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Free radical scavenging activities and bioactive substances of Jerusalem artichoke (*Helianthus tuberosus* L.) leaves

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ABSTRACT

The total phenolic content and radical scavenging activities of Jerusalem artichoke (*Helianthus tuberosus* L.) leaves were investigated. Results indicated that the ethyl acetate fraction contained the highest total phenolic content (266.69 ± 2.51 mg GAE/g dry extract) accompanied with strongest free radical scavenging abilities. Following an *in vitro* radical scavenging activity-guide fractionation procedure, six phenolic compounds which strongly quenched free radicals were separated from ethyl acetate fraction. Among them, 3-*O*-caffeoylquinic acid and 1,5-dicaffeoylquinic acid played a dominant role due to their strong free radical scavenging abilities and their high contents. The content of 3-*O*-caffeoylquinic acid in *n*-butanol fraction was 74.58 ± 1.05 mg/g, while 1,5-dicaffeoylquinic acid in ethyl acetate fraction was 104.51 ± 2.86 mg/g. The results imply that the leaves of Jerusalem artichoke might be a potential source of natural antioxidants.

1. Introduction

Oxygen-centred free radicals and other reactive oxygen species (ROS) can be generated as by-products during oxidative progresses of living organisms (Halliwell & Gutteridge, 1999). Many human diseases, including accelerated ageing, cancer, cardiovascular disease, neurodegenerative disease and inflammation, are linked to excessive amounts of free radicals (Moskovitz, Yim, & Chock, 2002). The antioxidants are necessary to cure these diseases. However, the synthetic antioxidants might be unsafe (Wanasundara & Shahidi, 1994; Wettasinghe & Shahidi, 1999). Therefore, more attentions are drawn to searching for natural antioxidants from medicinal and dietary plants to prevent oxidative damage.

The Jerusalem artichoke (JA, *Helianthus tuberosus* L.) in *Helianthus* genus and *Asteraceae* family is native to eastern North America and also cultivated widely across the temperate zone. With various pharmacological activities, such as aperient, cholagogue, diuretic, stomachic and tonic effects, its tuber is often used as food and folk medicine for the treatment of diabetes and rheumatism (Pan et al., 2009; Talipova, 2001). Besides, the extracts of the aerial part of this plant were also found to possess antimicrobial, antifungal and anticancer activities (Ahmed, El-Sakhawy, Soliman, & Abou-Hussein, 2005; Pan et al., 2009). Moreover, the JA leaf is a natural remedy for the treatment of bone fracture, skin wound, swelling and pain (Baba, Yaoita, & Kikuchi, 2005; Health Department and National Chinese Medicine Management Office, 1998). A previous study demonstrates that the leaves of JA contained a high amount of phenolic compounds (Yuan et al., 2008). According to chemical structures of these phenolic compounds, JA leaves should show good antioxidant properties. To the best of our knowledge, antioxidant activities of this plant have not previously been reported.

To evaluate the antioxidant activity and the corresponding constituents in the leaves of JA, the total phenolic contents of different fractions/extract of JA and their *in vitro* radical scavenging abilities were investigated in this study. Furthermore, radical scavenging abilities-guided fractionation was carried out to investigate the relationships between phenolic compounds and antioxidant activities.

2. Materials and methods

2.1. Chemicals and materials

Gallic acid and 3-*O*-caffeoylquinic acid were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 1,1′-diphenyl-2-picrylhydrazyl (DPPH) and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other analytical grade chemicals were obtained from Kemio Chemical Co. (Tianjin, China). The leaves of JA were collected from Yulin District (Shannxi, China) in October of 2008 and identified by Prof.

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Zhaopu Liu from the College of Resource and Environmental Science, Nanjing Agricultural University. The leaves were air-dried at room temperature.

2.2. Extraction and isolation

One kilogram of the dried leaves was powdered using a herb grinder (Joyoung, Shandong, China) and then refluxed twice with 20 l of 60% ethanol for 2 h. The extracts were concentrated under reduced pressure at 50 °C and then completely dried using a freeze drier. A total of 249 g crude extract (CE) was obtained. The CE (200 g) was diluted in 1.0 l water and successively extracted with petroleum ether (PE), ethyl acetate (EA) and *n*-butanol (NB). After removing the solvents, four fractions were obtained. The yields of PE, EA, NB and water fraction were 6.42, 53.11, 41.10 and 105.68 g, respectively. The EA fraction was subjected to further chromatographic separation on a 60–100 mesh polyamide resin column, eluting with a series of ethanol/water from 30:70 (v/v) to 100:0 (v/v) to afford six subfractions (EA_1–EA_6). Subfractions EA_3 and EA_4 were combined and further separated by preparative HPLC to give six compounds. The structures of compounds 1–6 were determined by mass spectrometry and NMR.

2.3. Measurement of total phenolic content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu reagent with gallic acid as a standard (Naczk & Shahidi, 1989). Basically, 0.5 ml test sample was mixed with 0.5 ml Folin–Ciocalteu reagent and 1.5 ml 10% sodium carbonate solution. After the mixture was reacted for 10 min at 75 °C, the absorbance was read at 760 nm, the absorbance at 760 nm was recorded. Results were expressed in milligram gallic acid equivalents per gram of dried sample.

2.4. DPPH radical scavenging ability assay

The DPPH radical scavenging ability was determined according to the method described by Amarowicz, Karamać, Weidner, Abe, and Shahidi (2002). An aliquot of 2.0 ml of 0.1 mM DPPH radical solution was mixed with 1.0 ml of test samples at various concentrations. Absorbance at 517 nm was measured after 10 min. BHT was employed as positive control. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{Scavenging ability } (\%) = [(A_0 - A_s)/A_0] \times 100.$$

where A_0 is the absorbance of the control, A_s is the absorbance of the tested sample.

2.5. $ABTS^+$ radical scavenging ability assay

The ABTS radical ($ABTS^+$) scavenging ability was measured according to the method described by Re et al. (1999). $ABTS^+$ was produced by reacting 0.0768 g ABTS salt with 0.0132 g potassium persulphate in 20 ml deionized water, stock solution was kept in dark for 14–16 h at room temperature. Prior to use, $ABTS^+$ stock solution was diluted with PBS (4 mM, pH = 7.4) to obtain the absorbency of 0.700 ± 0.004 measured at 734 nm. After 0.1 ml sample was mixed with 1.9 ml $ABTS^+$ solution and kept for 5 min at room temperature, the absorbance of reaction mixtures were monitored at 734 nm. BHT was used as positive control. The $ABTS^+$ radical scavenging ability was calculated according to the same equation as that in the DPPH assay.

2.6. Hydroxyl radical scavenging ability assay

The hydroxyl radical scavenging effect was evaluated based on the Fenton reaction described by Yu, Zhao, and Shu (2004). Firstly, 0.5 ml sample was mixed with 0.5 ml ferrous chloride (3 mM), 0.5 ml 1,10-phenanthroline (3 mM), 2 ml phosphate buffer (2.5 mM, pH = 7.4) to prepare the mixture. Then 0.5 ml of 0.1% hydrogen peroxide was added to the mixture to initiate the reaction. After 30 min incubation at 37 °C, the absorbance of the mixture was measured at 560 nm. BHT was used as positive control. Hydroxyl radical scavenging ability was expressed by the following equation:

$$\text{Scavenging ability } (\%) = [(A_s - A_1)/(A_0 - A_1)] \times 100\%.$$

where A_0 is the absorbance of control without test sample and H_2O_2, A_1 is the absorbance of control without test sample and A_s is the absorbance of the test sample.

2.7. HPLC analysis of phenolic compounds

HPLC analysis of phenolic compounds in the crude extract and fractions was performed on a Waters Alliance 2690 apparatus (Milford, MA, USA) using a Capcell Pak C_{18} column (250 mm × 4.6 mm, 5 μm, Shiseido Co. Ltd., Tokyo, Japan). The mobile phases consisted of methanol containing 0.5% acetic acid (A) and 0.5% acetic acid aqueous solution (B). Gradient elution was started with 30% of A and ascended to 50% of A in 45 min. The flow rate was kept at 0.8 ml/min while the column temperature was at 30 °C. Samples were filtered through a 0.22 μm filter prior to HPLC injection. The injection volume was 10 μl. The online UV spectra were recorded at 327 nm.

2.8. Statistical analysis

Data were reported as mean ± SD from triplicate determinations. Statistical analysis was performed with Student's *t*-test. A difference was considered statistically significant, when $P < 0.05$.

3. Results and discussion

3.1. Radical scavenging activities

3.1.1. DPPH radical scavenging ability

The DPPH radical assay is a suitable model for estimating radical scavenging activities of antioxidants (Sánchez-Moreno, 2002). Fig. 1A shows the dose–response curves of the DPPH radical scavenging activities of crude extract and fractions of the JA leaves. The SC_{50} values (the antioxidant concentrations corresponding to 50% radical scavenging efficiencies) of CE, PE fraction, EA fraction, NB fraction, water fraction and BHT were 56.85 ± 2.83, 304.74 ± 22.33, 11.01 ± 1.43, 36.21 ± 1.41, 86.11 ± 1.17 and 73.06 ± 1.57 μg/ml, respectively. The results indicate that the DPPH radical scavenging ability of the EA fraction was nearly sevenfold better than that of BHT. Scavenging abilities on DPPH radical show the following order: EA fraction > NB fraction > CE > BHT > water fraction > PE fraction ($P < 0.05$).

3.1.2. $ABTS^+$ radical scavenging ability

$ABTS^+$ is another widely used synthetic radical for both the polar and non-polar samples (Re et al., 1999). The $ABTS^+$ scavenging abilities of the crude extract and fractions of JA leaves are plotted in Fig. 1B. It can be seen that the EA fraction show the strongest $ABTS^+$ scavenging ability, even at the relatively low concentrations from 1.8 to 12 μg/ml ($P < 0.05$). The SC_{50} values of CE, PE fraction, EA fraction, NB fraction, water fraction and BHT were 11.78 ± 0.71,

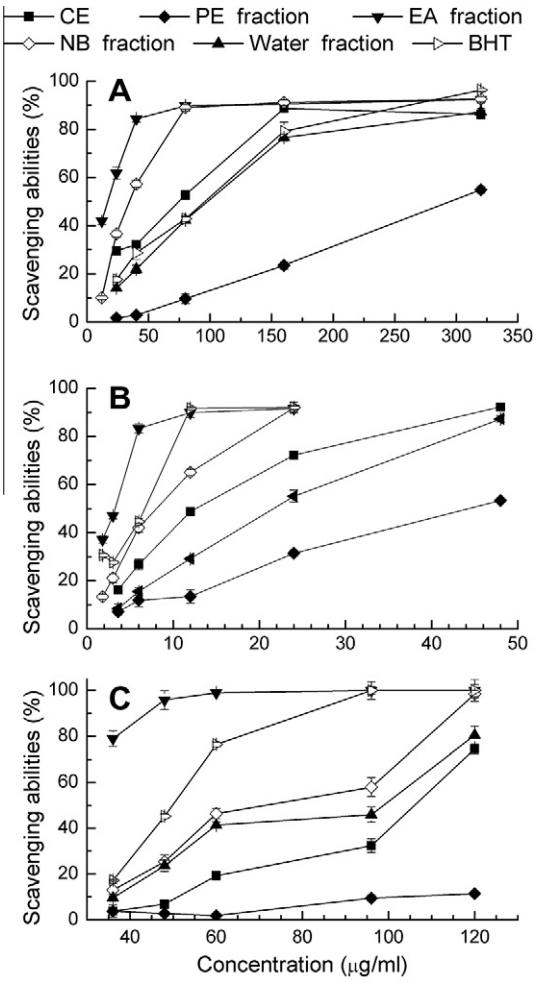


Fig. 1. Radical scavenging activities of BHT, different fractions and crude extract of the leaves of Jerusalem artichoke. (A) DPPH radical scavenging assay; (B) $ABTS^{+}$ radical scavenging assay; (C) hydroxyl radical scavenging assay. Values are mean ± SD ($n = 3$) of three determinations.

50.33 ± 2.44, 2.54 ± 0.09, 6.58 ± 0.18, 16.47 ± 0.26 and 4.48 ± 0.15 μg/ml, respectively. According to the SC_{50} values, the order of the $ABTS^{+}$ scavenging abilities is: EA fraction > BHT > NB fraction > CE > water fraction > PE fraction ($P < 0.05$).

3.1.3. Hydroxyl radical scavenging ability

Hydroxyl radicals are highly reactive and short-lived to damage virtually adjacent biomolecules (Sakanaka, Tachibana, & Okada, 2005). Its radical scavenging abilities of extract/fractions of JA leaves were investigated and shown in Fig. 1C. Similar to DPPH and $ABTS^{+}$ assays, the hydroxyl radical scavenging abilities increased with the concentration increase of the test samples. The SC_{50} values of CE, PE fraction, EA fraction, NB fraction, water fraction and BHT are 100.09 ± 4.28, 1025.33 ± 90.31, 23.98 ± 1.97, 63.36 ± 1.83, 78.27 ± 4.58 and 45.58 ± 2.14 μg/ml, respectively. According to these SC_{50} values, the order of hydroxyl radicals scavenging abilities is: EA fraction > BHT > NB fraction > water fraction > CE > PE fraction ($P < 0.05$).

3.2. Total phenolic content (TPC)

The antioxidant activities of plant extracts are closely related to their total phenolic contents (Rathee, Hassarajani, & Chattopadhyay, 2007). Therefore, it is important to evaluate the total phenolic in the crude extract and fractions of the JA leaves. The calibration curve for gallic acid was expressed by the equation $Y = 0.139X + 0.0354$ ($r = 0.9988$) with the concentrations of gallic acid ranging from 0.008 to 0.048 mg/ml. According to this calibration equation, the TPC of extract/fractions of JA leaves were calculated as shown in Table 1. The highest level of TPC was found in the EA fraction, with an amount of 266.69 ± 2.51 mg/g, followed by NB fraction, CE, water fraction and PE fraction ($P < 0.05$). This tendency of TPC follows their corresponding radial scavenging abilities.

Meanwhile, the tuber of JA was extracted using the same method with the leaves. The yields of the tuber and leaf extracts were 24.4% and 24.9% (w/w), respectively. A significant difference ($P < 0.05$) was observed in the total phenolic content of the JA tuber and leaf extracts, i.e., 22.40 ± 0.63 and 101.07 ± 1.61 mg GAE/g of dry extract, respectively. Amount of TPC in the JA leaves was found to be about fourfold higher than that in the tubers and the yields of TPC of the JA leaf and tuber were 2.52% and 0.55%, respectively. As a rule of thumb, the JA leaves should possess higher free radical scavenging activities than its tubers. This result was consistent with the pervious study, in which the blueberry leaves were more active than the fruits (Naczk, Amarowicz, Zadernowski, Pegg, & Shahidi, 2003).

3.3. Separation and identification of phenolic compounds

According to the aforesaid results, the EA fraction contained the most amount of phenolic compounds and exhibited the strongest radical scavenging activities. Therefore the EA fraction was further separated to afford the antioxidant compounds. With the aid of preparative HPLC described in Section 2.2, six compounds were isolated from the EA fraction. Among them, compound **1** was identified as 3-*O*-caffeoylquinic acid (3-CQA) by comparison of UV and retention time with reference compound; while compounds **2**–**6** were identified as caffeic acid (**2**), 3,5-dicaffeoylquinic acid (3,5-DiCQA, **3**), 1,5-dicaffeoylquinic acid (1,5-DiCQA, **4**), 4,5-dicaffeoylquinic acid (4,5-DiCQA, **5**) and 3,5-dicaffeoylquinic acid methyl ether (3,5-DiCQAME, **6**), respectively, by comparing their UV, MS, ^{1}H and ^{13}C NMR spectral data with the data reported in previous studies (Lee, Kim, Kim, Lee, & Kang, 2010; Peng, Mei, Jiang, Zhou, & Sun, 2000; Tolonen, Joutsamo, Mattlla, Kämäräinen, & Jalonen, 2002). Their structures are shown in Fig. 2. The sesquiterpene lactones from the aerial parts of JA and phenols from the tubers of JA have been reported during the last 50 years (Spring, 1991; Tchoné, Bärwald, Annemüller, & Fleischer, 2006). However, there has been no report about the phenolic compounds from the leaves of JA. In this study, the phenolic compounds from the leaves of JA are separated, among which compounds **3**–**6** were isolated from this plant for the first time.

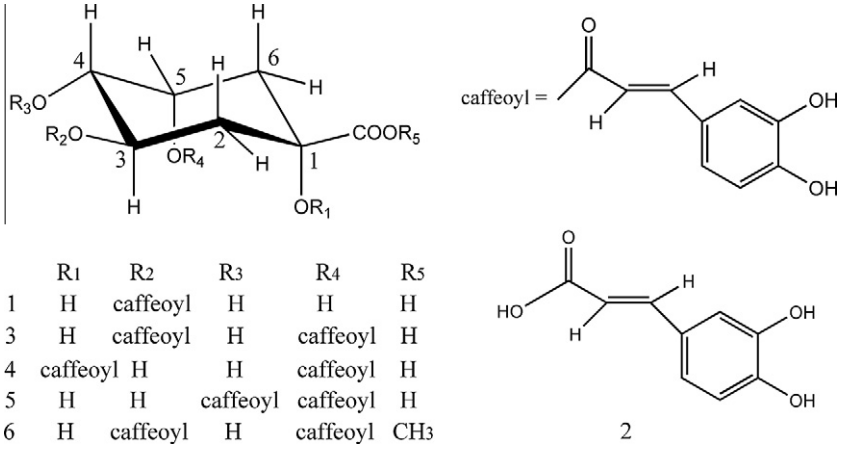
3.4. HPLC analysis and structure–activity relationship of phenolic compounds

In order to understand the composition of antioxidants in the JA leaves, the crude extract and various fractions of JA leaves were analysed by HPLC. Excellent separation was achieved under the optimized conditions described in Section 2.7 and the HPLC chromatogram of the crude extract is shown in Fig. 3. With the aid of standards of six phenolic compounds separated above, the predominant compounds **1**–**6** in Fig. 3 were identified and quantified. From their contents in each fraction (Table 1), it can be seen that 3-CQA and 1,5-DiCQA play a dominant roles as far as their contents are concerned in the crude extract. All these 6 compounds were enriched in EA and NB fraction but not in PE fraction. The contents of compounds **2**–**5** reached their highest in EA fraction. Among them, the content of 1,5-DiCQA attained the highest at 104.51 ± 2.86 mg/g; while the content of 3-*O*-caffeoylquinic acid arrives at the highest of 74.58 ± 1.05 mg/g in NB fraction. For the mentioned phenolic

Table 1
Contents of total phenolics and phenolic compounds of Jerusalem artichoke leaf extract and fractions (mg/g dried sample).[a]

Sample[b]	Total phenolics[c]	3-CQA	Caffeic acid	3,5-DiCQA	1,5-DiCQA	4,5-DiCQA	3,5-DiCQAME
CE	101.07 ± 0.63	30.42 ± 0.37	2.12 ± 0.09	1.47 ± 0.15	8.27 ± 0.54	2.17 ± 0.21	0.53 ± 0.05
PE fraction	31.89 ± 0.42	2.11 ± 0.09	1.56 ± 0.03	0.42 ± 0.03	1.59 ± 0.09	0.41 ± 0.09	ND[c]
EA fraction	266.69 ± 2.51	19.84 ± 0.61	13.83 ± 0.44	27.18 ± 1.59	104.51 ± 2.86	19.29 ± 0.44	20.67 ± 0.83
NB fraction	135.72 ± 1.07	74.58 ± 1.05	3.36 ± 0.33	2.21 ± 0.19	15.87 ± 1.09	3.41 ± 0.21	0.81 ± 0.12
Water fraction	52.45 ± 1.44	20.64 ± 0.76	ND	ND	7.94 ± 0.39	ND	ND

[a] Values are expressed as mean ± SD of triplicate measurements, ND means not detected.
[b] CE, crude extract; PE, petroleum ether; EA, ethyl acetate; NB, *n*-butanol.
[c] As gallic acid equivalents.



	R1	R2	R3	R4	R5
1	H	caffeoyl	H	H	H
3	H	caffeoyl	H	caffeoyl	H
4	caffeoyl	H	H	caffeoyl	H
5	H	H	caffeoyl	caffeoyl	H
6	H	caffeoyl	H	caffeoyl	CH_3

Fig. 2. Structures of six phenolic compounds isolated from the leaves of Jerusalem artichoke: 3-*O*-caffeoyquinic acid (**1**), caffeic acid (**2**), 3,5-dicaffeoyquinic acid (**3**), 1,5-dicaffeoylquinic acid (**4**), 4,5-dicaffeoyquinic acid (**5**) and 3,5-dicaffeoyquinic acid methyl ether (**6**).

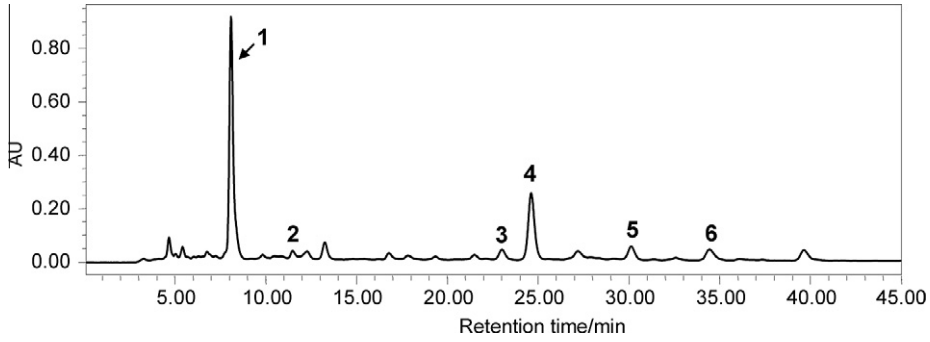


Fig. 3. HPLC chromatography of crude extract of the leaves of Jerusalem artichoke: 3-*O*-caffeoyquinic acid (**1**), caffeic acid (**2**), 3,5-dicaffeoyquinic acid (**3**), 1,5-dicaffeoylquinic acid (**4**), 4,5-dicaffeoyquinic acid (**5**) and 3,5-dicaffeoyquinic acid methyl ether (**6**).

compounds, dicaffeoylquinic acids have received more attention in recent years. Besides their known antioxidant and radical scavenging activity, the effect of reducing inflammation, as an antispasmodic and an inhibitor of the reproduction of the human immunodeficiency virus were reported (Stange, Midland, Holmes, Sims, & Mayer, 2001). The presence of such high amount of dicaffeoylquinic acids in this plant suggests that JA is a crop with potential health benefits. Thus, our study may help attract interest in future utilization of the leaves of JA for therapeutic purposes.

The radical scavenging activities of six dominant phenolic compounds were also evaluated and their SC_{50} values are summarized in Table 2. Based upon the estimated SC_{50} values, the DPPH radical scavenging abilities of the phenols decreased in the order of **3** = **6** > **4** = **5** > **1** > **2** > BHT ($P < 0.05$), with SC_{50} values ranging from 5.02 ± 0.11 to 243.53 ± 5.24 μM. Their $ABTS^{+}$ scavenging abilities follow the order of **3** > **6** > **5** > **4** > **1** > **2** > BHT ($P < 0.05$). The activities of compounds **3** and **6** (SC_{50} = 2.79 ± 0.04 and 3.19 ± 0.02 μg/ml) were higher than the others. The hydroxyl radical scavenging abilities can be ranked as **3** > **5** = **4** > **6** > **1** = **2** > BHT ($P < 0.05$); compound **3** being the most active with the lowest SC_{50} value of 11.70 ± 0.14 μg/ml. According to the obtained SC_{50} values, the

Table 2
Radical scavenging activities of the isolated phenolic compounds from the leaves of Jerusalem artichoke.

Compounds	SC_{50} (μM)		
	DPPH	ABTS	OH
1	11.97 ± 0.11	6.27 ± 0.85	57.85 ± 0.82
2	12.72 ± 0.11	8.94 ± 0.39	59.66 ± 3.49
3	5.08 ± 0.21	2.79 ± 0.04	22.67 ± 0.27
4	6.34 ± 0.23	4.21 ± 0.15	27.96 ± 1.03
5	6.67 ± 0.06	3.56 ± 0.08	25.34 ± 1.32
6	5.02 ± 0.11	3.19 ± 0.02	33.09 ± 1.06
BHT	243.53 ± 5.24	14.96 ± 0.51	151.94 ± 7.15

structure–activity relationships of phenolic compounds were also investigated. The radical scavenging abilities of compounds **3**–**6** were nearly twice that of compounds **1**–**2**, which could be ascribed to the presence of one more caffeoyl group. The caffeoyl group is the key active site exhibiting antiradical activities in the separated phenolic compounds. In addition, the radical scavenging activities of tested phenolic compounds are slightly affected by the position of the caffeoyl group on quinic acid. In the three adopted radical scavenging assays, isomers **3**, **4**, **5** exhibited similar radical scavenging activities, but compound **3** (3,5-dicaffeoyquinic acid) showed a little higher activity than the other two. Overall, all the tested phenolic compounds showed stronger radical scavenging activities than the crude extract and different fractions in all the tested radical scavenging assays. Therefore, the separated phenolic compounds are clearly responsible for the radical scavenging activities of the JA leaves.

4. Conclusions

This study is the first report on the radical scavenging activities and phenolic compositions of the leaves of JA. The results indicated that JA leaf extract/fractions exhibit excellent radical scavenging ability in all assays employed. Among them, the EA fraction was the most active. Phytochemical investigation of the EA fraction led to the separation of six phenolic compounds. HPLC analysis indicated that a higher concentration of 3-*O*-caffeoylquinic acid and 1,5-dicaffeoyqunic acid was present in the leaves of JA and were the major factors responsible for the radical scavenging activities. Overall, the leave of *H. tuberosus* L. is a promising source of natural antioxidants.

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