JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Identification and Quantification of Phytochemical Composition and Anti-inflammatory and Radical Scavenging Properties of Methanolic Extracts of Chinese Propolis

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Supporting Information

ABSTRACT: Fifteen propolis samples collected from different regions of China were investigated and compared for their phytochemical composition and anti-inflammatory and radical scavenging properties. Eleven compounds including caffeic, *p*-coumaric, ferulic, isoferulic, and 3,4-dimethylcaffeic acids, pinobanksin, chrysin, pinocembrin, galangin, pinobanksin 3-acetate, and caffeic acid phenylethyl ester were quantified for the 15 propolis samples using a UHPLC method, whereas 38 compounds were identified by UPLC/Q-TOF-MS. The 15 propolis samples significantly differed in their total phenolic and total flavonoid contents, as well as their phytochemical profiles. The methanol extracts of propolis also showed significant anti-inflammatory effects in LPS-stimulated RAW 264.7 mouse macrophage cells at 10 μg propolis extract/mL concentration. Additionally, the propolis samples differed in their DPPH, ABTS cation, hydroxyl, and peroxide radical scavenging capacities and ferric reducing abilities. The results from this study may be used to improve the commercial production and consumption of Chinese propolis products.

KEYWORDS: *propolis, phenolic, flavonoid, anti-inflammation, radical scavenging, methanolic extract, chemical constituent*

INTRODUCTION

Propolis, a resinous and adhesive natural substance produced by honeybees, has been used in functional foods and folk medicines for several centuries. Previous studies have shown that propolis may possess several health benefits, including antioxidant, anticancer, anti-inflammatory, antibacterial, antiviral, antifungal, and immunomodulatory properties.[1−4] The health benefits were mainly attributed to flavonoids and phenolic acids, the two major classes of phytochemicals in propolis. In the past 10 years, propolis has attracted more and more attention and has been extensively used in functional foods and nutritional supplement products.[5,6]

It is well accepted that the chemical constituents and health properties of propolis greatly depend on several ecological factors, including geographical region, plant source, season, and method of harvesting.[7−10] For instance, Hamasaka et al.[11] reported that 14 propolis samples collected in different locations of Japan differed in their chemical compositions and antioxidant activities in 2004. Additionally, Chinese propolis samples were shown to be rich in phenolics, including phenolic acids and flavonoids, and had strong antioxidant activities measured as reducing power, β-carotene bleaching inhibition, and scavenging ability against DPPH and ABTS cation radicals.[12,13] Recently, our group isolated 5 new glycerol esters and tentatively identified 12 minor constituents using UPLC-Q-TOF-MS from Wuhan propolis.[14] All five isolated compounds showed significant anti-inflammatory activities on interleukin (IL)-1β, IL-6, and cyclooxygenase (COX)-2 mRNA expressions. To date, there is little information on the hydroxyl ($HO^{\bullet}$) or peroxide anion ($O_2^{\bullet-}$) radical scavenging capacity of propolis. Also noted was that few UHPLC analyses have been performed to quantitatively characterize the detailed chemical composition of propolis, although the previous studies generally employed HPLC to quantify flavonoids and phenolic compounds by comparing the retention times and UV spectra with those of the standard compounds.

In the present study, 15 Chinese propolis were evaluated for their total phenolic contents (TPC), total flavonoid contents (TFC), potential anti-inflammatory effects, scavenging capability against $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $HO^{\bullet}$, and $O_2^{\bullet-}$, and ferric reducing ability. The anti-inflammatory effects were measured as their ability to suppress IL-1β, IL-6, and COX-2 mRNA expressions in LPS-stimulated RAW 264.7 mouse macrophages. In addition, a rapid and effective UHPLC analysis method was developed and applied to quantify the major compounds in the Chinese propolis samples. Finally, the chemical profiles of Chinese propolis from different origins were compared on the basis of UPLC/Q-TOF-MS analysis. The results advanced our understanding of the different chemical compositions among propolis and promoted its better use in health food and dietary supplement.

Received: October 6, 2012
Revised: November 22, 2012
Accepted: November 23, 2012
Published: November 23, 2012

MATERIALS AND METHODS

Materials. Fifteen propolis samples were collected from various locations in China (Figure S1 in the Supporting Information) and stored at −20 °C before use. Iron(III) chloride, fluorescein (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), gallic acid, 1,3,5-tri(2-pyridyl)-2,4,6-triazine (TPTZ), DMSO, and 2-peproponal were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reference compounds caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, 3,4-dimethylcaffeic acid, caffeic acid 1,1-dimethylallyl ester, and caffeic acid phenylethyl ester were purchased from Sigma-Aldrich; pinocembrin was obtained from Shanghai ANPEL Scientific Instrument Co., Ltd. (Shanghai, China); quercetin, apigenin, isorhamnetin, chrysin, and galangin were obtained from Shanghai R&D Centre for Standardization of Chinese Medicines; pinobanksin, caffeic acid isopent-3-enyl ester, caffeic acid 2-methyl-2-butenyl ester, pinobanksin 3-acetate, *p*-coumaric acid benzyl ester, caffeic acid cinnamyl ester, and chrysin-7-methyl ether were isolated from propolis in our laboratory. The purities of isolated compounds were all >98% by HPLC analysis. The chemical structures of all these compounds as standards were also confirmed by ^{1}H NMR and HR-MS. Folin−Ciocalteu (FC) reagent was purchased from Ambrosia Pharmaceuticals (Shanghai, China). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from J&K Scientific (Beijing, China). Thirty percent H_2O_2 reagent, analytical grade acetone, methanol, ethyl ether, ethyl acetate, petroleum ether, sodium hydroxide, sodium nitrite, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Sinopharm (Beijing, China). Aluminum nitrate was obtained from Aladdin (Shanghai, China). HPLC grade formic acid, methanol, and acetonitrile were purchased from Merck (Darmstadt, Hesse-Darmstadt Germany). RAW 264.7 mouse macrophage was purchased from the Chinese Academy of Sciences (Shanghai, China). DMEM, fetal bovine serum, and 1× PBS were purchased from Gibco (Life Technologies, Carlsbad, CA, USA). TRIzol reagent was obtained from Invitrogen (Life Technologies). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was obtained from Millipore (Billerica, MA, USA). IScript Advanced cDNA Synthesis kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA), whereas AB Power SYBR Green PCR Master Mix was purchased from ABI (Applied Biosystems, Carlsbad, CA, USA). Ultrapure water was prepared by a Millipore ultra-Genetic polishing system with <5 ppb TOC and resistivity of 18.2 mΩ and was used for all experiments.

Sample Preparation. The frozen propolis samples were powdered using a mill. Approximately 1 g of propolis was extracted by 10 mL of pure methanol at room temperature. After the mixtures were sonicated (320 W, 40 kHz) for 2 h, the extracts were collected. The extracts were kept at 4 °C in the refrigerator until further analysis. The average methanolic extract of propolis yield of 15 samples was 75.5 ± 6.2%. For the quantitative and qualitative analyses, an accurately weighed mass of the propolis powder (1.0 g) was transferred into a 50 mL volumetric flask adjusted with methanol and sonicated for 30 min. The supernatant of sample solution was filtered through a 0.22 μm GHP membrane for UPLC/Q-TOF-MS analysis and UHPLC quantification.

Total Phenolic Contents (TPC). The TPC of each methanol extract was measured according to a laboratory procedure described previously.[15] Briefly, the reaction mixture consisted of 50 μL of sample extracts, 250 μL of Folin−Ciocalteu reagent, 750 μL of 20% sodium carbonate, and 3 mL of ultrapure water. Gallic acid was used as the standard. Absorbance was read at 765 nm after 2 h of reaction at ambient temperature. The results are reported as milligrams gallic acid equivalent (GAE) per gram of propolis.

Total Flavonoid Contents (TFC). Total flavonoid was determined using an aluminum colorimetric method described previously.[16] In brief, 150 μL of propolis extracts was mixed with 1.5 mL of 5% sodium nitrite, and 1 mL of 10% aluminum nitrate was added after 6 min. Then 4 mL of 4% sodium hydroxide was added into the mixture. The absorbance was read at 502 nm after 15 min of reaction at ambient temperature. The results are reported as milligrams quercetin equivalent (QE) per gram propolis.

Identification of Chemical Constituents by UPLC-Q-TOF-MS Analysis. Propolis samples were analyzed for their chemical profiles by using a Waters Xevo G_2 Q-TOF mass spectrometer (Milford, MA, USA). UPLC was performed at 40 °C using an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm i.d.; 1.7 μm; Waters), equipped with an Acquity UPLC VanGuard precolumn (5 mm × 2.1 mm i.d.; 1.7 μm; Waters). The elution gradient (eluent A, 0.1% formic acid; eluent B, acetonitrile) was 20% B for 1.3 min, 20−30% B in 0.9 min, 30−40% B in 6.6 min, 40−60% B in 3.3 min, 60−90% B in 3.4 min, and 90% B for 3.2 min. The flow rate was 0.5 mL min^{-1}, and the injection volume was 1.5 μL. MS conditions were as follows: capillary voltages for negative and positive ion modes, 2.8 and 3.0 kV, respectively; sampling cone voltages for negative and positive ion modes, 55.0 and 43.0 V, respectively; source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas flow, 500.0 L/h; cone gas flow, 50.0 L/h; scan range, m/z 80−1000; scan time, 0.3 s; and interscan time, 0.02 s. Data were collected and analyzed with Waters MassLynx v4.1 software.

Quantification of 11 Compounds in Propolis by UHPLC Analysis. The analytical UHPLC system was a Shimadzu LC-30AD series ultrahigh-performance liquid chromatograph equipped with a Shimadzu series diode array detector (Shimadzu Technologies, Kyoto, Japan). The UHPLC pumps, autosampler, column oven, and diode array system were monitored and controlled using the Shimadzu LC-solution computer program. The individual compounds in propolis were quantified according to the absorbance at 280 nm. Quantitative analysis was carried out on an Acquity BEH ODS-C_{18} column (Waters) (150 mm × 2.1 mm i.d., 1.7 μm). The temperature of the column oven was set to 45 °C. The mobile phases consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B). Gradient elution was as follows: start at 20% B; hold for 1.2 min; 1.2−2.0 min, increase via linear gradient to 30% B; 2.0−8.0 min, increase via linear gradient to 40% B; 8.0−11.0 min, increase via linear gradient to 60% B; 11.0−15.0 min, increase via linear gradient to 90% B; and hold for 2 min. The flow rate of the mobile phase was 0.5 mL/min, and the injection volume was 1.0 μL. All 11 compounds were quantified against external standards. Quantification was based on peak area. Calibration curves of the standards were made by diluting stock standards in methanol.

Inhibition of IL-1β, IL-6, and COX-2 mRNA Expression in RAW 264.7 Mouse Macrophage Cells. Solvent was removed from a known volume of the methanol extract for each propolis sample. The residue was redissolved in a known amount of DMSO (100 mg/mL) and diluted in the medium for cell treatment. RAW 264.7 mouse macrophages were cultured in 6-well plates and reached the confluence of 80%. The cells were pretreated with media containing propolis extracts for 24 h at an initial concentration of 0.1 mg propolis equiv/mL. After pretreatment, LPS was added at an initial concentration of 10 ng/mL, and cells were incubated at 37 °C under 5% CO_2 for another 4 h. After induction, culture medium was discarded and cells were collected to perform total isolation and real-time PCR.[17]

RNA isolation and real-time PCR were performed according to the previously published protocol.[18] After LPS induction, cells were washed with 1× PBS, and TRIzol reagent was added for total RNA isolation. An IScript Advanced cDNA Synthesis kit was used to reverse transcribe complementary DNA. Real-time PCR was performed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using AB Power SYBR Green PCR Master Mix. Primers used in this study were as follows: IL-1β (forward, 5′-GTTGACGGACCCCAAAAGAT-3′; reverse, 5′-CCTCATCCTGGAAGGTCCAC-3′); IL-6 (forward, 5′-CACGGCCTTCCCTACTTCAC-3′; reverse, 5′-TGCAAGTGCATCATCGTTGT-3′); COX-2 (forward, 5′-GGGAGTCTGGAACATTGTGAA-3′; reverse, 5′-GCACGTTGATTGTAGGTGGACTGT-3′). The mRNA amounts were normalized to an internal control, GAPDH mRNA (forward, 5′-AGGTGGTCTCCTCTGACTTC-3′; reverse, 5′-TACCAGGAAATGAGCTTGAC-3′). The following amplification parameters were used for PCR: 50 °C for 2 min, 95 °C for 10 min, and 46 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min.

DPPH Radical Scavenging Activity (DPPH). Hydrogen-donating activity was measured using DPPH radicals following a previously

Table 1. Total Phenolic Content (TPC), Total Flavonoid Content (TFC), DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ Scavenging Activities, Ferric Reducing Ability (FRAP), Hydroxyl Radical Scavenging Capacity (HOSC), and Oxygen Radical Absorbing Capacity (ORAC) of Propolis Collected in Different Regions of China[a]

sample[b]	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH (IC_{50})	ABTS (IC_{50})	FRAP (mmol TE/g)	HOSC (mmol TE/g)	ORAC (mmol TE/g)
A	184.71d ± 0.81	130.25g ± 2.47	**50.52h ± 0.81**	33.93h ± 0.46	**0.68g ± 0.02**	4.16c ± 0.14	5.93d ± 0.23
B	**87.11h ± 0.96**	**105.25h ± 1.06**	168.16b ± 1.41	117.24b ± 1.05	0.25j ± 0.01	2.74g ± 0.02	3.26g ± 0.02
C	207.82c ± 1.06	300.00b ± 7.78	26.04m ± 0.20	23.58j ± 0.43	**1.72a ± 0.05**	**3.81cd ± 0.13**	6.99b ± 0.14
D	223.32b ± 2.88	307.25b ± 3.89	28.82k ± 0.33	26.40i ± 0.20	1.31c ± 0.02	**3.81cd ± 0.18**	6.40c ± 0.28
E	113.5g ± 5.76	156.25f ± 2.47	53.09g ± 0.81	54.85f ± 0.40	0.53h ± 0.01	2.66g ± 0.09	2.84gh ± 0.05
F	211.57c ± 2.83	295.75b ± 4.60	74.94e ± 0.98	68.29e ± 0.44	0.47hi ± 0.02	4.40bc ± 0.20	**5.21e ± 0.04**
G	107.54g ± 1 0.36	179.00e ± 1.41	**173.38a ± 1.19**	**152.80a ± 0.71**	**0.20j ± 0.01**	**1.83h ± 0.04**	**2.56h ± 0.11**
H	228.43b ± 1.41	302.00b ± 8.49	45.92i ± 0.84	41.71g ± 0.45	0.72g ± 0.00	4.42bc ± 0.17	7.02b ± 0.12
I	**257.93a ± 2.32**	**351.25a ± 5.30**	**21.79n ± 0.24**	**21.45k ± 0.07**	1.40b ± 0.05	**5.00a ± 0.20**	**7.63a ± 0.24**
J	230.04b ± 0.56	341.50a ± 1.41	33.49j ± 0.23	32.78h ± 0.08	0.95e ± 0.03	4.68ab ± 0.20	6.35cd ± 0.16
K	118.96g ± 1.87	162.75ef ± 4.60	49.53h ± 0.34	**43.43g ± 0.22**	0.86f ± 0.01	3.13fg ± 0.11	2.74h ± 0.08
L	**180.93de ± 0.20**	200.00d ± 0.71	26.35m ± 0.39	22.48jk ± 0.52	1.16d ± 0.01	3.71de ± 0.03	5.13ef ± 0.19
M	149.14f ± 1.72	207.00cd ± 7.07	89.08c ± 0.78	78.84c ± 0.66	0.44i ± 0.01	3.18f ± 0.13	4.73f ± 0.10
N	156.11f ± 0.45	300.75b ± 1.77	84.08d ± 1.13	69.08e ± 1.61	0.52h ± 0.01	4.35bc ± 0.10	5.42e ± 0.06
O	175.21e ± 0.71	**223.50c ± 4.95**	69.37f ± 0.03	71.67d ± 0.64	0.63g ± 0.01	3.76de ± 0.18	5.03ef ± 0.03

[a]Data are reported on a per gram of propolis basis as the mean ± SD (n = 3). Values in the same column marked by different letters are significantly different (P < 0.05). GAE, gallic acid equivalent; QE, quercetin equivalent; TE, trolox equivalent. The minimal, maximal, and median values of each column are shown in bold type. IC_{50} values are the effective concentration (μg/mL) at which 50% of DPPH$^{\bullet}$ or ABTS$^{\bullet+}$ was scavenged. [b]Letters A–O stand for the propolis samples from Changbaishan, Jilin (A); Shenyang, Liaoning (B); Beijing (C); Shijiazhuang, Hebei (D); Zhengzhou, Henan (E); Jiaozuo, Henan (F); Yanan, Shaanxi (G); Qingdao, Shandong (H); Linyi, Shandong (I); Zibo, Shandong (J); Anqing, Anhui (K); Taixing, Jiangsu (L); Xiangshan, Zhejiang (M); Wuhan, Hubei (N); Pengshan, Sichuan (O).

reported protocol.[19] For each sample, different concentrations ranging from 0.6 to 500 μg/mL were prepared with methanol or 10% DMSO/methanol (v/v). The reaction mixtures in the 96-well plates consisted of sample (100 μL) and DPPH radical (100 μL, 0.2 mM) dissolved in methanol. The absorbance was measured at 517 nm against a blank. The percentage of scavenging activity was calculated as $[1-(A_1 - A_2)/A_0] \times 100\%$, where A_0 is the absorbance of the control, A_1 is the absorbance of the sample, and A_2 is the absorbance of blank that contained sample without DPPH radical. The scavenging activity of the samples was expressed as the IC_{50} value, the concentration required to scavenge 50% of DPPH radicals.

ABTS Cation Radical Scavenging Activity (ABTS). The ABTS cation radical scavenging activity assay was carried out via the ABTS cation radical decolorization.[19] The samples were prepared by using the same procedure as the DPPH assay. The ABTS cation radical was prepared by reacting 7 mM aqueous solution of ABTS (15 mL) with 140 mM potassium persulfate (264 μL) to obtain an ABTS working reagent with an absorbance of 0.70 ± 0.02 at 734 nm. The reaction mixtures consisted of sample (50 μL) and the ABTS methanol working solution (100 μL). The mixture was kept for 10 min in the dark, and the absorbance was taken at 734 nm against a blank. The scavenging capacity was calculated as $[1 - (A_1 - A_2)/A_0] \times 100\%$, where A_0 is the absorbance of the control (without sample), A_1 is the absorbance in the presence of the sample, and A_2 is the absorbance of sample without ABTS working solution. The IC_{50} value was calculated from the scavenging activities (percent) versus concentrations of respective sample curves.

Ferric Reducing Ability of Plasma Assay (FRAP). The ability to reduce ferric ions was measured using a modified method described previously.[20] Propolis extracts (0.2 mL) were added to 3.8 mL of FRAP regent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution, and 1 part of 20.0 mM $FeCl_3$ solution). Absorbance was read at 595 nm after 30 min of reaction at 37 °C. The results are reported as millimoles Trolox equivalents (TE) per gram of propolis.

Hydroxyl Radical Scavenging Capacity (HOSC). The HOSC values were measured using a previously published laboratory protocol.[21] The reaction mixture contained 170 μL of 9.28×10^{-8} M fluorescein, 30 μL of sample, blank, or standard, 40 μL of 0.1990 M H_2O_2, and 60 μL of 3.43 M $FeCl_3$. The fluorescence of the reaction mixture was measured every minute for 6 h at ambient temperature, with the excitation wavelength at 485 nm and the emission wavelength at 528 nm. HOSC values were expressed as millimoles of Trolox equivalents (TE) per gram of propolis.

Oxygen Radical Absorbance Capability (ORAC). The ORAC values were determined using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) according to a laboratory protocol described previously.[22] Thirty microliters of sample, blank, or standard solution was added to 225 μL of freshly prepared 81.63 nM fluorescence. The mixture was pipetted into a 96-well plate and preheated at 37 °C for 20 min. Then 25 μL of 0.36 M AAPH was added to the mixture. The reaction mixture was measured every minute for 2 h, with an excitation wavelength at 485 nm and an emission wavelength at 528 nm. ORAC values were reported as millimoles of TE per gram of propolis.

Statistical Analysis. Data are reported as the mean ± SD for triplicate determinations. One-way ANOVA and Tukey's test were employed to identify differences in means. Statistics were analyzed using SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL, USA). Statistical significance was declared at P < 0.05.

RESULTS AND DISCUSSION

Total Phenolic Contents and Total Flavonoid Contents of Chinese Propolis Samples. The propolis from Linyi, Shandong (I) had the greatest TPC of 257.93 mg gallic acid equivalents (GAE)/g propolis and TFC value of 173.90 mg quercetin equivalents (QE)/g propolis (Table 1). These TPC and TFC values were about 3-fold the lowest TPC and TFC values detected, respectively, indicating the significant variations of TPC and TFC in the 15 Chinese propolis samples. The TPC range was comparable to that of 197.6–409.2 mg GAE/g propolis using three different solvents (chloroform, acetone, and ethanol)[10] and 33–176 mg GAE/g propolis using ethanol as the solvent,[23] respectively. Interestingly, the TFC of 52.11–173.90 mg quercetin equivalents (QE)/g propolis from this study with methanol as extraction solvent was similar to the 8.3–188 mg QE/g propolis using ethanol as the solvent[12] and

greater than the 3.47−15.42 mg QE/g propolis using water as the extraction solvent.[13]

Identification of Chemical Compounds in Chinese Propolis Samples. Propolis is a complex mixture containing >300 compounds.[2] It is difficult to separate and identify the individual compounds including isomers or analogues in propolis. In this study, the chemical profiles of the Chinese propolis were examined using a UPLC/Q-TOF-MS. Forty compounds were detected in the methanol extract of the 15 tested Chinese propolis samples. Among them, 20 compounds were confirmed for their chemical structures according to MS data and retention time compared with those of standard compounds. An additional 18 compounds were tentatively identified by elucidation of their MS data, UV spectra, and structural information from the literature,[24] whereas another 2 compounds remained unknown (Table 2). Representative UPLC chromatograms at 280 nm of the 15 propolis samples are shown in Figure 1, and the entire UPLC data are provided in Figure S2 in the Supporting Information. Propolis from Wuhan, Hubei (N), showed a remarkable difference in the tested samples, the detailed chemical composition of which was reported in our recent research.[14] In addition, propolis samples from Changbaishan, Jilin (A), and Shengyang, Liaoning (B), were comparable but differentiated from the rest of the samples by a characteristic major peak (*p*-coumaric acid benzyl ester, **31**) at 11.14 min. The HPLC profiles of the two propolis samples (A and B) were similar to that collected from Heilongjiang province previously reported by Ahn et al.,[12] revealing that propolis from northeastern China might have a similar distinct chemical composition. The rest of the propolis samples had similar chemical profiles, except for the trace compounds and the relative abundance of the major compounds. The data from this study showed that Chinese propolis was rich in phenolic acids, flavonoids, and phenolic acid esters. Four flavonoids, chrysin (**20**), pinocembrin (**23**), galangin (**26**), and pinobanksin 3-acetate (**27**), were primary constituents in most tested Chinese propolis samples. This study also confirmed that Chinese propolis samples belonged to the poplar-type propolis.[25] Interestingly, cinnamylideneacetic acid, previously found in an ethanol extract of Chinese propolis,[12] was not detected in any propolis samples in the present study. Guo et al.[13] reported the contents of 23 compounds in a water extract of Chinese propolis from 26 locations, but 12 of them, including gallic acid, catechin, epicatechin, α-catechin, rutin, myricetin, fisetin, morin, naringenin, luteolin, genistein, and baicalin, were not found in our 15 samples, which may be partially explained by the different extraction solvents as well as the different collection locations and seasons. To the best of our knowledge, this is the first report on the identification of the chemical composition of Chinese propolis using high-resolution mass spectrometry.

Concentrations of the 11 Compounds in Chinese Propolis Samples. To compare the chemical composition of the Chinese propolis samples, the levels of 11 compounds, including 5 phenolic acids and a phenolic acid ester and 5 flavonoids, were determined by UHPLC in 15 min, on a per propolis weight basis (Table 3). The 11 compounds were caffeic acid (**1**), *p*-coumaric acid (**2**), ferulic acid (**3**), isoferulic acid (**4**), 3,4-dimethylcaffeic acid (**5**), pinobanksin (**10**), chrysin (**20**), pinocembrin (**23**), galangin (**26**), pinobanksin 3-acetate (**27**), and caffeic acid phenylethyl ester (**28**), and their structures are shown in Figure S3 in the Supporting Information. A typical UHPLC chromatogram is also provided in Figure S4 in the Supporting Information. The propolis sample from Linyi, Shandong (I), contained the greatest amounts of caffeic acid (**1**) at 16.68 mg/g, 3,4-dimethylcaffeic acid (**5**) at 10.17 mg/g, chrysin (**20**) at 44.40 mg/g, pinobanksin 3-acetate (**27**) at 55.06 mg/g, and caffeic acid phenylethyl ester (**28**) at 7.99 mg/g. It was noted that caffeic acid and its phenylethyl ester were found to be the best antioxidants in propolis from Anhui, China, on the basis of DPPH and ABTS cation radical scavenging capacities and FRAP values.[19] Propolis from Changbaishan, Jilin (A), had

Table 2. Characterization of Compounds Present in Propolis from Different Regions of China

	t_R (min)	UV λ_{max} (nm)	$[M - H]^-$	$[M + H]^+$	identification
1	0.78	322	179.0500	nd[a]	caffeic acid
2	1.10	309	163.0551	nd	*p*-coumaric acid
3	1.30	320	193.0660	nd	ferulic acid
4	1.43	323	193.0656	nd	isoferulic acid
5	2.88	321	207.0807	nd	3,4-dimethylcaffeic acid
6	3.51	365	301.0469	303.0504	quercetin
7	3.86	287	285.0885	287.0917	pinobanksin-5-methyl ether
8	3.96	308	315.0614	317.0657	quercetin-3-methyl ether
9	4.33	337	269.0584	271.0608	apigenin
10	4.46	291	271.0734	273.0764	pinobanksin
11	4.72	287	301.0832	303.0868	unknown compound
12	4.75	371	315.0625	317.0663	isorhamnetin
13	4.82	370	315.0617	317.0663	quercetin-X-methyl ether
14	4.93	290	269.0941	271.0972	pinocembrin-5-methyl ether
15	5.03	290	299.0671	301.0713	luteolin-5-methyl ether
16	5.33	354	329.0768	331.0819	quercetin-5,7-dimethyl ether
17	6.10	308	283.0733	285.0764	galangin-5-methyl ether
18	6.35	351	315.0620	317.0664	quercetin-X-methyl ether
19	7.24	354	329.0771	331.0816	quercetin-7-methyl-X-methyl ether
20	7.90	268	253.0641	255.0660	chrysin
21	8.04	326	247.1110	nd	caffeic acid isoprenyl ester
22	8.15	289	285.0882	287.0917	pinobanksin-7-methyl-ether
23	8.35	290	255.0791	257.0812	pinocembrin
24	8.47	328	247.1110	nd	caffeic acid isoprenyl ester
25	8.62	328	247.1109	nd	caffeic acid isoprenyl ester
26	8.72	265	269.0583	271.0604	galangin
27	9.24	293	313.0827	315.087	pinobanksin-3-*O*-acetate
28	9.87	328	283.1094	285.0761	caffeic acid phenylethyl ester
29	10.33	309	223.1117	225.1128	unknown compound
30	11.14	309	253.0998	255.1021	hydroxy-cinnamic acid benzyl ester
31	11.48	311	253.1001	255.1011	*p*-coumaric acid benzyl ester
32	12.44	328	295.1092	nd	caffeic acid cinnamyl ester
33	12.74	293	327.0976	329.1026	pinobanksin-3-*O*-propionate
34	15.14	268	nd	269.0816	chrysin-7-methyl ether
35	15.31	289	nd	271.0967	pinocembrin-7-methyl ether
36	15.52	310	279.1152	nd	*p*-methoxy-cinnamic acid cinnamyl ester
37	15.75	293	341.1131	343.1177	pinobanksin-3-*O*-(butyrate or isobutyrate)
38	16.82	292	355.1277	357.1328	pinobanksin-3-*O*-(pentanoate or 2-methylbutyrate)
39	17.61	279	293.2240	295.2270	methoxycinnamic acid cinnamyl ester
40	17.79	278	293.2234	nd	methoxycinnamic acid cinnamyl ester

[a]nd, not detected.

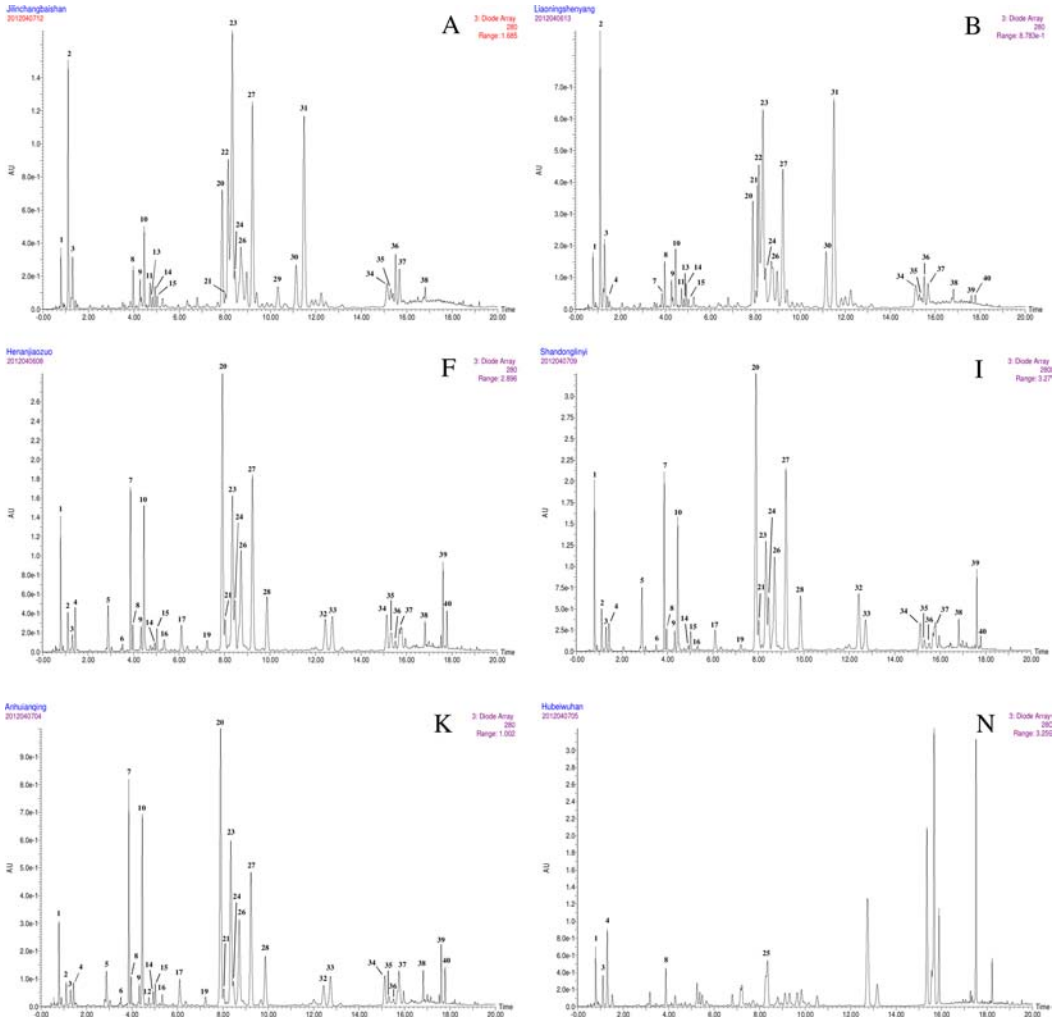

Figure 1. Representative UPLC chromatograms of Chinese propolis collected from different locations of China. Samples were from Changbaishan, Jilin (A); Shenyang, Liaoning (B); Jiaozuo, Henan (F); Linyi, Shandong (I); Anqing, Anhui (K); and Wuhan, Hubei (N), respectively.

the greatest amounts of *p*-coumaric acid (**2**) and pinocembrin (**23**) at 46.01 and 10.14 mg/g, respectively. Propolis from Wuhan, Hubei (N), had the greatest ferulic acid content of 17.77 mg/g (**3**). Unfortunately, it was not possible to compare these concentrations with those reported from the previous studies, because all of the previous studies reported individual compound concentration on a per total extract weight basis, without total extraction yield data.[11,12]

The total phenolic acids and flavonoids in propolis were also calculated by adding UHPLC analytical values of individual phenolic acids and flavonoids. The propolis from Linyi, Shandong (I), contained the greatest amount of total phenolic acids at 47.12 mg/g and total flavonoids at 176.93 mg/g, which were consistent with those detected by the colorimetric method. The propolis from Zhengzhou, Henan (E), and Wuhan, Hubei (N), had the lowest amounts of total phenolic acids (10.23 mg/g) and flavonoids (17.08 mg/g), respectively.

Effects of Propolis Extracts on IL-1β, IL-6, and COX-2 mRNA Expression. Chronic inflammation has been associated with a number of human chronic diseases, such as cardiovascular diseases, cancer, autoimmune diseases, and arthritis. Several cytokines including IL-1β, IL-6, and COX-2 are critical mediators involved in multiple inflammatory pathways. In the present study, the effects of propolis methanolic extracts on the expression of IL-1β, IL-6, and COX-2 mRNA were measured in LPS-stimulated RAW 264.7 mouse macrophage cells for the first time.

Individual propolis samples showed different inhibitory activities on IL-1β, IL-6, and COX-2 mRNA expression at an initial treatment concentration of 10 μg propolis extract/mL. As shown in Figure 2A, most propolis samples exhibited 100% inhibition on IL-1β mRNA expression, except Changbaishan, Jilin (A), Shenyang, Liaoning (B), and Wuhan, Hubei (N). An inhibitory effect on IL-6 mRNA expression was detected in all 15 proplis extracts, although the degree of inhibition differed among them. In this study, propolis samples from Shijiazhuang, Hebei (D), Jiaozuo, Henan (F), Linyi, Shandong (I), and Zibo, Shandong (J), showed the strongest inhibitory effects in suppressing IL-6 mRNA expression (Figure 2B). On the other hand, the 15 propolis samples, except Wuhan, Hubei (N), significantly suppressed the LPS-induced COX-2 mRNA expression (Figure 2C). The results suggested that propolis might have excellent anti-inflammatory activities at the concentration of 10 μg propolis extract/mL, and their anti-inflammatory activity might be selective. Furthermore, the Chinese propolis showed stronger anti-inflammatory effects than the four different fractions of *Engelhardia roxburghiana* extract under the same experimental conditions.[26] These results suggested potential application of the Chinese propolis as a dietary source of anti-inflammatory nutraceuticals because *E. roxburghiana* has been

Table 3. Levels (Milligrams per Gram Propolis) of 11 Compounds in Propolis Collected from Different Regions of China[a]

sample	1	2	3	4	5	10	20	23	26	27	28
A	4.06h ± 0.01	10.14a ± 0.03	3.90c ± 0.05	1.16m ± 0.00	0.95m ± 0.00	7.82i ± 0.02	10.99k ± 0.02	46.01a ± 0.39	15.74e ± 0.04	32.71h ± 0.08	1.42m ± 0.01
B	2.41i ± 0.01	6.17b ± 0.02	2.77f ± 0.04	1.07n ± 0.00	0.91m ± 0.00	3.44k ± 0.01	5.63m ± 0.02	20.55i ± 0.07	7.09j ± 0.05	12.22m ± 0.03	0.73n ± 0.00
C	7.26f ± 0.05	3.11f ± 0.02	1.45j ± 0.03	7.76a ± 0.06	8.86c ± 0.09	6.54j ± 0.03	23.22g ± 0.28	38.24d ± 0.37	23.14c ± 0.10	26.97i ± 0.17	6.61e ± 0.07
D	12.07b ± 0.11	3.45d ± 0.03	3.34e ± 0.03	3.52f ± 0.03	6.18e ± 0.05	18.18e ± 0.29	38.02b ± 0.39	28.37h ± 0.04	26.26a ± 0.28	46.75b ± 0.41	6.72de ±0.08
E	1.08j ± 0.00	1.01m ± 0.00	1.00k ± 0.00	1.23k ± 0.00	2.50j ± 0.01	8.68h ± 0.05	18.07h ± 0.10	19.55j ± 0.04	9.14h ± 0.05	11.36n ± 0.01	3.41j ± 0.02
F	10.12d ± 0.08	2.69h ± 0.02	2.74f ± 0.01	4.62c ± 0.03	6.02f ± 0.04	18.44de ±0.12	31.41d ± 0.28	29.18g ± 0.39	23.02 cd ±0.24	39.33f ± 0.38	5.77g ± 0.07
G	9.80e ± 0.09	1.16k ± 0.01	1.04k ± 0.01	2.82g ± 0.02	3.11i ± 0.03	3.63k ± 0.02	10.96k ± 0.13	13.90n ± 0.23	7.71i ± 0.08	9.92o ± 0.10	2.08k ± 0.04
H	12.17b ± 0.08	3.00g ± 0.02	3.53d ± 0.02	4.34d ± 0.03	8.51d ± 0.08	18.74d ± 0.19	33.43c ± 0.34	37.00e ± 0.44	25.39b ± 0.18	42.46d ± 0.48	6.51ef ±0.17
I	16.68a ± 0.05	3.57c ± 0.01	4.73b ± 0.01	3.96e ± 0.01	10.17a ± 0.02	21.86b ± 0.02	44.40a ± 0.36	29.88g ± 0.14	25.73b ± 0.14	55.06a ± 0.16	7.99a ± 0.02
J	11.09c ± 0.04	3.57c ± 0.01	3.30e ± 0.02	4.98b ± 0.02	9.97b ± 0.05	19.32c ± 0.13	38.24b ± 0.28	35.00f ± 0.36	23.13c ± 0.14	40.36e ± 0.24	7.46b ± 0.06
K	4.18h ± 0.02	1.33j ± 0.00	1.76i ± 0.00	1.81j ± 0.00	3.09i ± 0.00	12.78g ± 0.03	16.10i ± 0.02	15.88m ± 0.08	10.02g ± 0.05	14.98k ± 0.06	3.57j ± 0.07
L	7.17f ± 0.00	2.74h ± 0.00	2.48g ± 0.02	1.86j ± 0.00	3.54h ± 0.01	22.82a ± 0.03	28.51e ± 0.03	41.62c ± 0.01	22.64d ± 0.01	37.81g ± 0.01	4.61h ± 0.04
M	6.55g ± 0.03	1.77i ± 0.01	1.96h ± 0.02	2.35h ± 0.01	5.12g ± 0.03	12.61g ± 0.12	23.93f ± 0.17	17.46k ± 0.17	14.34f ± 0.08	26.02j ± 0.15	3.84i ± 0.05
N	7.12f ± 0.07	3.19e ± 0.02	17.77a ± 0.14	nd[b]	nd	nd	nd	17.08k ± 0.16	nd	nd	nd
O	4.18h ± 0.01	0.92n ± 0.00	0.93k ± 0.00	2.09i ± 0.00	1.99k ± 0.01	14.07f ± 0.04	13.99j ± 0.04	43.59b ± 0.22	25.02b ± 0.34	45.74c ± 0.15	7.02c ± 0.03

[a]Values are reported as the mean ± SD on a per propolis weight basis (n = 3). Values in the same column marked by the same letters are not significantly different ($P < 0.05$). Compounds: **1**, caffeic acid; **2**, p-coumaric acid; **3**, ferulic acid; **4**, isoferulic acid; **5**, 3,4-dimethylcaffeic acid; **10**, pinobanksin; **20**, chrysin; **23**, pinocembrin; **26**, galangin; **27**, pinobanksin 3-acetate; **28**, caffeic acid phenylethyl ester. See Table 1 for sample designations. [b]nd, not detected.

Figure 2. Effects of propolis extracts on (A) IL-1β, (B) IL-6, and (C) COX-2 mRNA expressions in RAW 264.7 mouse macrophage cells. Letters A–O stand for the propolis samples from Changbaishan, Jilin (A); Shenyang, Liaoning (B); Beijing (C); Shijiazhuang, Hebei (D); Zhengzhou, Henan (E); Jiaozuo, Henan (F); Yanan, Shaanxi (G); Qingdao, Shandong (H); Linyi, Shandong (I); Zibo, Shandong (J); Anqing, Anhui (K); Taixing, Jiangsu (L); Xiangshan, Zhejiang (M); Wuhan, Hubei (N); Pengshan, Sichuan (O). The final concentration was 10 μg propolis extract/mL in the initial culture media. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters above the bars represent significant differences ($P < 0.05$).

Table 4. Correlations between Individual Compounds and Antioxidant Activities[a]

	1	2	3	4	5	10	20	23	26	27	28
DPPH					−0.525	−0.671	−0.665	−0.56	−0.699	−0.632	−0.677
ABTS					−0.487	−0.63	−0.624	−0.58	−0.661	−0.609	−0.586
FRAP				0.599	0.611		0.591	0.472	0.648	0.519	0.644
HOSC	0.554				0.651	0.724	0.73	0.557	0.827	0.865	0.709
ORAC	0.665			0.636	0.741	0.567	0.726	0.636	0.875	0.847	0.727

[a]Correlation significant at the 0.001 level. Compounds: **1**, caffeic acid; **2**, *p*-coumaric acid; **3**, ferulic acid; **4**, isoferulic acid; **5**, 3,4-dimethylcaffeic acid; **10**, pinobanksin; **20**, chrysin; **23**, pinocembrin; **26**, galangin; **27**, pinobanksin 3-acetate; **28**, caffeic acid phenylethyl ester. DPPH, ABTS, FRAP, HOSC, and ORAC stand for DPPH radical scavenging capacity, ABTS radical scavenging activity, ferric reducing ability power, hydroxyl radical scavenging capacity and oxygen radical absorbance capability, respectively.

used in functional foods and supplemental products for its anti-inflammatory effect. To the best of our knowledge, this is the first report on potential anti-inflammatory activities of propolis extract through down-regulating cytokine expressions, although five propolis components have been reported for their significant inhibitory effects on IL-1β, IL-6, and COX-2 mRNA expression in LPS-stimulated RAW 264.7 mouse macrophage cells.[14]

DPPH and ABTS Cation Radical Scavenging Capacities. Methanol extracts of the propolis samples were investigated for their free radical scavenging capacities against DPPH and ABTS cation radicals. As shown in Table 1, all of the propolis extracts showed significant $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging capacities except those from Shenyang, Liaoning (B), and Yanan, Shaanxi (G). The propolis from Linyi, Shandong (I), had the strongest $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging capacities, with IC_{50} values of 21.79 and 21.45 μg propolis equivalents/mL, respectively. Additionally, the IC_{50} values of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging capacities showed superior negative correlations with TPC (R = −0.726, P < 0.001; and R = −0.695, P < 0.001, respectively) and inferior negative correlations with TFC (R = −0.537, P < 0.001; and R = −0.475, P = 0.001, respectively).

Ferric Reducing Ability of Plasma Assay. The FRAP values of the Chinese propolis were 0.20−1.72 mmol of TE/g propolis (Table 1). The ferric reducing ability of propolis sample collected in Beijing (C) was greater than that of the other samples. FRAP values had a significant correlation with TPC (R = 0.655, P < 0.001) and a weaker correlation with TFC (R = 0.546, P < 0.001), indicating that phenolic acids might play an important role in the ferric reducing ability of propolis.

Hydroxyl Radical Scavenging Capacity. The 15 propolis samples differed in their HOSC values under the experimental conditions (Table 1). Propolis from Linyi, Shandong (I), showed the strongest HOSC of 5.00 mmol of TE/g propolis, followed by that of 4.68 mmol of TE/g propolis observed for propolis from Zibo, Shandong (J). HOSC value had strong correlations with TPC (R = 0.859, P < 0.001) and TFC (R = 0.737, P < 0.001).

Oxygen Radical Absorbance Capability. Propolis from Linyi, Shandong (I), showed the greatest oxygen radical absorbance capability (Table 1). The ORAC values were 2.56−7.63 mmol of TE/g propolis for the 15 samples. ORAC values were correlated to TPC (R = 0.922, P < 0.001) and TFC (R = 0.770, P < 0.001). ORAC values were also correlated with FRAP and HOSC (R = 0.672 and 0.856, respectively, P < 0.001).

Correlations of Antioxidant Activity and Chemical Composition. Taken together, the correlations between TPC and TFC and DPPH, ABTS, FRAP, HOSC, and ORAC indicated that phenolic compounds played a more important role than flavonoids in DPPH, ABTS cation, hydroxyl, and oxygen radical scavenging capacities and ferric reducing activity. Propolis from Linyi, Shandong (I), with the greatest TPC and TFC possessed the strongest antioxidant activity, supporting the high correlation between the antioxidant activity and phenolics and flavonoids.

Correlations between individual compounds and antioxidant activity were also analyzed (Table 4). Concentrations of 3,4-dimethylcaffeic acid (**5**), chrysin (**20**), pinocembrin (**23**), galangin (**26**), pinobanksin 3-acetate (**27**), and caffeic acid phenylethyl ester (**28**) showed significant correlations with DPPH, ABTS cation, hydroxyl, and oxygen radical scavenging capacities and ferric reducing activity (P < 0.001), whereas *p*-coumaric acid (**2**) and ferulic acid (**3**) had no correlation with any of the five tested antioxidant activities. Caffeic acid (**1**), isoferulic acid (**4**), and pinobanksin (**10**) were correlated with two or four antioxidant activities. These data suggested that the individual compound might have a different contribution to the total antioxidant activity of propolis. Additional research is needed to further investigate whether a synergistic or additive effect may exist among individual propolis components in their antioxidant and maybe anti-inflammatory activities.

Together, the results indicated that Chinese propolis from different regions may serve as excellent natural antioxidants to reduce the risk of oxidation-related diseases. China is a vast country with different climate zones and plant distribution, which might lead to commercial propolis samples significantly differing in chemical compositions and health properties. The control of the botanic origin and sampling region is essential for standardization of propolis and related products.

In summary, the present study demonstrated the potential of propolis in suppressing chronic inflammation and reducing the risk of related human health problems. This study also showed the $HO^{\bullet}$ and $O_2^{\bullet-}$ scavenging properties of propolis under physiological pH. Propolis collected from various locations may differ in neutraceutical compositions, anti-inflammatory effects, and radical scavenging activities. A rapid analytical method has been developed to quantify the 11 compounds in Chinese propolis, and a total of 38 compounds were identified. This information may be important for quality control and quality assurance of propolis and related functional products for their potential health properties.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This research was supported by grants from SJTU 985-III disciplines platform and talent fund (Grants TS0414115001 and TS0320215001), a Special Fund for Agro-scientific Research in the Public Interest (Grant 201203069), and a grant from the Opening Foundation of Zhejiang Provincial Top Key Discipline (Wenzhou University) (100061200125).

Notes

The authors declare no competing financial interest.

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