NATURAL PRODUCTS

Quality Assessment and Antioxidant Activities of the Blossoms of *Inula Nervosa* Wall

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Abstract

Background: Currently, although *Inula nervosa* Wall is substantially investigated, little is understood about blossoms of *Inula nervosa* Wall (BINW).

Objective: In this work, we systematically investigated the antioxidant activity of the extract from BINW by various standard assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical ability, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt radical cation (ABTS), and ferric reducing antioxidant potential (FRAP).

Methods: Chemical compounds were tentatively identified through an UHPLC-QTOF-MS system. Furthermore, the contents of nine compounds were detected with UHPLC method coupled with photodiode array (PDA) detector. By carefully analyzing the quantitative data via clusters analysis and principal component analysis (PCA).

Results: Forty-six compounds were tentatively identified, and our results showed that nine compound samples in 21 batches of BINW collected from different areas could be differentiated and analyzed by a heatmap visualization. In addition, the contents of nine compounds (flavonoids, phenolic acids) exhibited a total of higher amounts and better antioxidant activities from Yunnan than those from the other three origins.

Conclusions: Our study not only developed a powerful platform to explain the difference between traditional Chinese medicines species that are closely related through the chemometric and chemical profiling, but also presented a useful method to establish quality criteria of BINW with multiple origins.

Highlights: To characterize the BINW in detail, we not only performed DPPH, FRAP, and ABTS assays to investigate its antioxidant activity, but also established UHPLC-QTOF-MS/MS- and UHPLC-PDA-based methods to comprehensively identify and qualitatively analyze its components.

An imbalance of the homeostasis between pro-oxidant and antioxidant could induce oxidative stress and generate toxic

radical species. Varieties of oxidative stress in most aerobic organisms can have many effects on different

biomacromolecules such as including DNA, proteins, and membrane lipids, which would induce various chronic disorders such as cancer, aging, inflammation, type 2 diabetes, and neurodegenerative diseases (1–5). Reactive oxygen species at low levels are essential for life, however excessive amounts could have a negative impact on human health. Hence, study of antioxidants of greater efficacy and lower toxicity was worthy of considerable attention. To evaluate antioxidant effects, there are many efforts spent in establishing relevant detection methods, among which 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS), ferric reducing antioxidant potential (FRAP), and 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power assays, were frequently employed (6–9).

Inula nervosa Wall (INW), commonly called “Xiaoheiyao” in China, is currently being extensively used as an expectorant and digestant in Chinese medicine. In addition, blossoms of INW (BINW) have been utilized as a popular kind of wine used by the Tong people, one of the minorities in China (10). Recently, reports indicated that many active compounds from INW, such as polysaccharides, phenolic acids, and flavonoids (11–13), exhibit numerous beneficial effects including anti-inflammatory, neuroprotective, antioxidant, hepatoprotective, and anti-cancer assistance (14–18). However, little is understood about the antioxidant effects from extracts of BINW and their bioactive components. Analytical techniques such as LC-MS, HPLC, and GC are the fundamental ways to analyze components in vivo and in vitro (19–24). Up until now, a series of analytical methods such as LC-MS, HPLC-PDA, and GC-MS has been used for qualitative and quantitative analysis of INW (25, 26). However, there were few compounds quantified because of the lack of good standards. To this end, this study will establish a series of good standards, aimed at quality assessment and antioxidant activities for these samples from different areas of BINW. First, an UHPLC-QTOF-MS/MS method is established to identify chemical constituents. Next, 21 batches of samples from different areas of BINW were used to determine the contents of multiple compounds and also to test the antioxidant capacities through DPPH, ABTS, and FRAP assay.

Materials and Methods

Chemicals and Reagents

The nine standard chemical compounds including kaempferol, luteolin, kaempferol-3-O-rutinoside, nepetin, 3,5-dicaffeoylquinic acid, chlorogenic acid, quercetin, 1,5-DQA: 1,5-dicaffeoylquinic acid, and thymol were bought from Chengdu DeSiTe Biotechnology Co., Ltd. (Chengdu, China). All compounds' purity was higher than 98% which was detected through HPLC-(PDA) detector analysis. HPLC-grade formic acid, MeOH, and EtOH were bought from Thermo Fisher Scientific (China) Co., Ltd. Distilled water was bought from Watsons Water (Hong Kong, China). DPPH, ABTS, FRAP, and Vitamin C were bought from Beijing Solarbio Science & Technology Co., Ltd.

Plant Materials and Sample Preparation

The samples of BINW, totaling 21 batches in all, were gathered from the Hebei, Shandong, Yunnan, and Hubei provinces and verified by Professor Jianping Gong from Jiangxi University of Traditional Chinese Medicine. Detailed information on the samples are shown in Supplemental Table S1.

Extraction of each dried sample was obtained from 0.5 g powder dissolved in a 50 mL solution ($\text{CH}_3\text{OH}:\text{H}_2\text{O}=7:3$). Later, the solution was refluxed for 30 min, and then centrifuged at 13 krpm for 10 min, which were finally used for further analyze.

A stock solution of nine standards was prepared by dissolving accurately weighed standards in methanol to make the concentrations of 184.0, 123.3, 645.5, 38.0, 36.3, 19.0, 62.0, 22.0, and 520.0 $\mu\text{g/mL}$ for chlorogenic acid (C1), 3,5-dicaffeoylquinic acid (C2), 1,5-dicaffeoylquinic acid (C3), kaempferol-3-O-rutinoside (C4), quercetin (C5), luteolin (C6), nepetin (C7), kaempferol (C8), and thymol (C9), respectively. The stock solution was then diluted to a series of working solutions in six levels by methanol solution, and all solutions were kept at 4°C.

Antioxidant Activities

All samples were diluted with methanol and then a series of extract concentrations were prepared. In addition, the antioxidant activities were tested via the methods of DPPH, FRAP, and ABTS.

DPPH Assay

DPPH activity assay was determined based on a previous report (27). Briefly, the solution of extracts (100 μL) were mixed with DPPH (100 μL) and the solutions were put it in the dark for 30 min to prepare a stock solution. Finally, the IC_{50} values were calculated based on the absorbance characterized at 517 nm.

FRAP Assay

Ferric activity assay was determined based on the Benzie and Strain method with minor modifications (28). Samples (5 mg/mL) were dissolved in a 1 mL solution containing potassium ferricyanide (1%) and phosphate buffer (0.2 M, pH = 6.6), following incubation at 50°C for 30 min. The mixed sample (1 mL) was then added to a stock solution including deionized water (1 mL), 10% trichloroacetic acid (1 mL), and 0.1% FeCl_3 (1 mL). Finally, the IC_{50} values calculated through absorbance were measured at 700 nm after depositing the solution in the dark for 10 min.

ABTS Assay

An ABTS solution was prepared by the traditional method. Extracts (100 μL) were mixed with the ABTS solution (100 μL) and put it in the dark for 30 min. The IC_{50} values calculated through absorbance measured 734 nm (29).

The Analytical Condition of the UHPLC-QTOF-MS/MS Experiments

The UHPLC analysis for BINW was performed with an LC-30 type instrument system from Shimadzu (Kyoto, Japan). The separation was carried out using a Welch HPLC column (Ultimate XB C₁₈: 100 mm × 2.1 mm, 1.7 μm) and the flow rate was fixed at 0.25 mL per/min. The temperature of the column was kept at 35°C, and the volume of the sample injected was 2 μL . The gradient elution system was established by CH_3CN (A solvent) and 0.1% aqueous formic acid (B solvent): 98% B (0–4 min), 98–95% B (4–6 min), 95–90% B (6–10 min), 90–82% B (10–22 min), 82–75% B (22–32 min), 75–30% B (32–42 min), 30–98% B (42–52 min), 98% B (52–55 min).

The UHPLC-QTOF-MS/MS was used by an AB SCIEX Triple TOF™ 5600+ system (Boston, CA, USA). The optimized MS condition was listed as follows: declustering potential (DP) and the ion spray voltage were fixed at ± 100 and ± 4500 V, respectively.

The temperature for ion source was set at 550°C; curtain gas, nebulizer gas (Gas 1) and heater gas (Gas 2) were set at 50, 50, and 25 psi, respectively. The collision energy (CE) was fixed at 40 and –35 eV for BINW, respectively.

UHPLC Chromatographic Conditions

The UHPLC system (Shimadzu, Kyoto, Japan) consisting of an LC-300type instrument system was applied to quantitative determination for different compounds. The separation experiment was carried out via an Agilent column (100 mm × 3.0 mm, 2.7 μm, Poroshell 120 C₁₈). The flow rate for the system was set at 0.30 mL/min with the temperature fixed at 35°C. The wavelength for the detection wavelength was set at 254 nm, and the sample volume for the injection was 5 μL. The mobile phase included CH₃CN (A solvent) and 0.1% CH₃COOH (B solvent), which were used as a gradient elution system with an equilibration time of 15 min, the details of which are shown below: 98–98% B (0–4 min), 98–95% B (4–6 min), 95–90% B (6–10 min), 90–80% B (10–20 min), 80–73% B (20–30 min), 73–75% B (30–37 min), 75–68% B (37–45 min), 68–42% B (45–68 min), 42–75% B (68–75 min), 75–98% B (75–82 min), and 98–98% B (82–90 min). The acceptable ranges of the linearity, detection, and quantification limit of precision, repeatability, stability, and accuracy were followed according to Pharmacopoeia guidelines.

Results and Discussion

Antioxidant Assay for the Extracts of BINW

The human body contains a variety of free radical, light radicals, superoxide anions, and peroxide radicals ozone, as well as various antioxidants. However, due to their diverse natures, a simplex antioxidant may exhibit different effects when suffering various free radicals. Therefore, diverse methods for antioxidant evaluation should be applied in assessing the capability of total antioxidants in substances comprehensively. Based on these reasons, the antioxidant activity of BINW was evaluated by using the FRAP, DPPH, and ABTS methods.

DPPH is an effective method for testing the antioxidant abilities of BINW. Based on the results obtained, the extracts of BINW from different areas also show different free radical scavenging abilities. The IC₅₀ values were: Yunnan samples 1.40 ± 0.03 mg/mL ~ 1.57 ± 0.01 mg/mL; Shandong samples 4.70 ± 0.04 mg/mL ~ 6.05 ± 0.00 mg/mL; Hebei samples 2.83 ± 0.01 mg/mL ~ 3.18 ± 0.03 mg/mL; and Hubei samples 3.63 ± 0.03 mg/mL ~ 4.01 ± 0.01 mg/mL. The tendency of IC₅₀ values calculated from ABTS and FRAP was similar to those from DPPH shown in Table 1. In short, it was clearly shown that the samples from Yunnan overall have better antioxidant activity than those from the other three areas.

Optimization of Extraction and Analysis Conditions

Different extraction solvents (95, 75, 50, and 25% methanol or ethanol) and extraction time (60 or 30 min) were applied to find the most efficient extraction method. The optimized method was reflux extraction by 70% methanol at room temperature for 30 min. The extracts of BINW were tested and evaluated in two ionization modes (positive and negative) by UHPLC-Q-TOF-MS/MS. In addition, several experiments on mobile phase combinations such as 0.1% formic acid with acetonitrile or methanol were conducted. The results indicated that CH₃CN containing formic acid (0.1%, v/v) showed better sufficient ionization and separation.

Table 1. Antioxidant activity of blossoms of *Inula nervosa* Wall [IC₅₀ value (mg/mL)]^a

Sample source	DPPH	FRAP	ABTS
Shandong, S1	4.70 ± 0.04	7.56 ± 0.02	19.90 ± 0.05
Shandong, S2	5.04 ± 0.02	6.56 ± 0.02	20.36 ± 0.08
Shandong, S3	4.89 ± 0.02	7.12 ± 0.03	21.15 ± 0.02
Shandong, S4	6.05 ± 0.00	7.50 ± 0.02	20.36 ± 0.06
Yunnan, S5	1.57 ± 0.01	1.34 ± 0.02	6.18 ± 0.03
Yunnan, S6	1.44 ± 0.02	1.16 ± 0.02	5.64 ± 0.03
Yunnan, S7	1.42 ± 0.02	1.41 ± 0.01	7.95 ± 0.03
Yunnan, S8	1.40 ± 0.03	1.43 ± 0.05	8.50 ± 0.07
Hebei, S9	2.96 ± 0.02	2.54 ± 0.04	11.40 ± 0.03
Hebei, S10	2.83 ± 0.01	2.85 ± 0.03	13.01 ± 0.06
Hebei, S11	2.94 ± 0.03	2.97 ± 0.07	13.07 ± 0.03
Hebei, S12	2.89 ± 0.02	3.08 ± 0.01	13.66 ± 0.07
Hebei, S13	3.16 ± 0.07	3.18 ± 0.07	14.87 ± 0.05
Hebei, S14	3.18 ± 0.03	3.16 ± 0.00	14.88 ± 0.02
Hebei, S15	3.14 ± 0.04	3.22 ± 0.00	13.14 ± 0.07
Hubei, S16	3.85 ± 0.02	4.13 ± 0.02	16.04 ± 0.02
Hubei, S17	3.63 ± 0.03	4.29 ± 0.04	16.06 ± 0.05
Hubei, S18	3.96 ± 0.07	4.43 ± 0.07	17.00 ± 0.03
Hubei, S19	3.77 ± 0.05	4.84 ± 0.05	17.17 ± 0.07
Hubei, S20	3.95 ± 0.04	4.86 ± 0.02	17.62 ± 0.03
Hubei, S21	4.01 ± 0.01	5.08 ± 0.03	18.92 ± 0.06
Vitamin C	0.12 ± 0.01	0.32 ± 0.01	1.23 ± 0.02

^a Each value in the table is presented as the mean ± SD (n = 3).

Identification and Characterization of the Compounds from BINW by the Combined Instrument System of UHPLC-QTOF-MS/MS

The basic peak chromatograms (BPC) of BINW in two ionization modes (positive and negative) are shown in Figure 1. Forty-six compounds were discovered and, preliminarily, evaluated, including 10 flavonoids, 8 phenolic acids, and another 28 compounds (including coumarins, anthraquinones, glycosides, etc.) (30–53). The retention time and fragment information were compared with the reference standard, and 11 compounds were successfully identified and confirmed by analyzing the mass results. The detailed MS data of identified compounds is listed in Table 2. For instance, there is a molecular ion [M – H] m/z 179.0347 for peak 12, and it split into an ion at m/z 161.0380 (Δm = 18) due to the splitting of H₂O. The fragment of m/z 135.0456, and 107.0511 were due to the loss of CO₂ and CO. Furthermore, taking peak 15 as another example, there is a protonated molecular ion [MH] at m/z 609.1854. Due to the cleavage of glucose bond, it disintegrates to ion at m/z 447.0970 (ΔM = 162). The fragments of m/z 285.0411 and 257.0275 were due to the loss of glucose and CO.

UHPLC-PDA Method Validation

The chromatogram of nine standard compound mixtures are shown in Figure 2A and a sample chromatogram is shown in Figure 2B. We first confirmed that all calibration curves exhibited good linearity within the test ranges of our test experiment. Then the LOD was determined in the ranges of 0.23–10.24 μg/mL as the LOQs were 0.77–34.13 μg/mL (Supplemental Table S2). The RSD data was less than 1.00% in terms of both intra- and inter-day precisions. Stability was analyzed in 24 h and the RSD results were in the range of 0.11–1.05%. Repeatability was evaluated and performed with 6 individual samples each (S13, Hebei) and RSD values did not reach 2.00%. The average recoveries

Figure 1. The basic peak chromatogram (BPC) of BINW in the negative (A) and positive (B) ion modes.

Figure 2. (A) Chromatogram of nine mixture standard compounds: Chlorogenic acid (C1), 3,5-Dicaffeoylquinic acid (C2), 1,5-Dicaffeoylquinic acid (C3), Kaempferol-3-O-rutinoside (C4), Quercetin (C5), Luteolin (C6), Nepetin (C7), Kaempferol (C8), and Thymol (C9). (B) Sample chromatogram.

Table 2. Structural information of compounds characterized of K. indica by negative UHPLC-QTOF-MS/MS

No.	tR, min	MF	Product ion	m/z	Calc. m/z	Diff., ppm	Fragment ions	Compounds	Ref.
1	1.91	C ₇ H ₆ O ₅	[M-H] ⁻	169.0341	169.0323	-2.7	151.5225, 125.6473, 107.3104	Gallic acid	(30)
2	2.08	C ₁₁ H ₁₄ N ₃ NaO ₃ S	[M-Na] ⁻	304.0312	304.0123	1.7	289.0431, 240.5433, 156.0342	Methyl orange	(31)
3	3.55	C ₁₀ H ₁₂ O ₄	[M+H] ⁺	197.0808	197.0809	0.6	137.0602, 122.0373, 109.0689	ω-Hydroxypropioguaiacone	(32)
4	3.63	C ₉ H ₁₁ NO ₂	[M-H] ⁻	164.0717	164.0729	4.5	147.0447, 103.0545	Phenylalanine	(33)
5	3.79	C ₁₇ H ₂₀ N ₄ O ₆	[M-H] ⁻	375.131	375.1316	1.5	339.1140, 327.1142, 213.0780, 177.0550, 149.0602, 122.0389	Riboflavin	(34, 35)
6 ^a	8.04	C ₁₆ H ₁₈ O ₉	[M-H] ⁻	353.0878	353.0896	4.1	191.0566	5-Caffeoylquinic acid	(36-38)
7	8.96	C ₁₂ H ₁₇ N ₄ OS.Cl	[M-H] ⁻	299.0739	299.0779	3.5	137.0246, 136.0148	Vitamin B1	(34, 35)
8	9.4	C ₁₆ H ₁₈ O ₉	[M-H] ⁻	353.0878	353.0891	3.8	191.0563, 179.0349, 135.0458	Chlorogenic acid	(36-38)
9	11.3	C ₈ H ₈ O ₃	[M-H] ⁻	151.0401	151.041	-4.2	136.0163, 109.0268, 108.0221	Isovanillin	(39, 40)
10	11.51	C ₇ H ₆ O ₂	[M-H] ⁻	121.0295	121.0311	3.4	91.0161	2-Hydroxybenzaldehyde	(39, 40)
11 ^a	11.92	C ₁₆ H ₁₈ O ₉	[M-H] ⁻	353.0866	353.0897	4.5	191.0567	scopolin	(39, 40)
12 ^a	12.05	C ₉ H ₈ O ₄	[M-H] ⁻	179.0349	179.0359	4.9	161.0380, 135.0456, 134.0373, 107.0511	Caffeic acid	(36-38)
13 ^a	12.62	C ₁₆ H ₁₈ O ₉	[M-H] ⁻	353.0878	353.0896	1.5	191.0565, 179.0352, 173.0461, 135.0458	4-Caffeoylquinic acid	(39, 40)
14	13.57	C ₁₇ H ₂₆ O ₉	[M-H] ⁻	373.1504	373.1522	4.7	327.1487, 165.0929, 164.0847, 161.0470, 123.0431, 113.0295, 85.0321	Dihydrosyringin	(41)
15 ^a	15.03	C ₂₇ H ₃₀ O ₁₆	[M-H] ⁻	609.1461	609.1505	2.7	447.0970, 285.0411, 255.0275	Rutin	(39, 40)
16	15.84	C ₁₃ H ₁₈ O ₆	[M-H] ⁻	269.1031	269.0949	-0.3	225.1032, 183.0935, 157.0870, 156.0812, 142.0643	Benzyl alcohol-β-D-glucoside	(42)
17	16.26	C ₁₅ H ₁₈ O ₄	[M-H] ⁻	261.1132	261.1144	4.5	217.01248, 146.9254	Coronopilin isomer	(43)
18	17.21	C ₁₀ H ₆ O ₄	[M+H] ⁺	193.0495	193.0496	0.5	178.0271, 150.0305, 133.0282, 122.0353, 107.0479, 105.0341	Isoscopoletin	(39, 40)
19 ^a	18.2	C ₂₁ H ₂₀ O ₁₂	[M-H] ⁻	463.0882	463.0905	2.8	301.0380, 300.0284	Isoquercitrin	(39, 40)
20 ^a	18.63	C ₁₅ H ₁₀ O ₇	[M-H] ⁻	301.0354	301.0373	3.6	273.0410, 255.0305, 159.0473	Quercetin	(39, 40)
21	19.11	C ₁₁ H ₁₆ O ₃	[M+H] ⁺	197.1172	197.1173	0.5	179.1091, 161.0935, 151.0771, 133.1012, 115.0545, 105.0704	Lolilide	(44)
22	19.59	C ₁₂ H ₂₂ O ₆	[M-H] ⁻	261.1343	261.1359	-2.5	243.1244, 207.0996, 201.1129	3-hexenol-β-D-glucoside	(45)
23	20.71	C ₂₁ H ₂₀ O ₁₂	[M-H] ⁻	463.0882	463.0912	-3	301.0373, 300.0293, 271.0265, 255.0316, 151.0030	Isoquercetin	(39, 46, 47)
24 ^a	21.29	C ₂₁ H ₂₀ O ₁₁	[M-H] ⁻	447.0933	447.0963	3.7	285.0422, 284.0336	Quercetin	(39, 46, 47)
25	21.94	C ₁₅ H ₂₀ O ₄	[M-H] ⁻	263.1032	263.1047	4.2	219.1043, 217.1249, 199.1102, 177.0923, 159.0826, 137.0612, 123.0525	Coronopilin	(43)
26	22.79	C ₁₅ H ₁₂ O ₆	[M-H] ⁻	287.0561	287.0573	4.1	259.0618, 201.0549, 125.0254	Carthamidin	(48)
27 ^a	23.21	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1195	515.1228	4.6	353.1242, 203.0354, 191.0563, 179.0353	3,5-Dicaffeoylquinic acid	(39, 46, 47)
28	24.48	C ₂₁ H ₂₀ O ₁₀	[M-H] ⁻	431.0984	431.1018	2.4	387.1867, 369.1814, 363.1814, 319.1947, 317.1775, 311.0578, 273.1909, 269.0472, 211.0427	Ermodin-1-O-β-D-glucoside	(49)
29	24.61	C ₁₅ H ₁₈ O ₄	[M+H] ⁺	263.1278	263.1284	2.2	245.1213, 227.1088, 199.1125, 173.0970, 165.0700, 129.0703, 105.0695	Nervolan C	(50)
30	25.98	C ₂₂ H ₂₂ O ₁₁	[M-H] ⁻	461.1089	461.1124	3.5	446.0905, 299.0546, 297.0435, 283.0261, 255.0296	Genistein	(39, 46, 47)
31	30.72	C ₁₅ H ₁₀ O ₆	[M-H] ⁻	285.0405	285.0409	1.5	199.0402, 195.9147, 181.1264, 151.0115, 133.0314, 117.0377	Luteolin	(39, 46, 47)
32	34.76	C ₁₇ H ₂₀ O ₅	[M+H] ⁺	305.1383	305.1396	4.1	263.1291, 245.1175, 227.1075, 217.1223, 199.1124, 181.1020, 171.1172, 143.0856, 129.0856, 105.0692	Bigelovin	(51)
33 ^a	35.04	C ₁₅ H ₁₀ O ₅	[M-H] ⁻	269.0456	269.0467	3.5	225.1136, 211.0332	Ermodin	(49)

(continued)

Table 2.. (continued)

No.	tR, min	M F	Product ion	m/z	Calc. m/z	Diff., ppm	Fragment ions	Compounds	Ref.
34	35.10	C ₁₄ H ₂₀ O ₅	[M-H] ⁻	267.1238	267.1258	2.7	223.1312, 179.0704, 161.0615, 133.0663, 121.0666, 87.0463	Colletalol	(52)
35	35.82	C ₁₆ H ₁₂ O ₆	[M-H] ⁻	299.0561	299.0586	3.5	284.0340, 256.0382, 199.0422, 146.9690	hydroxygenkwanin	(46)
36	37.41	C ₁₉ H ₃₂ O ₈	[M-H] ⁻	387.2024	387.1961	3.1	351.2175, 329.2358, 251.1253, 233.1192, 229.1457, 211.1364	Boscialin 4'-O-Glucoside	(39, 46, 47)
37 ^a	37.59	C ₁₅ H ₂₂ O ₄	[M-H] ⁻	265.1445	265.1458	2.3	221.1577	Inulicin	(50)
38	39.32	C ₁₇ H ₂₄ O ₅	[M-H] ⁻	307.1551	307.1576	3.6	102.9592	Acetyl inulinat	(50)
39	39.79	C ₁₅ H ₂₀ O ₃	[M-H] ⁻	247.1339	247.136	3.1	203.1456, 201.1300, 135.0809	Neobritannilactone B	(50)
40	41.49	C ₂₀ H ₃₈ O ₄	[M-H] ⁻	341.2697	341.2675	-2.3	213.1539, 165.1185, 164.1172	Nervoyan B	(50)
41	41.73	C ₁₆ H ₃₂ O ₂	[M-H] ⁻	255.2329	255.2345	2.2	186.945	palmitic acid	(39, 46, 47)
42	42.05	C ₂₁ H ₂₆ O ₆	[M+H] ⁺	375.1802	375.1803	0.1	357.1670, 273.1125, 195.0983, 145.0658	Sinapyl diangelate	(50)
43	42.14	C ₂₀ H ₂₄ O ₅	[M-H] ⁻	343.1551	343.1584	3.6	315.1604, 206.8956	Coniferyl diangelate	(50)
44	42.93	C ₉ H ₁₆ O ₂	[M-H] ⁻	155.1077	155.1097	2.5	136.9436, 127.0478, 113.1003, 109.0425	Allyl hexanoate	(53)
45	43.27	C ₁₆ H ₂₄ O ₂	[M-H] ⁻	247.1703	247.1724	3.8	231.1361	2,4,6-tri(propan-2-yl) benzoic acid	(46)
46	43.6	C ₂₀ H ₃₄ O ₂	[M+H] ⁺	307.2631	307.2631	3.1	192.0040, 173.1370, 91.0557	(13E)-Neocleroda-3,13-diene-15,18-diol	(46)

^a Confirmed by standard compounds.

were in the range of 96.05–103.01% while the RSD data was determined in the range of 0.45–2.74%. Our results showed that the analytical method established here is successfully used to quantify the multi-compounds in BINW (Supplemental Table S3).

Quantitative Analysis of the Nine Compounds in BINW

The contents of the nine compounds were tested in 21 BINW samples. As shown in Table 3, it can clearly be seen that the total contents of nine monomers in Yunnan province were the highest among these batches of samples from different origins (Figure 3). In addition, our results also suggested that 1,5-dicaffeoylquinic acid (C3) and thymol (C9) may be the basic and key constituents in BINW.

Discrimination of the BINW from Different Areas based on the Chemometrics

The quantitative data was analyzed both by IBM SPSS statistics and SIMCA-P 12.0 software to discriminate BINW samples from different areas. With the cluster analysis by SPSS, all data can be divided into four categories that corresponded to four different origins (Figure 4). Principal component analysis (PCA) was utilized to interpret the trend of the classification. The PCA scores plot (Figure 5) demonstrated a visible separation combined two constituents occupying 82.72% of the variance in total among 21 samples. Therefore, these data clearly demonstrated that chemometrics software was a useful instrument to classify the areas of BINW.

Distribution of the Nine Compounds in BINW from Different Areas

Although a clear discrimination of 4 groups of BINW samples was successfully confirmed and investigated, the distribution of the 9 compounds in 21 BINW samples was still unknown. Therefore, after Pareto calibration, the mean centered method was used to analyze the visualization of thermogram for unsupervised clustering, and the situation of these nine monomers can be clearly understood. As shown in Figure 6, BINW samples were obviously discriminated by hierarchical clustering analysis (HCA). The distribution of nine compounds in the four groups of BINW samples are deserving of notice. The compounds of kaempferol-3-O-rutinoside (C4), quercetin (C5), and luteolin (C7) could be marked to distinguish Yunnan samples from the other three area samples. Otherwise, chlorogenic acid (C1) and 3,5-dicaffeoylquinic acid (C2) could be taken into consideration in Shandong samples for the difference between the other three areas samples. Due to the variation of findings in the chemical constituents from the different areas samples, our results could explain the significant discrimination among these samples from Yunnan, Shandong, Hebei, and Hubei, thus probably influencing their pharmacological activities which could be further determined in the future.

Conclusions

In this paper, in an effort characterize BINW in detail, we not only performed DPPH, FRAP, and ABTS assays not only to investigate antioxidant activity but also to establish UHPLC-QTOF-MS/MS- and UHPLC-PDA-based methods to comprehensively identify and qualitatively analyze their components. Interestingly, our results showed that 46 compounds in total,

Table 3. The contents (mg/g) of the 9 marker compounds in 21 samples of BINW

Sample	Location	C1	C2	C3	C4	C5	C6	C7	C8	C9	Total contents
S1	Shandong	1.46 ^a	1.33	7.07	0.36	0.48	0.16	0.88	0.16	5.54	17.44
S2	Shandong	1.42	1.15	6.93	0.36	0.40	0.17	0.90	0.16	5.64	17.13
S3	Shandong	1.41	1.40	7.24	0.35	0.43	0.25	0.93	0.15	5.51	17.67
S4	Shandong	1.39	1.42	7.49	0.36	0.49	0.28	0.91	0.16	6.09	18.58
S5	Yunnan	5.70	7.25	19.89	1.43	1.49	0.77	1.49	0.38	16.11	54.50
S6	Yunnan	5.03	6.95	20.35	1.45	1.51	0.77	1.48	0.38	15.43	53.35
S7	Yunnan	5.28	7.11	20.34	1.48	1.53	0.82	1.53	0.40	16.04	54.52
S8	Yunnan	5.34	7.49	20.35	1.56	1.53	0.84	1.52	0.37	15.64	54.64
S9	Hebei	2.95	2.53	12.97	0.96	0.96	0.31	1.26	0.21	8.87	31.02
S10	Hebei	2.82	2.84	13.00	0.99	0.81	0.31	1.27	0.22	8.76	31.02
S11	Hebei	2.93	2.96	13.06	0.95	0.84	0.33	1.31	0.24	8.90	31.52
S12	Hebei	2.88	3.07	13.65	1.07	0.84	0.34	1.25	0.22	9.23	32.56
S13	Hebei	3.05	3.17	12.86	1.10	0.89	0.33	1.27	0.23	9.36	32.26
S14	Hebei	3.07	3.15	12.88	1.11	0.93	0.33	1.30	0.22	9.04	32.03
S15	Hebei	3.03	3.11	13.13	1.16	0.99	0.33	1.29	0.19	9.18	32.41
S16	Hubei	3.84	4.32	16.83	1.17	1.09	0.47	1.05	0.13	12.88	41.78
S17	Hubei	3.87	4.28	16.75	1.21	1.07	0.46	1.07	0.11	12.75	41.56
S18	Hubei	3.95	4.42	16.99	1.23	1.07	0.48	1.07	0.11	13.09	42.41
S19	Hubei	3.86	4.23	17.16	1.20	1.09	0.52	1.10	0.11	13.38	42.66
S20	Hubei	3.94	4.35	17.21	1.23	1.12	0.52	1.12	0.12	13.54	43.14
S21	Hubei	3.90	4.27	17.11	1.22	1.16	0.51	1.11	0.10	13.00	42.38

^aValues represent means $\pm$ SD, n = 9. **Figure 3.** The total contents of 9 compounds in 21 samples. **Figure 4.** The diagram of cluster analysis of 21 batches of BINW samples.

including 10 flavonoids, 8 phenolic acids, and another 28 compounds, were tentatively identified. In addition, our results also confirmed that UHPLC-PAD as a rapid, accurate, and efficient method could be employed for qualitative analysis of 21 batches of BINW. Furthermore, our results also indicated that nine compounds could be marked as the quality control compounds for BINW. Together with quantitative data by the clusters analysis via PCA, our results show that a heatmap visualization could be used to analyze and discriminate the 21 batches of BINW from different areas through the quality control of 9 compounds. Lastly, our results also indicated that the Yunnan samples had the highest contents of the nine compounds (flavonoids, phenolic acids) and the best antioxidant activity among these samples from four different origins, which unambiguously indicated that the higher the total amount of flavones and phenolic

Figure 5. The PCA score plot of 21 batches of BINW samples based on the quantitative data of 9 compounds using Pareto scaling with mean centering. The green circle corresponds to Shandong province (S1–S4), blue square corresponds to Yunnan province (S5–S8), red triangle corresponds to HeBei province (S9–S15), and the yellow pentagram corresponds to Hubei province (S16–S21).

Figure 6. Hierarchical clustering analysis heatmap of nine compounds between Yunnan, Shandong, Hebei, and Hubei samples.

acids in the sample is, the better the antioxidant activity it shows. Taken together, our study not only developed a powerful platform to explain the different traditional Chinese medicines species that are closely related through chemometric and chemical profiling, but also presented a useful method to establish quality criteria of BINW with multiple origins. It is envisioned that the methods developed here could be extended to the many compound identifications and discriminations from other sources in the future.

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Conflict of interest

There is no conflict in this section.

Supplemental information

[Supplemental information](#) is available on the J. AOAC Int. website.

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