weight (mg RE/g DW).

HPLC analysis

The analyses were performed according to the method described by Feng and Xu (2014). Separation was carried out using a reversed-phase C₁₈ column (Alltech, Allsphere ODS-2, 5 μm, 250 mm × 4.6 mm) and operated at 30 °C. The mobile phase consisted of a binary mixture of solvent A (0.1% acetic acid in water) and B (acetonitrile) with a linear gradient program as follows: starting from 10% to 30% B over 20 min, from 30% to 40% over 10 min, from 40% to 60% B over 10 min, from 60% to 80% over 5 min and then isocratic for 5 min set at 1 mL/min of flow rate. The detection wavelength was set at 254 nm. The extracts and standard compounds were analyzed under the same analysis conditions, and all of the above experiments were replicated three times. Identification of the main phenolic compounds (chlorogenic acid, caffeic acid, rosmarinic acid, oridonin, and ponocidin) was performed by comparisons to the retention time and UV spectra of authentic standards; then the quantitative data was calculated from their linear calibration curves under analysis conditions. Results of the analyses for the main phenolic compounds were expressed as micrograms per gram extracts.

DPPH assay and ABTS assay

The scavenging rate of the sample on DPPH radicals was determined according to the method as previously described (Xu et al., 2012). Briefly, each of sample solutions was serially diluted to various concentrations in ethanol respectively, and then a 0.5 mL of samples was mixed with 2.5 mL of 60 μM DPPH dissolved in ethanol. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was measured at 517 nm against a solvent blank. The scavenging rate on DPPH radicals was calculated according to the formula, scavenging rate (%) = $[(A_0 - A_1)/A_0] \times 100$, where A₀ is the absorbance of the control solution, A₁ is the absorbance in the presence of samples in DPPH solution. The scavenging activity of the sample on DPPH radicals was expressed by EC₅₀ value that is the effective concentration at which free radicals are scavenged by 50% and is obtained by interpolation from regression analysis.

ABTS Free radical scavenging activity was determined according to the method as previously described (Xu et al., 2012). Briefly, ABTS cation radical was generated

by a reaction of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate. The reaction mixture was allowed to stand in the dark at room temperature for 16-24 h before use and used within 2 days. The ABTS⁺ solution was diluted with methanol to an absorbance of 0.700 ± 0.050 at 734 nm. One hundred microliters of the diluted samples was mixed with 2.0 mL of diluted ABTS⁺ solution. The mixture was allowed to stand for 6 min at room temperature and the absorbance was immediately recorded at 734 nm. The scavenging rate and EC₅₀ value were calculated using the equation described for DPPH assay.

Ferric reducing antioxidant power (FRAP) assay

The reducing ability was determined by using FRAP assay described by Xu et al. (2012). Briefly, the FRAP reagent was freshly prepared from 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM FeCl₃ solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C in a water bath prior to use. Then 0.1 mL of samples was mixed with 1.8 mL of FRAP reagent and 3.1 mL ultra pure water. The absorption of the reaction mixture was measured at 593 nm after incubation for 30 min at 37 °C. The standard curve was constructed using FeSO₄ solution (100-1000 µM), and FRAP value was expressed as micromoles Fe(II) per gram sample.

Antibacterial activity

Oxford cup assay was carried out according to the method described by Diao et al. (2013) with minor modifications. Briefly, a suspension (200 µL of 10^6 CFU/mL) of bacteria spread on nutrient agar (NA) medium. Oxford cups (6 mm in diameter) were placed on the inoculated agar, and then 100 µL of sample was added with a micropipette. The antibacterial activity was evaluated by measuring the diameter of zones of inhibition (ZOI) after 24 h of incubation at 37 °C. Tests were performed in triplicate.

Two fold serial dilutions of samples were prepared in sterile NB medium. To each tube 100 µL of the exponentially growing bacterial cells was added to give a cell concentration of approximately 1×10^7 CFU/mL. The tubes were incubated at 37 °C for 24 h and then examined for evidence of the growth. The Minimum inhibitory

concentration (MIC) was determined according to the method described by Diao *et al.* (2013). Ten microliters of Gentamicin (4 mg/mL) were used as positive control.

Statistical analysis

All experiments were conducted three times independently and the experimental data were expressed as mean \pm standard deviation. Analysis of variance and Duncan's multiple range tests were carried out to determine significant differences ($p < 0.05$) between the means by DPS 7.05.

References

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Table S1 The content (mg/g extracts) of several phenolic compounds in *I. rubescens* extracts

	Chlorogenic acid	Caffeic acid	Rosmarinic acid	Oridonin	Ponicidin
ND	11.5±1.5 b	36.6±2.5 b	47.8±3.1 b	253.4±10.2 b	75.1±3.6 b
HD	8.8±1.0 c	31.4±1.6 c	40.1±2.6 c	244.4±12.5 b	71.4±2.2 b
ID	4.1±0.4 d	22.8±2.4 d	27.5±1.8 d	201.4±14.1 c	60.6±2.4 c
VD	10.6±0.8 bc	35.2±3.3 bc	45.1±3.5 bc	276.8±5.4 a	88.4±3.1 a
FD	18.2±1.6 a	43.3±2.6 a	56.2±2.7 a	289.5±12.2 a	94.1±4.3 a

Values represent means of three independent replicates \pm SD. Different lowercase letters within a column indicate statistically significant differences ($p < 0.05$) among different drying methods.

Table S2 The antioxidant activities of different extracts from *I. rubescens*

	DPPH (EC ₅₀ , mg/mL)	ABTS (EC ₅₀ ,mg/mL)	FRAP (μmol Fe(II)/g)
ND	2.9±0.4 bc	21.5±1.3 bc	114.1±5.2 ab
HD	3.6±0.2 b	25.3±2.6 b	111.5±4..5 bc
ID	4.8±0.5 a	33.4±2.4 a	93.2±4.2 c
VD	3.5±0.3 b	26.5±1.6 b	126.5±10.4 ab
FD	2.1±0.2 c	16.2±2.1 c	133.4±9.3 a

Values represent means of three independent replicates ± SD. Different letters within a column indicate significant differences at $p < 0.05$.

Table S3 The ZOI (mm) and MIC (mg/mL) of different extracts from *I. rubescens*

	<i>S. aureus</i>		<i>B. subtilis</i>		<i>E. coli</i>		<i>S. dysenteriae</i>	
	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC
ND	20.3±1.0 a	5	18.3±1.3 a	10	7.2±0.4 a	ND	6.5±0.7 a	ND
HD	19.6±1.4 a	5	17.1±1.1 a	10	7.0±0.5 a	ND	7.6±0.8 a	ND
ID	11.5±1.7 b	10	10.0±1.5 b	20	6.5±0.6 a	ND	6.6±0.6 a	ND
VD	18.2±1.4 a	10	16.4±1.4 a	10	6.5±0.7 a	ND	7.0±0.2 a	ND
FD	22.4±3.1 a	5	19.8±2.6 a	10	7.3±0.4 a	ND	7.4±0.5 a	ND
Gentamicin	25.5±2.3 a	<0.1	27.4±2.2 a	<0.1	21.6±1.8 a	<0.1	19.5±2.3	<0.1

Values represent means of three independent replicates \pm SD. Different letters within a column indicate significant differences at $p < 0.05$ for ZOI of different treatments. ND, no detect.

Table S4 Correlation analysis of different compounds and bioactivity

	Antibacterial activity (ZOI, mm)	Antioxidant activity		
		DPPH (EC ₅₀ , mg/mL)	ABTS (EC ₅₀ ,mg/mL)	FRAP (μ mol Fe(II)/g)
Chlorogenic acid	0.8853*	0.9807**	0.9687**	0.9094*
Caffeic acid	0.9405*	0.9364*	0.9216*	0.9369*
Rosmarinic acid	0.9495*	0.9297*	0.9164*	0.9293*
Oridonin	0.8876*	0.8050	0.7750	0.9972**
Ponicidin	0.7840	0.7972	0.7566	0.9882**
Total phenolics	0.9797**	0.8104*	0.9053*	0.9059*
Total flavonoids	0.9539*	0.9238*	0.9120*	0.9126*

Significantly different: ** $p < 0.01$, * $p < 0.05$.

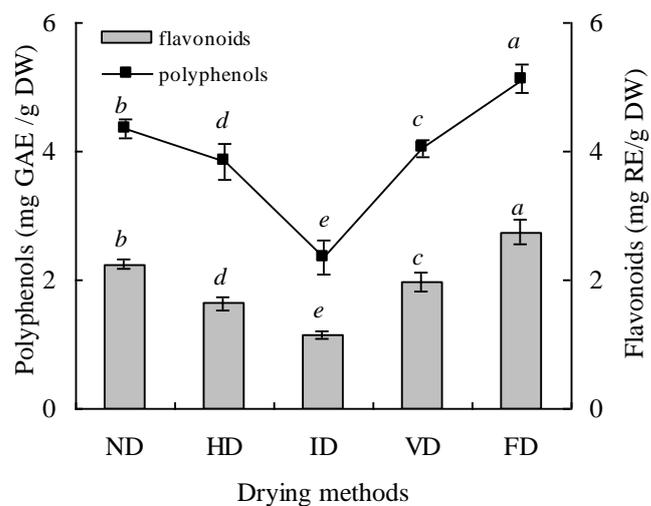


Figure S1. The contents of total phenolics and flavonoids of *I. rubescens* extracts. The results are expressed as mean \pm standard deviation of three independent experiments performed in duplicate. Different letters indicate statistically significant differences between different treatments.