


# Identification of Free Radical Scavengers from Brazilian Green Propolis Using Off-Line HPLC-DPPH Assay and LC-MS

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**Abstract:** Brazilian green propolis is known as an appreciable natural antioxidant with abundant polyphenolic compounds. For quality control, a fingerprint-efficacy study of Brazilian green propolis was carried out in this work. Chemical fingerprints of Brazilian green propolis from 22 different sources were determined by HPLC and investigated by similarity analysis. The fingerprint-efficacy relationships between chemical fingerprint and DPPH radical-scavenging activity were established. The results showed that 14 characteristic common peaks were identified, and 9 compounds were discovered with free radical-scavenging activities. Caffeoylquinic acids and artemillin C might be the major effective components for quality control of Brazilian green propolis due to their specificity and strong antioxidant activity. This study provides new markers for the quality assessment of Brazilian green propolis and its derived products.

**Keywords:** antioxidant activity, Brazilian green propolis, chromatographic fingerprint, HPLC-DPPH, quality control

## Introduction

Propolis is used as a construction material and defense protection for beehives and also maintains the health of a bee colony, it possesses many biological activities, such as antibacterial, antifungal, antitumoral, antioxidative, immunomodulatory, and other beneficial activities (Ghisalberti 1979; Marcucci 1995). More than 500 compounds have been identified in propolis, including flavonoids, phenolic acids, terpenoids, aromatic aldehydes and alcohols, lignins, fatty acids, amino acids, vitamins, sugars, and microelements (Zhang and Hu 2009, 2012, Zhang and others 2013a; Huang and others 2014). The chemical composition and biological activities of propolis vary with bee species, plant origin, geographic location, seasonality, and storage condition (Silici and Kutluca 2005; Teixeira and others 2010; Toreti and others 2013).

Brazilian green propolis, derived from *Baccharis dracunculifolia* DC., has been the most thoroughly studied regarding its composition and biological activity. Its characteristic constituents have found to be prenylated phenylpropanoids, caffeoylquinic acids, and diterpenoid acids (Salatino and others 2005). Many analytical separation techniques have been used to analyze these compounds, including TLC (thin-layer chromatography), high-performance liquid chromatography (HPLC), gas chromatography (GC), and chromatography combined with mass spectrometry (MS; Midorikawa and others 2001; Bankova 2005; de Sousa and others 2007; Matsuda and de Almeida-Muradian 2008; Mayworm and others 2015). For the past several years, chromatographic fingerprints have been used to characterize the chemical profiles to identify plant origin, authentication, and quality. Major compounds are considered as markers in the quality assessment, regardless of their effectiveness. Till now there has been little work completed evaluating the relationships between biological activity and chemical properties. So, it is imperative and urgent

to find the main effective components in fingerprints that reflect the therapeutic effects of propolis.

Some researchers have indicated that anti-oxidative or radical-scavenging activities may play a key role for cardioprotective effects (Daleprane and Abdalla 2013), neuroprotective activity (Nakajima and others 2007), and other activities. In recent years, on-line and off-line HPLC-DPPH/ABTS assays have been performed to investigate the chromatographic-pharmacodynamics relationship of various traditional Chinese medicines, fruits, and vegetables, such as blueberry (Sun and others 2012), tea (Zhang and others 2013b), *Mentha spp.* (Kosar and others 2004), *Epimedium spp.* (Ding and others 2011), and *Hibiscus esculentus* Linn. (Shui and Peng 2004); and the determination of bioactive fingerprint was achieved by combining fingerprint chromatography analysis with bioactivity evaluations to better present both the chemical profiles and active ingredients.

This study was undertaken to investigate characteristic chemical fingerprints and the antioxidant material basis of Brazilian green propolis. Separation and identification of characteristic constituents was accomplished by using HPLC-UV and HPLC-ESI-MS/MS. Meanwhile, an off-line HPLC-DPPH method was developed to screen and assess free radical scavengers in Brazilian green propolis.

## Materials and Methods

### Chemicals and reagents

HPLC-grade methanol was purchased from Merck (Darmstadt, Germany); analytical grade acetic acid and absolute ethanol were purchased from Chemical Reagent Factory of Zhejiang Univ. (Hangzhou, China). Ultra-pure water was purified by the Yjd-upws Ultra-Pure water system (Hangzhou, China).

1,1-Diphenyl-2-picrylhydrazyl, caffeic acid, chlorogenic acid, *p*-coumaric acid, and kaempferol ( $\geq 98.0\%$ ) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.), kaempferide, isochlorogenic acid A, and isochlorogenic acid C were obtained from Ningbo Haishu Apexocean Biochemicals Co., Ltd. (Ningbo, China), Artemillin C (purity  $\geq 98\%$ ) was purchased from Wako

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(Wakayama, Japan). Quercetin and gallic acid were purchased from Sangon Biotech (Shanghai, China).

### Propolis sample preparation

Twenty-two raw Brazilian green propolis samples were kindly provided by Hangzhou Bee Words Bee Industry Stock Corp. Ltd. (Nanjing, China), and Fengnaibao Benpu (Nanjing) Health-care Food Co., Ltd (Hangzhou, China).

The frozen propolis samples (0.5 g) were extracted with 15 mL of 95% hydro-alcoholic solution in an ultrasonic water bath for 45 min. The extracts were filtered and the residues were re-extracted twice under the same conditions. Then the filtrates were combined and kept at 4 °C overnight to remove insoluble matter. Afterwards the solutions were transferred to a volumetric flask and adjusted to 50 mL with 95% ethanol and finally filtered through a 0.22 µm membrane filter into a vial for HPLC analysis.

### Determinations of total flavonoids and total phenolics

Total flavonoids content (TFC) was measured by aluminum ion chromogenic method with minor modifications (Park and others 1997): 2.8 mL of 95% ethanol was added to 0.2 mL propolis extract (5 mg/mL), and then mixed with 0.2 mL (100 g/L) aluminum nitrate and 0.2 mL (9.8 g/L) potassium acetate and diluted to 10 mL with distilled water. The absorbance was measured at 415 nm in a Shimadzu 2550 UV-vis spectrophotometer after 1 h at room temperature. Quercetin was employed as analytical standard in concentrations ranging from 2 to 20 µg/mL and the results were expressed as mg/g.

The amount of total phenolics was determined by the modified Folin–Ciocalteu method (Woisky and Salatino 1998). Generally, 1 mL Folin–Ciocalteu reagent was added to 1 mL of a 0.5 mg/mL propolis extract, and then 5 mL (1 mol/L) sodium carbonate was mixed in and adjusted to 10 mL with distilled water. The absorbance was measured at 760 nm after 1 h in the dark. Gallic acid was employed as a reference in concentrations ranging from 1 to 10 µg/mL and the results were expressed as mg/g.

### Determination of DPPH<sup>•</sup> radical-scavenging activity

The DPPH<sup>•</sup> antioxidant activity was determined according to the modified method Yamaguchi and others (1998). In brief, 120 µL DPPH<sup>•</sup> working solution was mixed with 120 µL propolis extract in a 96-well plate. The absorbance of the reaction solutions was read at 517 nm after incubating for 30 min in the dark. The results were expressed as IC<sub>50</sub> (µg/mL, the concentration of scavenging 50% DPPH<sup>•</sup> radical).

### HPLC procedures

Chromatographic analysis was performed with Agilent 1200 Series (Agilent Technologies, Inc., Santa Clara, Calif., U.S.A.) equipment. The LC system was equipped with a quaternary pump G1311A, an online vacuum degasser G1322A, an auto-sampler G1314B, and a Thermos-tatted Column Compartment G1316A. Separation was achieved on a Sepax HP-C18 column (150 × 4.6 mm, 5 µm; Sepax Technologies, Inc., Newark, Del., U.S.A.) and maintained at 33 °C. The mobile phase consisted of both organic phase A, methanol and aqueous phase B, 0.1% acetic acid at a constant flow rate of 1 mL/min. The gradient elution was adjusted as follows: 15% to 25% (A) from 0 to 10 min, 25% to 40% (A) from 10 to 25 min, 40% to 60% (A) from 25 to 55 min, 60% to 75% (A) from 55 to 75 min, and finally 75% to 85% (A) from 75 to 90 min. Each propolis sample (5 µL) was injected through an automatic sampler system and monitored at 280 nm.

Methodology was validated through precision, repeatability, and stability tests. Repeatability was determined by the analysis of the same sample with 6 parallel processes, although precision and stability tests were carried out every 3 h in a day and every day on 6 consecutive days, respectively.

### Evaluation of chromatographic fingerprints

The software “Similarity Evaluation System for Chromatographic Fingerprint of TCM” published by the Chinese Pharmacopoeia Commission (Version 2004A) was employed to synchronize and make qualitative and quantitative comparisons among all propolis samples. The reference fingerprint was produced by the system using the median method from the general comparison of the chromatograms of 22 propolis extracts, meanwhile the similarity values of each propolis extract and reference fingerprint were also determined using this software.

### HPLC-ESI-MS/MS analysis

Mass spectrometry was performed with an Agilent 6430 QQQ MS (Agilent Technologies, Inc.) instrument equipped with an electronic spray ionization (ESI) interface with the following operating conditions: drying gas (N<sub>2</sub>) flow rate, 9.0 L/min; drying gas temperature, 350 °C; nebulizer, 35 psig; capillary, 4000 V; fragmentor voltage, 135 V. All the operations, acquisition, and analysis of data were controlled by Agilent LCMS-MS MassHunter Acquisition Software Version B.03.01 (Agilent Technologies, Inc.) and operated under MassHunter Workstation Software Version B.03.01 (Agilent Technologies, Inc.). The mass spectra were analyzed in both positive and negative ion mode and recorded across the range *m/z* 100 to 1000 for scan mode.

### Off-line HPLC-DPPH assays

Off-line HPLC-DPPH assay was developed based on a previous method developed by Toshiya with some modifications (Masuda and others 2003). A total of 500 µL of freshly prepared (3 mg/mL) DPPH<sup>•</sup> ethanol solution (500 µL) was added into the same volume (10 mg/mL) of propolis ethanol extract. The mixture was fully blended and kept at 25 °C in the dark for 1 h. After filtration through a 0.22 µm filter, 5 µL of the mixed solution was injected into the HPLC and analyzed as described above. Blank control of propolis extract with equivoluminal ethanol and DPPH with ethanol were also analyzed. Appropriate concentration of DPPH ethanol solution, suitable reaction ratio, and reaction time were optimized.

### Statistical data analysis

Data are expressed as mean ± SD and each value is representative of at least 3 independent experiments. Multivariate correlation analysis was used for the evaluation of the spectrum–effect relationships by SPSS statistics software (SPSS for Windows 18.0, SPSS Inc., Chicago, U.S.A.). Statistical analysis (Student's *t*-test) was also performed to determine significant differences.

## Results and Discussion

### Contents of total flavonoids, total phenolics, and antioxidant activity

The amounts of total phenolics and total flavonoids varied widely in different Brazilian green propolis samples, ranging from 87.5 to 148.6 mg/g and 38.4 to 67.6 mg/g, respectively, all samples showed a good free radical-scavenging activity with IC<sub>50</sub> values varying from 93.51 to 190.27 µg/mL (Table 1). The relationships

**Table 1** Total phenolic, total flavonoid contents, and antioxidant activity in Brazilian green propolis.

Sample no.	Total phenolics (mg/g, GAE)	Total flavonoids (mg/g, QE)	DPPH <sup>•</sup> scavenging activity (IC <sub>50</sub> , µg/mL)
1	148.55 <sup>a</sup>	64.99 <sup>a</sup>	108.303 <sup>gh</sup>
2	128.29 <sup>b</sup>	67.60 <sup>a</sup>	108.44 <sup>gh</sup>
3	122.47 <sup>c</sup>	58.46 <sup>b</sup>	116.86 <sup>fg</sup>
4	122.67 <sup>c</sup>	53.74 <sup>bcd</sup>	128.68 <sup>de</sup>
5	102.14 <sup>g</sup>	46.17 <sup>def</sup>	154.22 <sup>b</sup>
6	87.53 <sup>i</sup>	39.48 <sup>ef</sup>	186.87 <sup>a</sup>
7	114.71 <sup>de</sup>	50.78 <sup>bcd</sup>	104.04 <sup>h</sup>
8	98.01 <sup>gh</sup>	40.66 <sup>ef</sup>	127.23 <sup>de</sup>
9	115.26 <sup>de</sup>	52.24 <sup>bcd</sup>	93.51 <sup>i</sup>
10	116.89 <sup>d</sup>	45.49 <sup>def</sup>	104.75 <sup>h</sup>
11	111.12 <sup>ef</sup>	49.22 <sup>cd</sup>	121.01 <sup>ef</sup>
12	99.64 <sup>gh</sup>	48.53 <sup>cd</sup>	162.60 <sup>b</sup>
13	116.22 <sup>d</sup>	56.36 <sup>bc</sup>	140.16 <sup>c</sup>
14	107.11 <sup>f</sup>	51.75 <sup>bcd</sup>	140.81 <sup>c</sup>
15	96.13 <sup>i</sup>	38.35 <sup>f</sup>	104.12 <sup>h</sup>
16	95.24 <sup>i</sup>	47.40 <sup>cde</sup>	162.40 <sup>b</sup>
17	97.39 <sup>gh</sup>	49.75 <sup>bcd</sup>	135.85 <sup>cd</sup>
18	99.67 <sup>gh</sup>	39.30 <sup>ef</sup>	134.72 <sup>cd</sup>
19	100.38 <sup>gh</sup>	49.73 <sup>bcd</sup>	157.17 <sup>b</sup>
20	108.52 <sup>f</sup>	40.15 <sup>ef</sup>	113.68 <sup>fg</sup>
21	109.71 <sup>f</sup>	56.10 <sup>bc</sup>	158.51 <sup>b</sup>
22	110.95 <sup>ef</sup>	50.49 <sup>bcd</sup>	190.27 <sup>a</sup>

Note: Data are shown as the mean ( $n = 3$ ). Different superscript lowercases indicate significant differences with each other ( $P < 0.05$ ). GAE, gallic acid equivalent; QE, quercetin equivalent.

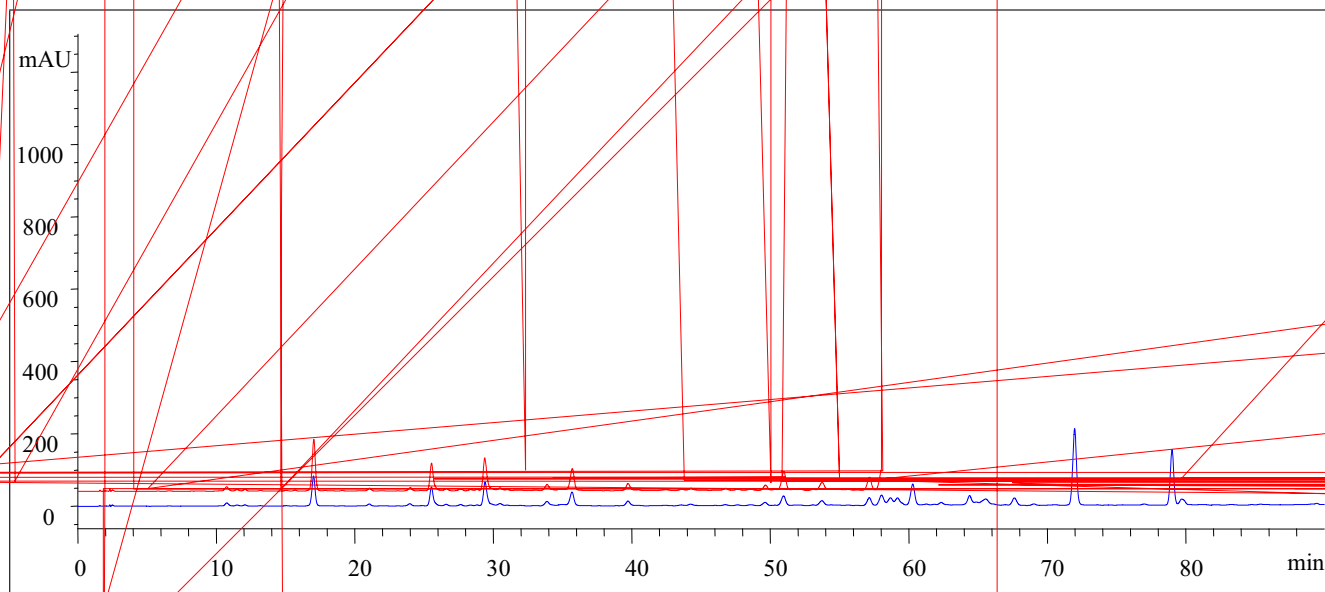
between DPPH<sup>•</sup> scavenging activity (IC<sub>50</sub>) and total phenolics, total flavonoids were also analyzed, and a significant negative correlation was observed with total phenolics ( $R^2 = -0.506$ ,  $P < 0.01$ ), although no significant correlation with total flavonoids was observed ( $R^2 = -0.185$ ,  $P > 0.05$ ). The data indicated that it is the phenolics that contribute to the antioxidant activity, which is

in accordance with Mello and Hubinger (2012), who reported that the pronounced antioxidant activity of Brazilian green propolis is mainly attributed to the high levels of phenolic contents.

### The characteristic chemical fingerprint

**HPLC chromatogram.** The HPLC chromatograms of different samples were similar: 18 common fingerprint peaks were observed in all propolis samples, although variation in the intensities of peak areas was quite significant (Figure 1).

**Identification of characteristic peaks.** The characteristic common peaks were identified by comparing their chromatographic behavior, UV spectra, and MS information to those of reference compounds or referring to previous studies (Midorikawa and others 2001; Sawaya and others 2004). Chlorogenic acid (peak 1), caffeic acid (peak 2), *p*-coumaric acid (peak 3), 3,5-dicaffeoylquinic acid (peak 4), 4,5-dicaffeoylquinic acid (peak 5), 3,4,5-tricaffeoylquinic acid (peak 7), kaempferol (peak 8), kaempferide (peak 13), and artepillin C (peak 16) were determined by comparison with available standards. Although another 5 characteristic peaks were initially identified as aromadendrin-4'-methyl ether (peak 6, RT 35.761 min, [M-H]<sup>-</sup>301, MS<sup>2</sup> [*m/z*] 213, 172), 3-prenyl-4-hydroxycinnamic acid (peak 10, RT 51.009 min, [M-H]<sup>-</sup>231, MS<sup>2</sup> [*m/z*] 133,187), betuletol (peak 14, RT 58.713 min, [M-H]<sup>-</sup>329, MS<sup>2</sup> [*m/z*] 314), 3-prenyl-4-dihydro-cinnamoyloxycinnamic acid (peak 17, RT 79.072 min, [M-H]<sup>-</sup>363, MS<sup>2</sup> [*m/z*] 319, 187, 149, 131), and 2,2-dimethoxy-8-prenyl-chromene-6-propenoic acid (peak 18, RT 79.929 min, [M-H]<sup>-</sup>297, MS<sup>2</sup> [*m/z*] 253, 149) based on published data, respectively. The chemical structures of all identified compounds are showed in Figure 2. The polyphenolic compounds above identified are mainly prenylated derivatives of *p*-coumaric acid (peaks 10, 16, 17, 18) and caffeoylquinic acids (peaks 1, 4, 5, 7), which were not found in other types of propolis, proved to be the markers of Brazilian green propolis (Salatino and others 2005).



**Table 2** Content of 9 pol phenolic compounds in Brazilian green propolis samples.

Sample	Content (mg/g)								
	Chlorogenic acid	Caffeic acid	<i>p</i> -Coumaric acid	3,5-Dicaffeoyl quinic acid	4,5-Dicaffeoyl quinic acid	3,4,5-Tricaffeoyl quinic acid	Kaempferol	Kaempferide	Artepillin C
1	1.98	0.75	10.42	8.69	18.20	2.81	1.49	12.73	26.81
2	3.78	0.97	11.44	12.54	25.64	3.76	1.66	12.60	35.94
3	3.97	1.16	11.22	9.97	21.89	7.16	1.92	11.09	21.66
4	4.90	0.76	10.14	15.55	31.99	4.95	1.86	15.97	60.75
5	4.57	0.67	10.01	15.12	30.58	4.79	1.74	18.79	81.01
6	4.53	1.36	14.13	15.32	31.57	5.46	2.27	15.93	24.80
7	3.93	0.99	10.82	12.19	25.61	3.96	1.67	12.54	36.05
8	6.07	0.54	5.93	15.36	30.96	5.12	1.25	13.57	53.07
9	3.19	0.45	5.74	10.55	23.26	3.34	0.94	9.45	41.14
10	4.51	1.05	7.45	11.71	24.74	4.09	1.51	11.10	40.20
11	4.71	1.11	10.75	16.33	33.40	5.50	2.19	14.78	26.50
12	4.16	1.29	13.86	13.94	28.91	5.12	2.18	14.45	27.61
13	4.76	0.80	10.65	14.00	28.71	4.29	1.47	14.02	65.07
14	3.24	0.80	9.53	10.36	23.18	3.34	1.80	13.37	40.83
15	4.48	0.70	9.82	14.69	30.18	4.89	1.73	17.09	74.22
16	3.21	3.35	13.17	18.45	24.31	7.73	4.11	20.10	35.81
17	3.36	0.91	10.44	10.93	22.58	3.42	1.59	12.68	39.25
18	4.72	0.69	9.55	14.44	29.46	4.74	1.36	17.57	72.76
19	4.23	0.86	9.94	13.04	26.76	4.38	1.81	14.28	42.06
20	4.56	0.72	10.16	13.42	27.11	4.28	1.28	12.87	59.74
21	6.36	1.34	11.61	14.68	31.15	6.58	2.38	13.03	26.12
22	3.58	1.11	13.07	12.87	25.35	7.55	2.11	14.54	32.81

Note: Data are shown as the mean ( $n = 3$ ).

**Table 3** Decreased rate of peak areas in Brazilian green propolis samples.

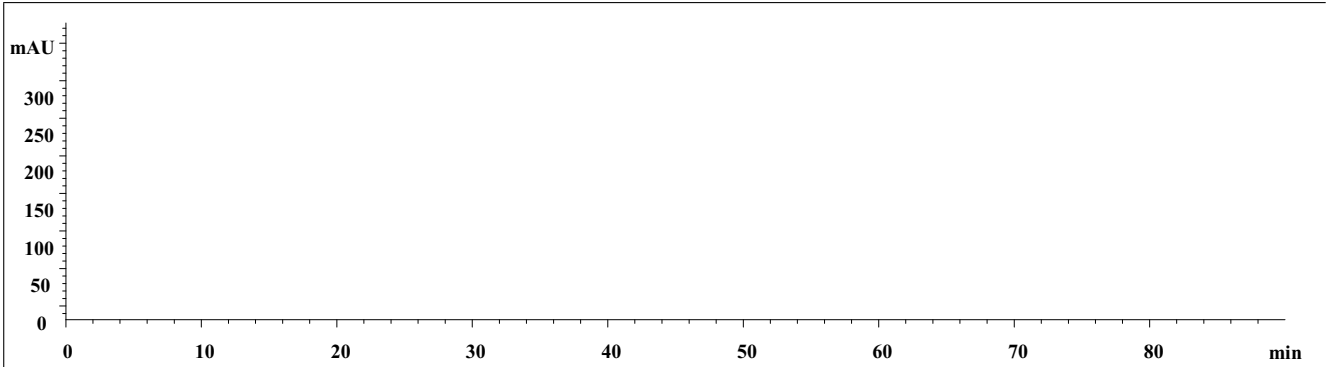
Sample	Decreased rate of peak areas (%)																	
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9	Peak 10	Peak 11	Peak 12	Peak 13	Peak 14	Peak 15	Peak 16	Peak 17	Peak 18
1	49.82	45.65	4.46	76.57	77.08	-1.03	88.25	85.02	-40.80	-5.95	-2.30	3.31	76.85	81.85	12.35	47.53	1.00	1.27
2	51.64	48.12	5.45	77.90	77.82	-2.14	80.70	59.80	-32.90	-7.03	-4.01	2.04	79.56	84.46	11.51	33.43	0.74	1.04
3	60.69	52.96	4.31	78.44	81.24	-0.78	88.80	75.68	-37.90	-6.54	-3.05	2.09	84.60	86.51	8.91	44.69	1.10	0.26
4	37.01	43.31	4.82	72.15	73.23	-1.54	82.87	73.67	-36.87	-3.30	-1.36	3.39	78.68	78.63	12.96	48.32	1.62	1.81
5	51.30	45.84	3.43	73.30	73.84	-3.18	100.00	100.00	-14.97	-1.43	-1.97	2.63	74.73	76.70	9.06	35.67	1.02	1.19
6	51.14	44.96	3.55	72.26	72.38	-5.16	83.34	100.00	-58.49	-4.28	-6.24	2.02	61.69	65.22	2.50	27.39	1.58	0.84
7	55.07	47.32	4.22	74.70	75.54	-6.29	81.92	100.00	-15.43	-7.04	-1.345	6.72	73.27	80.45	9.83	31.95	1.28	1.19
8	57.34	48.41	2.40	74.27	75.11	-4.87	84.95	100.00	-50.82	-3.46	-3.60	4.36	68.45	77.02	3.72	37.96	0.65	2.30
9	52.74	43.73	2.75	71.52	71.72	-5.46	83.07	74.64	-29.33	-4.21	-3.27	3.07	61.35	66.26	4.00	28.39	1.84	2.42
10	54.21	47.17	4.26	71.04	71.86	-4.36	83.34	79.15	-47.25	-10.27	-10.75	5.12	55.79	62.08	7.67	24.35	1.90	0.88
11	41.06	51.55	4.75	74.62	75.46	-3.81	85.37	100.00	-77.21	-3.28	-2.68	6.23	71.64	75.73	2.63	41.61	1.17	2.95
12	53.56	46.96	4.41	74.33	75.06	-2.65	79.10	61.27	-68.89	-7.95	-10.04	5.49	74.73	78.95	7.46	49.59	0.22	0.95
13	52.63	45.58	3.77	69.93	71.19	-5.58	84.94	100.00	-41.70	-3.78	-0.87	9.41	66.98	71.96	2.03	34.09	1.14	1.37
14	53.92	47.03	6.81	70.05	70.04	-0.41	79.51	86.28	-23.24	-2.38	-6.90	7.17	65.75	71.45	8.17	31.59	3.77	0.25
15	47.20	41.33	3.42	67.86	68.96	-2.50	74.96	70.30	-66.36	-0.05	-5.21	4.11	70.77	71.56	3.09	37.08	1.79	2.79
16	57.61	50.68	4.29	76.99	76.77	-1.44	91.81	100.00	-45.55	-4.05	-6.94	4.35	62.50	70.22	0.53	30.14	1.37	1.23
17	49.61	43.68	4.30	70.41	71.35	-0.32	86.10	100.00	-42.31	-3.86	-5.99	1.73	66.98	68.93	1.04	29.73	2.02	1.87
18	54.43	48.45	3.29	72.99	73.77	-0.28	89.81	100.00	-47.83	-2.02	-5.22	0.29	58.93	64.37	1.28	31.29	0.27	0.35
19	53.83	48.37	5.22	72.19	72.72	-2.10	90.27	100.00	-46.83	-3.53	-5.32	1.07	60.45	65.88	1.56	32.64	0.42	2.82
20	51.86	46.29	2.40	70.39	69.74	-0.28	87.44	100.00	-39.76	-5.84	-3.28	0.27	58.83	63.24	2.30	28.38	0.77	0.37
21	54.87	52.30	2.93	70.32	71.55	-3.05	90.16	100.00	-40.64	-5.30	-3.69	0.53	60.27	68.01	3.201	29.57	3.66	2.33
22	50.84	48.38	5.37	68.39	68.94	-1.93	86.48	100.00	-52.06	-0.53	-3.28	0.66	56.66	61.34	6.92	34.83	2.84	0.38

Nine phenolic compounds were further quantified by comparison with standard substances (Table 2). The most abundant compound was artepillin C, chlorogenic acid, and its derivative, kaempferide and kaempferol.

**Determination of antioxidant polyphenolic compounds.** To identify the potential antioxidant compounds, multivariate correlation analysis was performed between peak area of the 18 characteristic peaks and IC<sub>50</sub> values (Table 3). Results showed that chlorogenic acid, caffeic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, 3,4,5-tricaffeoylquinic acid,

kaempferol, kaempferide, and artepillin C presented a significant relation with the radical-scavenging activity, which suggested that these 8 compounds contributed much to the antioxidant activity.

To validate the anti-oxidative activities of different compounds, the off-line DPPH assays were also performed. As expected, after giving sufficient reaction time, the peak areas of chlorogenic acid, caffeic acid, kaempferol, 3,4,5-tricaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, betulet, kaempferide, and artepillin C were significantly decreased after being reacted with DPPH (Figure 2). The decreasing trend of



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