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Isolation and Characterization of Five Glycerol Esters from Wuhan Propolis and Their Potential Anti-Inflammatory Properties

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Supporting Information

ABSTRACT: Five new glycerol esters including 2-acetyl-1-coumaroyl-3-cinnamoylglycerol (**13**), (+)-2-acetyl-1-feruloyl-3-cinnamoylglycerol (**14**), (−)-2-acetyl-1-feruloyl-3-cinnamoylglycerol (**15**), 2-acetyl-1,3-dicinnamoylglycerol (**16**), and (−)-2-acetyl-1-(*E*)-feruloyl-3-(3″(*ζ*),16″)-dihydroxy-palmitoylglycerol (**17**) were isolated from methanolic extract of Wuhan propolis. The chemical structures of the five new compounds were confirmed by ^{1}H and ^{13}C NMR and HR-MS spectra. Twelve minor constituents in the Wuhan propolis extract were tentatively identified by UPLC−Q-TOF-MS, according to their characteristic UV spectrum, retention times, and accurate MS data. The anti-inflammatory activities of the five new compounds were studied in lipopolysaccharide (LPS)-stimulated RAW 264.7 mouse macrophage cells. All five compounds exhibited remarkable inhibitory effects on interleukin (IL)-1*β*, IL-6, and cyclooxygenase (COX)-2 mRNA expressions at 10 and 100 *μ*M, suggesting that Wuhan propolis may serve as a potential anti-inflammatory functional food ingredient or nutraceutical.

KEYWORDS: *anti-inflammation, glycerol esters, ultraperformance liquid chromatography quadrupole time-of-flight mass spectrometry, Wuhan propolis*

INTRODUCTION

Propolis is a natural resinous substance collected by honeybees from tree buds and exudates of various botanical sources, which was originally used to seal holes in honeycombs and protect the entrance against intruders. Propolis has been used as a traditional remedy since ancient times.[1] Previous research has indicated its several biological activities, such as antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, anticancer, and immunomodulatory activities.[1−3] Due to its broad range of bioactivities, the purified extract of propolis has been widely used in beverages, nutritional supplements, and health foods.[4−6] Flavonoids, aromatic acids, aromatic acid esters, benzophenones, and diterpenes were recognized as major chemical constituents in propolis.[2] It is well accepted that the chemical composition of propolis is mainly affected by the specific flora of the area in which it is collected,[7−13] and its bioactivities are determined by the composition and level of the chemical compounds.

A previous study showed that twenty Chinese propolis samples significantly differed in their concentrations of total phenolics, flavonoids, and other individual compounds, as well as their DPPH and ABTS cation radical scavenging capacities and inhibitory activities of linoleic acid oxidation.[11] Usia et al.[14] reported the isolation of two new flavonoids and twelve known compounds from Chinese propolis. Among the isolated compounds, benzyl caffeate and phenethyl caffeate possessed potential antiproliferative activities. Another study investigated the cytotoxic constituents of Chinese propolis collected from Henan province.[15] A pair of new flavanol racemates and a new flavanol racemic mixture were isolated, showing cytotoxicity against the human HeLa cervical cancer cells. Recently, water extract of propolis collected from twenty-six locations of China were compared for their total polyphenol, flavanone, flavone-flavonol, and soluble carbohydrate contents, and their antioxidant activities.[16] The twenty-six propolis samples had obvious differences in their total chemical contents, and differed in their reducing power and DPPH radical scavenging abilities. These previous studies indicated the potential variation among propolis products collected from different locations.

Interestingly, a preliminary HPLC analysis showed that the HPLC chromatogram for Wuhan propolis was very different from that for Chinese propolis samples from different origins reported previously.[11] As a continuation of our research on Chinese propolis,[17] this research was performed to further investigate the chemical composition and potential health properties of Wuhan propolis. In the present study, we describe the isolation and structure elucidation of five new compounds, and the tentative identification of twelve additional minor compounds in Wuhan propolis using UPLC−Q-TOF-MS. The anti-inflammation activities of the isolated compounds were also tested for their inhibitory effect on IL-1*β*, IL-6, and COX-2 mRNA expressions in lipopolysaccharide-stimulated mouse RAW 264.7 macrophage cells. The results will be used to promote the value-added utilization of Wuhan propolis in improving human health.

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MATERIALS AND METHODS

General Experimental Procedures. Melting point was determined on an XT4A digital micromelting point apparatus (Tianjin, China). Optical rotations were measured with a Jasco P-2000 polarimeter (Tokyo, Japan) in $CHCl_3$ or CH_3OH at 25 °C. UV data were recorded on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). IR spectra were determined with a Nicolet 6700 FT-IR spectrophotometer (Madison, WI, USA). NMR spectra were obtained on a Bruker Advance DRX-500 spectrometer (Rheinstetten, Germany), and the chemical shifts are given in δ (ppm) values with reference to TMS. The coupling constants (J values) are reported in hertz. Analytical UPLC and HRMS were carried out on Waters Acquity UPLC−TOF-MS Premier mass spectrometer (Milford, MA, USA). Semipreparative HPLC was conducted using an Agilent 1260 liquid chromatograph system (Palo Alto, CA, USA) which was set up with a quaternary pump, a diode array detector, and Agilent column Zorbax Eclipse XDB-C_{18} (250 mm × 9.4 mm i.d., 5 μm). The detection wavelength was set at 210 and 280 nm. Silica gel (200−300 mesh, Qingdao Marine Chemical Co. Ltd.) and ODS (Fuji Silysia Chemical Ltd.) were used in open column chromatography fractionations. All solvents used for isolation and UPLC−Q-TOF-MS analysis were of analytical grade and chromatographic grade, respectively.

Propolis Material and Reagents. The propolis was collected from Wuhan, Hubei Province, China, in May 2010 and stored at −20 °C before use. A voucher specimen (No. 20100913) has been deposited in our laboratory, School of Agriculture and Biology, Shanghai Jiao Tong University. Mouse RAW 264.7 macrophage cell line was obtained from Chinese Academy of Sciences (Shanghai, China). Lipopolysaccharide (LPS, from *Escherichia coli* O111:B4) was purchased from Millipore (Billerica, MA, USA).

Sample Preparation and UPLC−Q-TOF-MS Analysis. An accurately weighed mass of the Wuhan propolis powder (0.1 g) was transferred into a 25 mL volumetric flask adjusted with 100% methanol and sonicated for 45 min. The mixture was kept overnight. The supernatant of sample solution was filtered through a 0.22 μm membrane for UPLC−Q-TOF-MS analysis (Waters Micromass Q-TOF Premier mass spectrometer).

UPLC was performed at 40 °C using an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm), with a 5 mm × 2.1 mm i.d. guard column of the same material (Waters, Milford, MA, USA). The elution gradient (eluent A, 0.1% formic acid; eluent B, acetonitrile) used was as follows: 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, and the injection volume was 1.5 μL. MS conditions were as follows: capillary voltages for negative and positive ion modes, 2.8 kV and 3.0 kV; sampling cone voltages for negative and positive ion modes, 55.0 and 43.0 V; 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; interscan time, 0.02 s. Data were collected and analyzed with Waters MassLynx v4.1 software.

Extraction and Isolation. The frozen propolis samples (40 g) were powdered by a mill and extracted three times with methanol (1 L) in an ultrasonic water bath for 1.5 h each time. The combined extract was concentrated under vacuum; the residue (20 g) was subjected to column chromatography (200−300 mesh silica gel, 500 g). Fractions 1−15 were obtained through gradient elution with petroleum ether−acetone (50:1, 40:1, 30:1, 25:1, 20:1, 15:1, 10:1, 9:1, 8.75:1.25, 8.5:1.5, 8.25:1.75, 8:2, 7.75:2.25, 7.5:2.5, 7:3, each 1 L) on the basis of TLC analysis. Fraction 3 was purified by recrystallization from methanol to afford **16** (35.2 mg). Fraction 8 was subjected to semipreparative HPLC (MeCN−H_2O, 55:45, 4 mL/min) to afford **15** (10.0 mg). Fraction 10 was purified by semipreparative HPLC (MeCN−H_2O, 55:45, 4 mL/min) to give **13** (15.2 mg) and **14** (20.7 mg) (Figure 1). Fraction 11 was separated by semipreparative HPLC (MeCN−H_2O, 70:30, 4 mL/min) to give **17** (7.6 mg).

Structures of New Compounds. *2-Acetyl-1-coumaroyl-3-cinnamoylglycerol, **13**.* Gum: $[\alpha]_D^{25}$ +2.1 (c 0.06, CH_3OH); UV

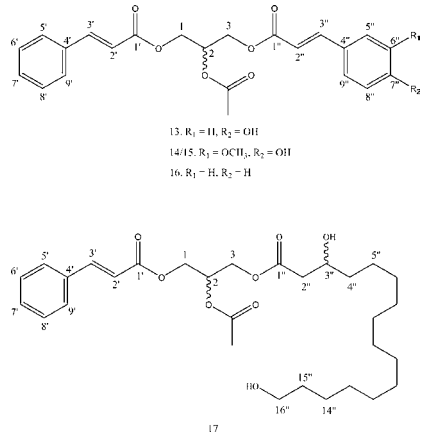

Figure 1. Chemical structures of compounds **13**−**17**.

(CH_3OH) λ_{max} (log ε) 284 (3.52) nm; IR (KBr) ν_{max} 3435, 1718, 1630, 1515, 1270, 1238, 1165 cm^{-1}; ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Table 1; HRESIMS $[M - H]^-$ m/z 409.1272 (calcd for $C_{23}H_{21}O_7$, 409.1287).

*(+)-2-Acetyl-1-feruloyl-3-cinnamoylglycerol, **14**.* Gum: $[\alpha]_D^{25}$ +1.5 (c 0.11, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 282 (4.16) nm; IR (KBr) ν_{max} 3432, 1714, 1635, 1515, 1274, 1236, 1163 cm^{-1}; ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Table 1; HRESIMS $[M - H]^-$ m/z 439.1376 (calcd for $C_{24}H_{23}O_8$, 439.1393).

*(−)-2-Acetyl-1-feruloyl-3-cinnamoylglycerol, **15**.* Gum: $[\alpha]_D^{25}$ −8.5 (c 0.28, CH_3OH); UV, ^1H, and ^{13}C NMR spectroscopic data are fully identical to those of **14**; HRESIMS $[M - H]^-$ m/z 439.1380 (calcd for $C_{24}H_{23}O_8$, 439.1393).

*2-Acetyl-1,3-dicinnamoylglycerol, **16**.* White power: mp 104−106 °C; $[\alpha]_D^{25}$ 0 (c 0.10, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 272 (3.77) nm; IR (KBr) ν_{max} 3410, 1744, 1716, 1635, 1315, 1236, 1168 cm^{-1}; ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Table 1; HRESIMS $[M + NH_4]^+$ m/z 412.1762 (calcd for $C_{23}H_{26}NO_6$, 412.1760).

*(−)-2-Acetyl-1-(E)-feruloyl-3-(3″(ζ),16″)-dihydroxy-palmitoylglycerol, **17**.* Gum: $[\alpha]_D^{25}$ −12.5 (c 0.40, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 280 (2.67) nm; IR (KBr) ν_{max} 3630, 2850, 1745, 1720, 1632, 1510, 1470, 1070 cm^{-1}; ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Table 2; HRESIMS $[M + H]^+$ m/z 535.3274 (calcd for $C_{30}H_{47}O_8$, 535.3271).

Anti-Inflammation Effects of Compounds 13−17 in Mouse RAW 264.7 Macrophage Cells. To determine the anti-inflammation activities of compounds **13**−**17**, mouse RAW 264.7 macrophages were cultured in 6 well plates overnight and reached the confluence of 80%. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% amphotericin B/streptomycin/penicillin and incubated at 37 °C with 5% CO_2 in ambient air. Each compound (10 μM and 100 μM) was added to the cells 24 h prior to induction, respectively. After 24 h incubation, LPS was added into the medium at the initial concentration of 10 ng/mL. After induction, culture medium was discarded and cells were collected to perform total RNA isolation and real-time PCR.[18]

RNA isolation and real-time PCR were performed according to the previously published protocol.[19] 4 h post LPS induction, cell was washed with 1× PBS and TRIzol reagent was added for total RNA isolation. IScript Advanced cDNA Synthesis kit was used to reverse transcribe cDNA. Real-time PCR was performed on ABI 7900HT Fast Real-Time PCR System using AB Power SYBR Green PCR Master Mix. Primers used in this study were as follows: IL-1β (forward, 5′-GTTGACGGACCCCAAAAGAT-3′; reverse, 5′-CCTCATCCTG-GAAGGTCCAC-3′); IL-6 (forward, 5′-CACGGCCTTCCCTACTT-CAC-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′-

Table 1. ^{1}H and ^{13}C NMR Data for Compounds 13−16[a]

	13		14/15		16	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	4.50 (2H, dd, J = 12.0, 4.0)	63.7	4.49 (2H, dd, J = 12.0, 4.0)	63.7	4.41 (2H, dd, J = 12.0, 4.5)	62.6
2	5.38 (1H, dd, J = 6.0, 4.0)	71.0	5.38 (1H, dd, J = 6.0, 4.0)	71.0	5.35 (1H, dd, J = 6.0, 4.5)	69.4
3	4.38 (2H, dd, J = 12.0, 6.0)	63.5	4.38 (2H, dd, J = 12.0, 6.0)	63.5	4.31 (2H, dd, J = 12.0, 6.0)	62.6
1′		168.0		168.0		166.6
2′	6.55 (1H, d, J = 16.0)	118.2	6.53 (1H, d, J = 16.0)	118.2	6.39 (1H, d, J = 16.0)	117.3
3′	7.72 (1H, d, J = 16.0)	147.0	7.70 (1H, d, J = 16.0)	147.0	7.65 (1H, d, J = 16.0)	145.9
4′		135.6		135.6		134.3
5′	7.60 (1H, m)	129.4	7.57 (1H, m)	129.3	7.46 (1H, m)	128.3
6′	7.40 (1H, m)	130.0	7.39 (1H, m)	130.0	7.32 (1H, m)	129.1
7′	7.40 (1H, m)	131.7	7.39 (1H, m)	131.7	7.32 (1H, m)	130.7
8′	7.40 (1H, m)	130.0	7.39 (1H, m)	130.0	7.32 (1H, m)	129.1
9′	7.60 (1H, m)	129.4	7.57 (1H, m)	129.3	7.46 (1H, m)	128.3
1″		168.6		168.6		166.6
2″	6.35 (1H, d, J = 15.5)	114.5	6.38 (1H, d, J = 15.5)	114.7	6.39 (1H, d, J = 16.0)	117.3
3″	7.64 (1H, d, J = 15.5)	147.3	7.63 (1H, d, J = 15.5)	147.5	7.65 (1H, d, J = 16.0)	145.9
4″		127.0		127.6		134.3
5″	7.45 (1H, d, J = 8.5)	131.3	7.18 (1H, d, J = 2.0)	111.7	7.46 (1H, m)	128.3
6″	6.79 (1H, d, J = 8.5)	116.9		149.4	7.32 (1H, m)	129.1
7″		161.4		150.8	7.32 (1H, m)	130.7
8″	6.79 (1H, d, J = 8.5)	116.9	6.80 (1H, d, J = 8.0)	116.5	7.32 (1H, m)	129.1
9″	7.45 (1H, d, J = 8.5)	131.3	7.06 (1H, dd, J = 8.0, 2.0)	124.4	7.46 (1H, m)	128.3
4″-OCH_3			3.86 (3H, s)	56.4		
$COCH_3$		172.0		172.0		170.3
$COCH_3$	2.09 (3H, s)	20.8	2.09 (3H, s)	20.9	2.05 (3H, s)	21.1

[a] δ in ppm, J in Hz. Compounds **13**−**15** were recorded in CD_3OD; compound **16** was recorded in $CDCl_3$.

Table 2. ^{1}H and ^{13}C NMR Data for Compound 17[a]

	δ_H	δ_C
1	4.42 (1H, dd, J = 12.0, 4.5)	62.3
	4.32 (1H, dd, J = 12.0, 6.0)	
2	5.34 (1H, m)	69.2
3	4.39 (1H, dd, J = 12.0, 4.5)	62.6
	4.26 (1H, dd, J = 12.0, 6.0)	
1′		166.5
2′	6.43 (1H, d, J = 16.0)	117.2
3′	7.70 (1H, d, J = 16.0)	146.0
4′		134.2
5′	7.53 (1H, m)	128.3
6′	7.39 (1H, m)	129.1
7′	7.39 (1H, m)	130.7
8′	7.39 (1H, m)	129.1
9′	7.53 (1H, m)	128.3
1″		172.6
2″	2.53 (1H, dd, J = 16.5, 3.5)	41.5
	2.44 (1H, dd, J = 16.5, 9.0)	
3″	4.01 (1H, m)	68.2
4″	1.52 (1H, m)	36.7
	1.42 (1H, m)	
5″	1.44 (2H, m)	25.6
6″-13″	1.25 (16H, brs)	29.5−29.7
14″	1.32 (2H, m)	25.9
15″	1.55 (2H, m)	32.9
16″	3.62 (2H, t, J = 6.5)	63.1
4″-OCH_3		
$COCH_3$	170.3	
$COCH_3$	2.10 (3H, s)	21.0

[a] δ in ppm, J in hertz. Compound **17** was recorded in $CDCl_3$.

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.

Statistical Analysis. Data were reported as mean ± SD for triplicate determinations on an "as-it-is" botanical weight basis. 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). Correlation was analyzed using a two-tailed Pearson's correlation test. Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Structural Identification of Compounds 13−17 from Wuhan Propolis. Compound **13** was obtained as a colorless

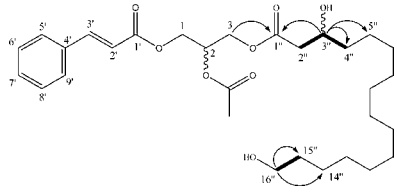

Figure 2. Key $^{1}H-^{1}H$ COSY and HMBC correlations of compound **17**.

gum with $[\alpha]_D^{25}$ +2.1° (c 0.06, CH_3OH). The molecular formula of **13** was determined as $C_{23}H_{22}O_7$ according to negative-ion HR-ESI-MS data and ^{13}C NMR. The IR spectrum showed absorption bands typical for hydroxyl (3435 cm^{-1}) and ester carbonyl (1718 cm^{-1}) groups, and aromatic rings (1630, 1515 cm^{-1}). The MS spectrum gave a characteristic product ion at m/z 349.1064 due to loss of a fragment of $C_2H_4O_2$, suggesting the existence of an acetyl group in **13**. The ^{1}H NMR of **13**

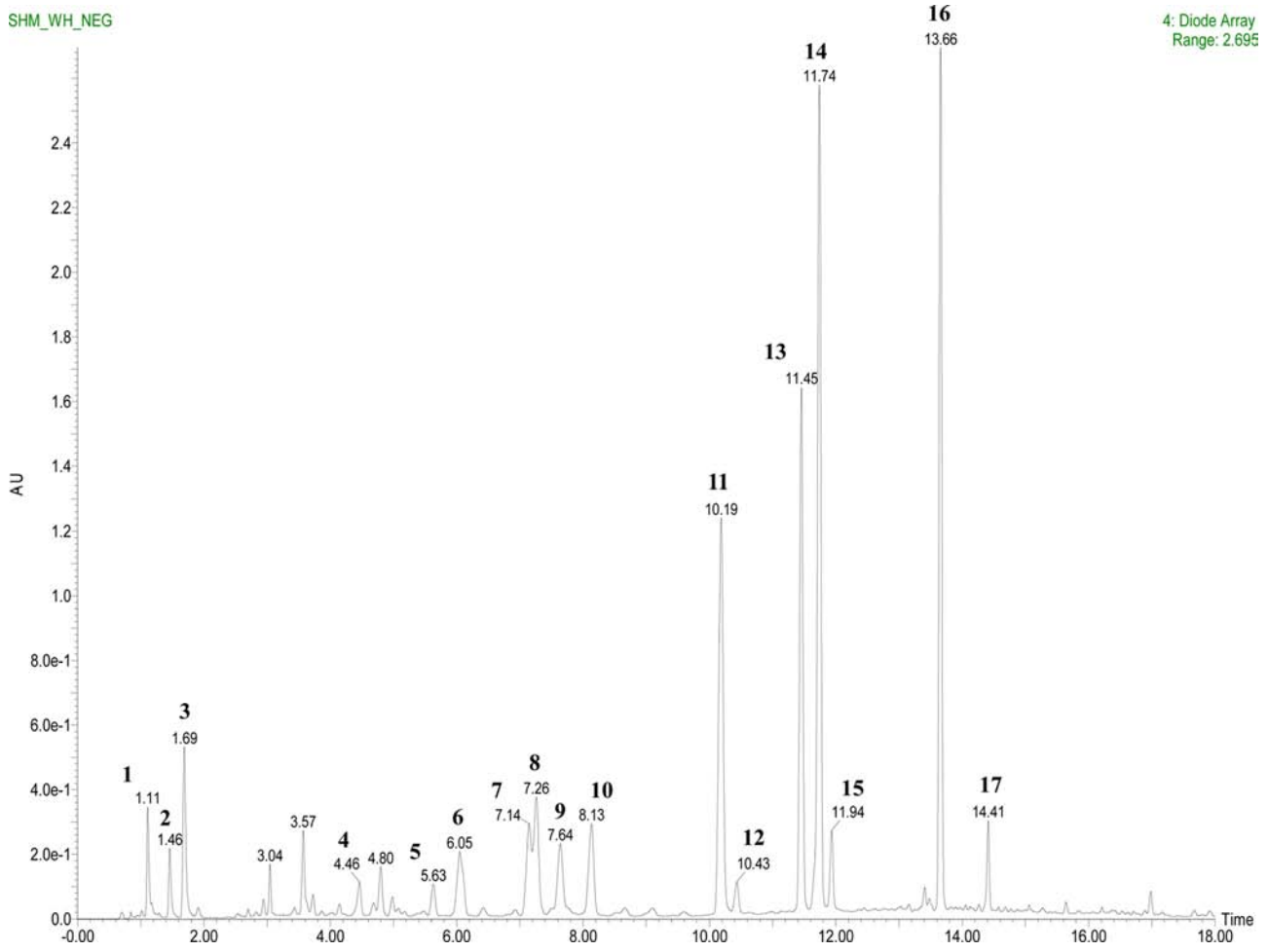

Figure 3. UPLC chromatographic spectra of Wuhan propolis.

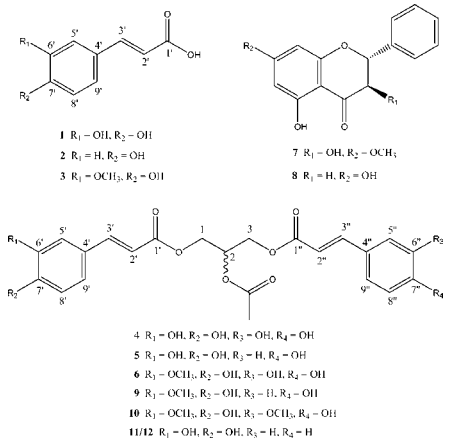

Figure 4. Chemical structures of compounds **1**−**12** tentatively identified by UPLC−Q-TOF-MS.

(Table 1) exhibited characteristic proton signals of an (*E*)-coumaroyl group [four aromatic protons at δ 7.45 (2H, d, J = 8.5 Hz) and 6.79 (2H, d, J = 8.5 Hz) as an AA′XX′ system, two *trans* olefinic protons as an AX system at δ 7.64 (d, J = 15.5 Hz) and 6.35 (d, J = 15.5 Hz)], an (*E*)-cinnamoyl moiety [five aromatic protons between δ 7.40 and 7.60, two *trans* olefinic protons at δ 7.72 (d, J = 16.0 Hz) and 6.55 (d, J = 16.0 Hz)], an acetyl group (3H, *s*, 2.09), two methylene groups [δ 4.50 (2H, dd, J = 12.0, 4.0 Hz) and δ 4.38 (2H, dd, J = 12.0, 6.0 Hz)], and a methine proton at δ 5.38 (1H, tt, J = 6.0, 4.0 Hz). Comparison of the ^{1}H and ^{13}C NMR data of **13** with those of 2-acetyl-1,3-dicoumaroylglycerol suggested that the structure of **13** is closely related to it,[20] except for the composition of aromatic acid. The ^{1}H and ^{13}C NMR data revealed that an (*E*)-cinnamoyl group at C-3 of **13** was replaced by an (*E*)-coumaroyl group at the corresponding position of 2-acetyl-1,3-dicoumaroylglycerol. Comprehensive HMQC and HMBC analysis also confirmed the above results, so the structure of compound **13** was elucidated as (+)-2-acetyl-1-(*E*)-coumaroyl-3-(*E*)-cinnamoylglycerol.

Compound **14** was isolated as a colorless gummy solid with $[\alpha]_D^{25}$ +1.5° (*c* 0.11, MeOH). Its HR-ESI-MS exhibited a negative pseudomolecular ion, compatible with the molecular formula $C_{24}H_{24}O_8$. Comparison of the IR, ^{1}H NMR, and ^{13}C NMR data of **14** with those of **13** suggested that the structure of **14** is closely related to that of **13**, which is a glycerol ester containing an acetyl group, an (*E*)-feruloyl group, and an (*E*)-cinnamoyl group. Based on 2D-NMR data, compound **14** was identified as (+)-2-acetyl-1-(*E*)-feruloyl-3-(*E*)-cinnamoylglycerol.

Compound **15** was isolated as a colorless gum with $[\alpha]_D^{25}$ 8.5° (*c* 0.28, MeOH). Its HR-ESI-MS exhibited a negative pseudomolecular ion in accord with the molecular formula $C_{24}H_{24}O_8$. The spectroscopic and spectrometric data of **15** were fully identical to those of **14**. Considering the negative

Table 3. Characterization of Additional Compounds in Wuhan Propolis

	MF	t_R (min)	UV λ_{max} (nm)	main negative ion	main positive ion	identification
1	$C_9H_8O_4$	1.11	320	179.0334 $[M - H]^-$		caffeic acid
2	$C_9H_8O_3$	1.46	310	163.0385 $[M - H]^-$		*p*-coumaric acid
3	$C_{10}H_{10}O_4$	1.69	320	193.0493 $[M - H]^-$		ferulic acid
4	$C_{23}H_{22}O_{10}$	4.46	330	457.1145 $[M - H]^-$, 397.0929 $[M - C_2H_4O_2 - H]^-$	459.1291 $[M + H]^+$, 476.1560 $[M + NH_4]^+$, 481.1111 $[M + Na]^+$	2-acetyl-1,3-dicaffeoylglycerol
5	$C_{23}H_{22}O_9$	5.63	320	441.1185 $[M - H]^-$	443.1343 $[M + H]^+$, 460.1607 $[M + NH_4]^+$, 465.1164 $[M + Na]^+$	2-acetyl-1-caffeoyl-3-coumaroylglycerol
6	$C_{24}H_{24}O_{10}$	6.05	330	471.1306 $[M - H]^-$, 411.1086 $[M - C_2H_4O_2 - H]^-$	473.1455 $[M + H]^+$, 490.1725 $[M + NH_4]^+$, 495.1281 $[M + Na]^+$	2-acetyl-1-feruloyl-3-caffeoylglycerol
7	$C_{16}H_{14}O_5$	7.14	290	285.0770 $[M - H]^-$	287.0932 $[M + H]^+$	pinobanksin 7-methyl ether
8	$C_{15}H_{12}O_4$	7.26	290	255.0658 $[M - H]^-$	257.0814 $[M + H]^+$	pinocembrin
9	$C_{24}H_{24}O_9$	7.64	318	455.1337 $[M - H]^-$, 395.1124 $[M - C_2H_4O_2 - H]^-$	457.1498 $[M + H]^+$, 474.1761 $[M + NH_4]^+$, 479.1321 $[M + Na]^+$	2-acetyl-1-feruloyl-3-coumaroylglycerol
10	$C_{25}H_{26}O_{10}$	8.13	326	485.1440 $[M - H]^-$, 425.1227 $[M - C_2H_4O_2 - H]^-$	487.1616 $[M + H]^+$, 504.1881 $[M + NH_4]^+$, 509.1437 $[M + Na]^+$	2-acetyl-1,3-diferuloylglycerol
11	$C_{23}H_{22}O_8$	10.19	285	425.1220 $[M - H]^-$, 365.1022 $[M - C_2H_4O_2 - H]^-$	427.1402 $[M + H]^+$, 444.1667 $[M + NH_4]^+$, 449.1237 $[M + Na]^+$	(+)-2-acetyl-1-caffeoyl-3-cinnamoylglycerol
12	$C_{23}H_{22}O_8$	10.43	285	425.1212 $[M - H]^-$	427.2700 $[M + H]^+$, 444.1670 $[M + NH_4]^+$, 449.1226 $[M + Na]^+$	(−)-2-acetyl-1-caffeoyl-3-cinnamoylglycerol

optical rotation for **15**, its structure was identified as (−)-2-acetyl-1-(*E*)-feruloyl-3-(*E*)-cinnamoylglycerol.

Compound **16** was obtained as an amorphous white powder, whose molecular formula was deduced to be $C_{23}H_{22}O_6$ by HR-ESI-MS. The spectroscopic and spectrometric data of **16** were quite similar to those of **13**, except for the presence of an (*E*)-cinnamoyl group at C-3 of **16** instead of an (*E*)-coumaroyl substituted at C-3 in **13**. The structural assignment of **16** was fully achieved by interpretation of 2D NMR including HMQC and HMBC. Consequently compound **16** was elucidated as 2-acetyl-1,3-dicinnamoylglycerol.

Compound **17** was obtained as an amorphous white powder with $[\alpha]_D^{25}$ −12.5° (*c* 0.40, $CHCl_3$), whose molecular formula was deduced to be $C_{30}H_{46}O_8$ by HR-ESI-MS. The ^1H and ^{13}C NMR spectra of **17** displayed many similarities with those of **16**, except that the signal of (*E*)-cinnamoyl group in **16** was replaced by the signal of fatty acid moiety in **17**. The full NMR assignments and connectivities of fatty acid moiety were determined by $^1H-^1H$ COSY, HSQC, and HMBC spectroscopic data analysis. The selected important HMBC correlations are shown in Figure 2. The proton at δ 4.01 (H-3″), attached to a carbon at δ 68.2, showed HMBC correlations with C-1″, C-4″, and C-5″, indicating the location of the oxymethine at C-3″. In the $^1H-^1H$ COSY spectrum, H-3″ showed cross peaks with H-2″ and H-4″, which also indicated that hydroxylation occurs on C-3″. Obvious HMBC correlations between H-16″ (δ 3.62) with C-14″ and C-15″, together with a clear cross peak between H-15″ and H-16″, were observed, indicating a hydroxyl group submitted on C-16″. However, the absolute configuration of C-3″ was not determined for its low yield. Taken together, compound **17** was identified as (−)-2-acetyl-1-(*E*)-cinnamoyl-3-(3″(ζ),16″)-dihydroxy-palmitoylglycerol.

Tentative Identification of the Additional Twelve Compounds in Wuhan Propolis Extract. According to UPLC−Q-TOF-MS analysis (Figure 3), seven structurally similar glycerol derivatives (**4**, **5**, **6**, and **9**−**12**), three phenolic acids (**1**, **2**, and **3**), and two flavonoids (**7** and **8**) were identified from the Wuhan propolis extract (Figure 4, Table 3). Compounds **1** (t_R = 1.11 min), **2** (t_R = 1.46 min), and **3** (t_R = 1.69 min) possessed characteristic UV spectra of phenolic acids and showed $[M - H]^-$ ions at *m*/*z* 179.0334, 163.0385, and 193.0493, correspondingly assigned as caffeic, *p*-coumaric, and ferulic acids. Compound **4** (t_R = 4.46 min) gave an $[M - H]^-$ ion at *m*/*z* 457.1145 followed by successive loss of a characteristic acetyl group to produce an ion peak at *m*/*z* 397.0929. Meanwhile, an $[M + H]^+$ ion at *m*/*z* 459.1291 was observed in positive ion mode. Considering its characteristic UV max absorption at 320 nm, compound **4** was tentatively deduced as 2-acetyl-1,3-dicaffeoylglycerol. Compound **5** (t_R = 5.63 min) produced a negative ion with $[m/z]^-$ 441.1185 and a positive ion with $[m/z]^+$ 443.1343, in accord with the molecular formula $C_{23}H_{22}O_9$. Similarly, compound **5** was tentatively characterized as 2-acetyl-1-caffeoyl-3-coumaroylglycerol. Compound **6** (t_R = 6.05 min) was tentatively characterized as 2-acetyl-1-feruloyl-3-caffeoylglycerol based on the negative ions at $[m/z]^-$ 471.1306 and 411.1086, and a positive ion with $[m/z]^+$ 473.1455. As expected, the retention behaviors of compounds **4**−**6** displayed a similar sequence to compounds **1**−**3**: caffeic acid derivative eluted first, then the *p*-coumaric acid derivative, and finally the ferulic acid derivative. Compound **7** (t_R = 7.14 min) produced a negative ion with $[m/z]^-$ 285.0770 and a positive ion with $[m/z]^+$ 287.0932, in accord with the molecular formula $C_{16}H_{14}O_5$. Considering also its characteristic UV max absorption at 290 nm, compound **7** was tentatively deduced as pinobanksin 7-methyl ether. The methoxy group cannot be at C-5 in the ring A; in fact, 5-methoxy pinobanksin was isolated by our group,[17] which has a retention time different from that of compound **7**. Compound **8** (t_R = 7.26 min) was unambiguously identified as pinocembrin by comparison with its retention time and UV and MS spectra of the authentic standard. Compounds **9** and **10** exhibited UV maxima at 318 and 326 nm, respectively, characteristic of phenolic acid derivatives. They had the molecular formula of $C_{24}H_{24}O_9$ and $C_{25}H_{26}O_{10}$, respectively, and all showed a characteristic product ion due to loss of an acetyl group. So compounds **9** and **10** were tentatively identified as 2-acetyl-1-feruloyl-3-coumaroylglycerol and 2-acetyl-1,3-diferuloylglycerol. Compounds **11** (t_R = 10.19 min) and **12** (t_R = 10.43 min) showed very similar UV and MS spectra. In the high resolution mass spectra, **11** and **12** showed $[M + NH_4]^+$ ions at *m*/*z* 444.1667 and 444.1670 and $[M - H]^-$ ions at *m*/*z* 425.1220

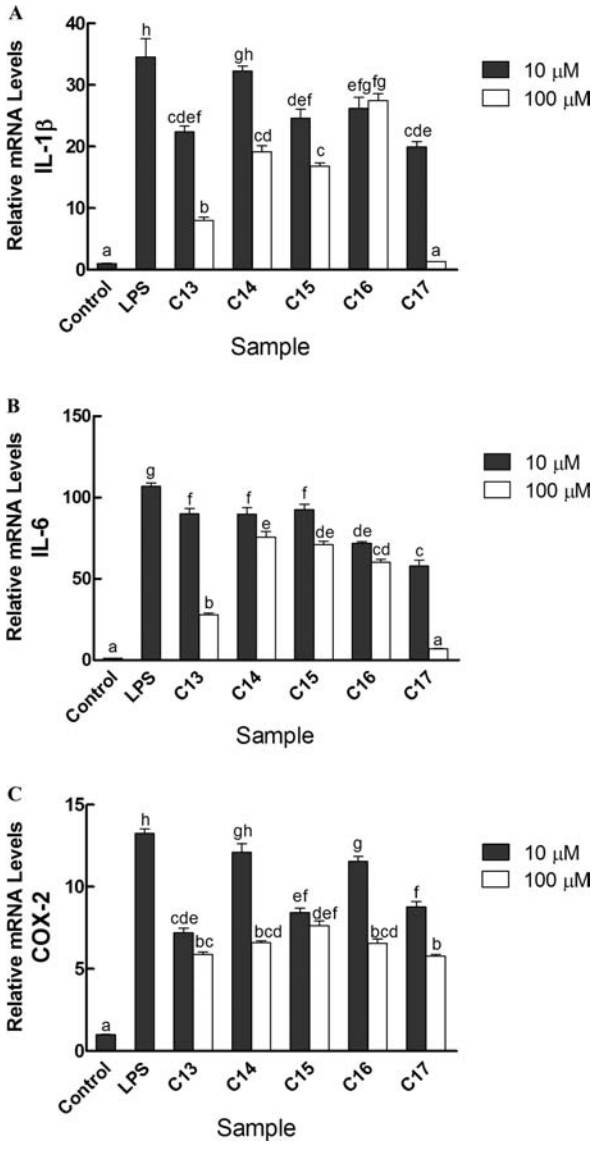

Figure 5. Effects of compounds **13**−**17** on mRNA expressions of (A) IL-1β, (B) IL-6, and (C) COX-2 in mouse RAW 264.7 macrophage cells. C13 stands for compound **13**, C14 for compound **14**, C15 for compound **15**, C16 for compound **16**, and C17 for compound **17**. Each column represents the mean ± SD (n = 3). Columns marked by different letters are significantly different from each other ($P \leq 0.05$).

and 425.1212, respectively, suggesting that compounds **11** and **12** were isomers. Considering also the similar chromatographic behavior of **14** and **15**, compounds **11** and **12** were putatively characterized as (+)-2-acetyl-1-caffeoyl-3-cinnamoylglycerol and (−)-2-acetyl-1-caffeoyl-3-cinnamoylglycerol. Among them, compounds **6**, **11**, and **12** have not been previously reported from nature, which should be confirmed by future phytochemical work. This study suggested that Wuhan propolis may differ from other Chinese propolis products and have glycerol esters as the primary constituents.

As described in previous publications,[21,22] one group of glycerol esters were identified as characteristic constituents of bud exudate of *Populus* species. So the botanical origin of this Wuhan propolis sample was inferred to be the genus *Populus*.

Anti-Inflammatory Effect of Compounds 13−17 on the mRNA Expression of Inflammatory Cytokines. Chronic inflammation has been linked to the initiation and progression of several aging associated chronic human diseases, including cardiovascular diseases, cancer, and arthritis. IL-1β, IL-6, and COX-2 are three important inflammation mediators, which may substantially alter cardiovascular function and enhance local inflammation through macrophage regulation.

In this study, the effects of 100% methanol extract of Wuhan propolis (see Figure S1 in the Supporting Information) and five isolated compounds **13**−**17** on IL-1β, IL-6, and COX-2 mRNA expressions were examined. A dose-dependent inhibitory effect was observed in this study (Figure 5). At 10 μM initial concentration, compounds **13**, **15**, **16**, and **17** showed inhibitory effect on IL-1β mRNA expression, while all five tested compounds were able to significantly reduce IL-1β mRNA expression at 100 μM concentration (Figure 5A). Compound **16** showed similar inhibitory effect on IL-1β expression at both concentrations. Among the five compounds, **17** showed the strongest inhibitory effect on IL-1β mRNA expression on a per same molar concentration basis.

All five compounds also exhibited inhibition on IL-6 mRNA expression (Figure 5B). Compound **17** had the strongest inhibitory activity, which inhibited 46 and 93% of the LPS-stimulated IL-6 mRNA expression at 10 and 100 μM, respectively. Compound **13** also showed remarkable inhibition on IL-6 gene expression at 100 μM. In addition, all five compounds slightly or moderately reduced the expression of COX-2 in LPS-stimulated macrophage cells (Figure 5C).

The primary structural difference of compounds **13**−**17** is the substituent group of phenolic acid or fatty acid moiety at the C-1 or C-3 of the glycerol residue (Figure 1). Compounds **13** and **17** exhibited much stronger inhibition on the mRNA expression of IL-1β, IL-6, and COX-2 than compounds **14**−**16** at 100 μM. Interestingly, compounds **14** and **15**, optical isomers, significantly differed in their inhibition on IL-1β and COX-2 mRNA expression at 10 μM initial concentration. Compound **17** possessed the strongest anti-inflammatory activity on three pro-inflammatory factors at both testing concentrations, except for COX-2 at 10 μM. These data suggested that the presence of fatty acid group at the C-3 of the glycerol backbone might significantly enhance anti-inflammatory activities.

The in vitro and in vivo anti-inflammatory activities of propolis and related products have been widely studied. Paulino et al.[23] reported the anti-inflammatory activities of a standard ethanol extract G1 from Brazilian green propolis in mouse model and LPS-stimulated RAW 264.7 cells. The data supported that the propolis showed an anti-inflammatory effect through inhibition of iNOS gene expression. Another study by Tan-no et al.[24] indicated that the ethanol extract of Brazilian propolis was able to inhibit nitric oxide production in mouse paw edema model. Later in 2007, the anti-inflammatory activity of an ointment containing different concentrations of propolis extract (3−7%) was examined using carrageenan-induced hind paw edema in rats.[25] Their results showed that 5 and 7% propolis-containing ointment could significantly inhibit the edema. Furthermore, artepillin C, a major compound in the Brazilian green propolis, was investigated for its anti-inflammatory effect, absorption, and bioavailability in mice.[26] Artepillin C showed anti-inflammatory effects mediated, at least in part, by prostaglandin E2 and nitric oxide inhibition through NF-κB modulation, and exhibited bioavailability with a maximal absorption peak after 1 h when administered orally. Our results were well in accordance with these previous reports that propolis extract or individual compound obtained from

propolis showed significant anti-inflammatory activities, suggesting that Wuhan propolis may be used as potential anti-inflammation ingredients in functional foods and dietary supplements to promote human health.

■ ASSOCIATED CONTENT

Supporting Information

Figure depicting anti-inflammatory effects of Wuhan propolis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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