In comparison with vitamin C and butylated hydroxytoluene, the antioxidant capacity of aqueous extracts from buds and flowers of Lonicera japonica Thunb.

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Abstract

OBJECTIVE: To assess the antioxidant capacity of aqueous extracts of buds and flowers of Lonicera japonica Thunb. (BLJ and FLJ) using in vitro assays.

METHODS: We assessed the in vitro antioxidant activities of aqueous extracts of BLJ and FLJ and compared with that of classical antioxidants vitamin C and butylated hydroxytoluene, using several well-established methods including the 1,1-diphenyl-2-picrylhydrazyl assay, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) assay, reducing power assay, fluorescence recovery after photo-bleaching assay, β-carotene bleaching assay, ferric thiocyanate assay, and thiobarbituric acid method.

RESULTS: The aqueous extracts of both BLJ and FLJ had similarly potent antioxidant capacity. There were no significance differences between BLJ and FLJ in all the assays.

CONCLUSION: The aqueous extracts of both BLJ and FLJ have antioxidant activity with comparable efficacy. These findings suggest that both BLJ and FLJ may have the potential as natural antioxidants.

INTRODUCTION

Many medicinal plants have antioxidant properties. These plants have been traditionally used in folk medicine as natural remedies for their preventive and/or therapeutic effects in cardiovascular diseases, inflammatory disorders, and cancer. These plants have also been utilized as food additives based on the preservative effects of their antioxidant and antimicrobial properties. Oxidation provides the energy for the human body for numerous activities such as exercising, breathing, digestion, metabolism, and blood circulation. In fact, a certain degree of oxidation can provide the necessary energy to maintain health and the production and elimination of free radicals within the bodies should be maintained at an equilibrium because excessive oxidation may be hazardous.

Free radicals are unstable molecules that can damage the cells of the human body. The oxidative stress is the result of an imbalance between the production of free radicals and the body’s antioxidant defense system. The excessive free radicals in the human body could increase lipid peroxides, which are believed to contribute...
to the pathogenesis of a variety of degenerative diseases. Antioxidants can regulate various oxidative reactions that naturally occur in tissues to help maintain redox balance. Lonicera japonica Thunb., also known as Jinyinhuah or Rendong, is commonly used in the Traditional Chinese Medicine (TCM). Buds of Lonicera japonica Thunb. have been used for the treatment of exopathogenic wind-heat, epidemic febrile diseases, sores, carbuncles, furuncles, and infectious diseases. It is also used as additives in food, cosmetics, and soft beverages for its specific activities.

In the Pharmacopoeia of the People’s Republic of China, Jinyinhuah is recorded as a caprifoliaceae honeysuckle, Lonicera japonica Thunb.. While the dried buds of Lonicera japonica Thunb. is the form of Lonicera japonica Thunb. commonly used in the TCM, the opened flowers of Lonicera japonica Thunb. (FLJ) have not been tested for their efficacy in the treatment of specific conditions and its pharmacological effects are unclear.

In the present study, we comprehensively evaluated the antioxidant activity of the aqueous extracts of BLJ and FLJ using several well-established in vitro assays.

MATERIALS AND METHODS

Drugs and reagents
Reference standards cyanoside and chlorogenic acid (both with purity > 98%) were purchased from Chengdu MUST Bio-Technology Co., Ltd. (Sichuan, China). HPLC grade methanol and acetonitrile were from Honeywell Burdick & Jackson (Morristown, NJ, USA). Phosphoric acid was from Dikma Technologies Inc. (Beijing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Vitamin C was from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Linoleic acid was from Alfa Aesar (Ward Hill, MA). 2, 2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) di-ammonium salt (ABTS), 2-thiobarbituric acid (TBA), trichloroacetic acid, potassium persulfate, 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ), and β-carotene were from Sigma. Butylated hydroxytoluene (BHT) was from the National Medicine Group Chemical Reagent Co., Ltd. (Beijing, China). All other chemicals used in this study were of analytical grade.

Plant materials and sample pretreatment
BLJ and FLJ were obtained from Fengqiu country, Henan province, China and they were authenticated by Professor Lidong Zhou at Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College. A voucher specimen (No. 20160001) was deposited in the National Compound Bank of Traditional Chinese Academy of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing.

The BLJ (10 g) and FLJ (10 g) were extracted three times in boiling water for 30 min respectively. The filtrate was first evaporated via a rotary evaporator to a volume of about 500 mL, and then 500 mL of absolute ethanol was added. The resulting solution was filtrated and then evaporated to a volume of about 30 mL prior to lyophilization.

Preparation of standards and samples for ultra performance liquid chromatography (UPLC) analysis
The standards were dissolved in 50% methanol to make the final standard solution of cyanoside (4.75 µg/mL) and chlorogenic acid (55 µg/mL). Corresponding to 100 mg materials, the extracts of FLJ (46.0 mg) and BLJ (31.4 mg) were dissolved in 50% methanol in an ultrasonic bath at room temperature for 30 min. The solutions of BLJ and FLJ were filtered through 0.22 µm membrane. A 3 µL aliquot of the filtrate and standard solution were injected into the UPLC system for analysis.

UPLC analysis
Waters Acquity UPLC™ system (Waters, MA, USA) was equipped with a PDA detector and an auto sampler. An ACQUITY UPLC HSS T3 column (2.1 × 150 mm, 1.8 µm) was used for the separation at 30 °C. The mobile phase consisted of A (water containing 0.4% phosphoric acid, v/v) and B (acetonitrile). The flow rate was 0.20 mL/min and the detection wavelength was 350 nm. The conditions of the linear gradient elution were as follows: 0-2 min, 5%-16% B; 2-4 min, 16%-20% B; 4-8 min, 20%-50% B; and 8-10 min, 50%-100% B.

DPPH radical scavenging activity assay
The DPPH radical scavenging effect was analyzed as described elsewhere. Equal volumes (1 mL) of the extract solution (in 65% ethanol) at various concentrations and 0.2 mM DPPH (in 65% ethanol) were mixed. After incubation for 1 h at room temperature in the dark, the absorbance value was measured at wavelength 517 nm using a microplate reader spectrophotometer (Bio Tek, VT, USA). The concentrations of BHT and VC (as positive controls) were identical to those of the experimental samples. Radical scavenging activity was determined using the following equation: [(As-Ai)/As] × 100% (As = Absorbance of DPPH alone, Ai = Absorbance of DPPH in the extract solution at various concentrations).

ABTS radical scavenging activity assay
The ABTS radical scavenging activity of the BLJ and FLJ extracts were determined as described elsewhere. A solution of ABTS radical cation was prepared by reacting 7 mM ABTS with 2.45 mM potassium persulfate (K_{2}S_{2}O_{8}) at room temperature in the dark for 16 h.

The mixture was then diluted with deionized water to
obtain an absorbance of 0.700 ± 0.005 at a wavelength of 734 nm. The sample solution (100 μL) at a range of concentrations was mixed with the ABTS− solution (100 μL) in 96-well plates. After incubation at 30 °C for 30 min in the dark, the absorbance at a wavelength of 734 nm was immediately recorded. BHT and VC were used as controls. The activity of ABTS− radical scavenging was calculated using the same method as that for DPPH.

**Reducing power assay**

The reducing power of the extracts was examined using the method described by Oyaizu. The BLJ and FLJ extracts were diluted to a range of concentrations in 65% ethanol. Equal volumes (1 mL) of the extract solution phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide [K₂Fe(CN)₆] were added to a 10 mL centrifuge tube and mixed. The mixture was then kept in a 50 °C in a water bath for 20 min before 1 mL of 10% trichloroacetic acid was added to stop the reaction. The mixture made as such was centrifuged at 3000 rpm for 10 min, after which 0.5 mL of the supernatant was collected and mixed with 50 mL of deionized water and 0.1 mL of 0.1% ferric chloride (FeCl₃). The absorbance was measured at wavelength 700 nm after 5 min at room temperature. BHT was used as reference.

**Ferric ion reducing antioxidant power (FRAP) assay**

The total antioxidant capacity of the extracts was determined using a method that measures ferric reducing ability of plasma. Thirty-milliliter of acetate buffer (0.1 M, pH 3.6) was mixed with 3 mL of TPTZ (10 mM, in 40 mM HCl) and 3 mL of FeCl₃·6H₂O (20 mM) as the FRAP working solution. Then, 30 μL of the extract solution at various concentrations and 90 μL of deionized water were added to 900 μL of the FRAP working solution. The absorbance was recorded at a wavelength of 593 nm after incubating at 37 °C for 10 min. BHT was used as reference antioxidant.

**β-Carotene bleaching assay**

The β-carotene bleaching activity of the BLJ and FLJ extracts were evaluated using the method of Shon et al. A β-carotene solution was prepared by dissolving 3 mg of β-carotene in 10 mL of chloroform. Then, 4 mL of the β-carotene solution, 80 mg of purified linoleic acid, and 800 mg of Tween 80 were then pipetted into a 500 mL round-bottom flask. After removing chloroform by vacuum, 200 mL of distilled water was added to the flask with vigorous shaking. Three milliliters of the above emulsion and 0.2 mL of BLJ and FLJ extracts (400 μg/mL) were then mixed in different test tubes and incubated in a 50 °C water bath. The absorbance of the mixtures was then measured at 30 min intervals for 2 h at a wavelength of 470 nm. BHT was used as reference antioxidant. Lipid peroxidation (LPO) inhibition was calculated as follows:

\[ \frac{(As-Ai)}{As} \times 100\% \]

**Antioxidant activity assay in a linoleic acid system using ferric thiocyanate (FTC)**

The FTC assay was adapted from Osawa and Namiki, with minor modifications. The BLJ and FLJ extracts dissolved in 2 mL of 65% ethanol were mixed with 2 mL of 2.5% linoleic acid in absolute ethanol, 4 mL of phosphate buffer (0.05 M, pH 7.0) and 2 mL distilled water, and kept in the dark at 40 °C. 50 μL aliquot of this solution and 50 μL of FeCl₃ (0.02 M, in 3.5% HCl) were added to 4.85 mL of 75% ethanol. After 3 min, the absorbance was determined at a wavelength of 500 nm. Controls without extracts and standards containing BHT and VC in place of extracts were subjected to the same procedure. The absorbance was measured at 24 h intervals until the absorbance of the control reached a constant maximum value.

**TBA assay**

The TBA content of the BLJ and FLJ extracts was assayed using the method of Kikuzaki and Nakatani. One milliliter of the sample solution prepared for the FTC assay was taken and mixed with 2 mL of 20% trichloroacetic acid and 2 mL of 0.67% TBA. The mixture was incubated in a boiling water bath for 10 min. Upon cooling, the mixture was centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured at a wavelength of 532 nm. The inhibition rate was determined using the following equation:

\[ \frac{(Ac-Ao)}{Ac} \times 100\% \]

**Statistical analysis**

Data analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). All experiments were done in triplicate and results are reported as mean ± standard deviation (±). Differences between groups were examined using a one-way analysis of variance and least significant difference. P < 0.05 was considered to be significant.

**RESULTS**

**Comparison of chemical components of the BLJ and FLJ extracts**

The peaks of the BLJ and FLJ extracts were very similar, and the results of quantitative analysis showed that the two extracts contained comparable levels of cynaroside (0.017% in FLJ and 0.016% in BLJ) and chlorogenic acid (0.806% in FLJ and 0.903% in BLJ) (Figure 1).

**DPPH and ABTS free radical scavenging activity**

The radical scavenging activities of DPPH and ABTS in various concentrations of BLJ and FLJ are presented in Figure 2. In both DPPH and ABTS scavenging as-
says, VC and BHT showed the highest scavenging activity. In the DPPH assay, the scavenging ability of both BLJ and FLJ increased with higher concentrations at almost the same rates (A). The scavenging ability of the BLJ and FLJ extracts in the ABTS assay slightly differed from that of the DPPH assay. A positive correlation between the concentration and scavenging ability of the BLJ and FLJ extracts was observed (B).

**Reducing power and FRAP assay**

The reducing power of BLJ and FLJ extracts increased in a dose-dependent manner; for comparison on the basis of equal concentrations, the BLJ and FLJ extracts had about one-fifth to one-fourth of the potency observed for BHT (Figure 3A). The aqueous extracts of both BLJ and FLJ also dose-dependently increased the total antioxidant capacity manner (Figure 3B).

**β-Carotene bleaching assay**

This method is based on the reaction of β-carotene with free radicals that result from hydroperoxides from linoleic acid. The rate of discoloration of β-carotene increased in the absence of antioxidants. Figure 4 shows that the antioxidant capacities of FLJ and BLJ extracts were 21% ± 4% and 27% ± 3%, respectively, relative to 98% ± 2% for the positive control BHT. No significant differences in antioxidant capacity were found between the FLJ and BLJ extracts.

**Lipid peroxidation inhibition activity**

The present study conducted two different assays to measure lipid peroxidation. In the FTC assay, the oxidation of linoleic acid generates peroxides that react with Fe³⁺ to form Fe⁵⁺. Fe⁵⁺ ions form a thiocyanate complex with SCN⁻, and the complex shows maximum absorbance at a wavelength of 500 nm. The absorbance of the BLJ and FLJ extracts was higher than that of BHT but lower than that of VC.

In the TBA assay, TBA reacts with MDA to form a red complex that can be determined spectrophotometrically at wavelength 532 nm. Similar to the results of the FTC assay, percentinhibitions of lipid peroxidation by TBA assay with VC, BHT, BLJ, and FLJ were 13.6814% ± 2.2130%, 78.9226% ± 0.4530%, 51.6217% ± 3.1405%, and 32.7243% ± 0.8207%, respectively (Table 1).
Figure 2 Antioxidant activity of BLJ and FLJ determined by the DPPH and ABTS radical scavenging assay
A: DPPH; B: ABTS. DPPH: 1,1-Diphenyl-2-picrylhydrazyl; ABTS: 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). VC: vitamin C; BHT: butylated hydroxytoluene; FLJ: flowers of Lonicera japonica Thunb.; BLJ: buds of Lonicera japonica Thunb.

Figure 3 Antioxidant activity of BLJ and FLJ as determined by reducing power and FRAP assay
A: reducing power assay; B: FRAP assay. FRAP: ferric ion reducing antioxidant power; BHT: butylated hydroxytoluene; FLJ: flowers of Lonicera japonica Thunb.; BLJ: buds of Lonicera japonica Thunb.
cause the pharmacological effects and health benefits of BLJ and FLJ, including antibacterial, antiviral, hepatic protective, antipyretic, and anti-inflammatory activities. It is reported that BLJ has several pharmacological effects with similar levels and activities. Two indicator compounds are presented in both BLJ and FLJ extracts with similar levels and activities.

Food. Chlorogenic acid and cynaroside are bioactive components and thus used as indicators to characterize the quality of BLJ and FLJ in the Chinese Pharmacopoeia (2015 Edition). UPLC analysis shows that the quality of BLJ and FLJ in the Chinese Pharmacopoeia is determined by the presence of these compounds. FLJ remains completely unknown, in the present study, we, for the first time, report that both BLJ and FLJ have potent antioxidant activity after using six complementary methods to assess their ability to scavenge free radicals and to inhibit lipid peroxidation. The antioxidant activity (total antioxidant capacity) of plant extracts can be determined by several in vitro methods. There are two general types of assays that are widely used for different antioxidant studies. The first group consists of assays that are associated with electron or radical scavenging, including the DPPH and ABTS assay, reducing power and FRAP assay. These assays are based on reductive reaction. The other group of assays is associated with lipid peroxidation, which includes the β-carotene bleaching assay and thiobarbituric acid assay. The present work has shown that the BLJ and FLJ extracts exhibit similarly potent DPPH and ABTS scavenging activity in a dose-dependent manner within the range of concentrations. The reducing ability of a compound generally depends on the presence of reductases, which exhibit antioxidative potential by breaking the free radical chain and donating a hydrogen atom. The reducing power of a compound may therefore serve as an indicator of its potential antioxidant activity. The FRAP assay is quick and easy to perform, and the reaction is reproducible and linearly related to the molar concentrations of the antioxidant(s). In the present study, high concentrations of the BLJ and FLJ aqueous extracts showed that comparable reduction capacities were positively related to their concentrations.

Lipid peroxidation, a consequence of oxidative stress, consists of a series of free radical-mediated chain reactions and is associated with several types of biological damages, some of which are known to play a key role in aging process and to contribute to the pathogenesis of several major degenerative diseases. In the present study, we utilized two types of in vitro assays to assess the antioxidative capacity of the aqueous extracts of BLJ and FLJ, which is related to lipid peroxidation. One is to monitor the early stage of lipid peroxidation (β-carotene bleaching and FTC assay), and the other is to monitor the final stage of lipid peroxidation (TBA assay). The β-carotene bleaching Assay is one of the most commonly used techniques for assessing lipid peroxidation. Radicals generated from the autoxidation of linoleic acid cause β-carotene bleaching, which is retarded by antioxidants. The ferric thiocyanate method measures the amount of peroxides produced during the initial stages of oxidation which are the primary products of oxidation. The TBA assay is one of the most popular assays for studies related to lipid peroxidation, and it is still widely used in the evaluation of antioxidant activities of various natural products. The results of the β-carotene bleaching, FTC and TBA assays suggest that the BLJ and FLJ aqueous extracts have potent antioxidative effects in preventing lipid peroxidation.

**DISCUSSION**

In China, the buds and stem of Lonicera japonica Thunb. are used in the TCM therapies and as healthy food. Chlorogenic acid and cynaroside are bioactive components and thus used as indicators to characterize the quality of BLJ and FLJ in the Chinese Pharmacopoeia (2015 Edition). UPLC analysis shows that the two indicator compounds are presented in both BLJ and FLJ extracts with similar levels and activities. It is reported that BLJ has several pharmacological effect, including antibacterial, antiviral, hepatic protective, antipyretic, and anti-inflammatory activities. Because the pharmacological effects and health benefits of BLJ and FLJ remain completely unknown, in the present study, we, for the first time, report that both BLJ and FLJ have potent antioxidant activity after using six complementary methods to assess their ability to scavenge free radicals and to inhibit lipid peroxidation.

**Figure 4** Antioxidant activity of BLJ and FLJ measured by the β-carotene bleaching assay. BHT: butylated hydroxytoluene; FLJ: flowers of Lonicera japonica Thunb.; BLJ: buds of Lonicera japonica Thunb.

**Figure 5** Antioxidant activity of BLJ and FLJ measured by the FTC assay. FTC: ferric thiocyanate; VC: vitamin C; BHT: butylated hydroxytoluene; FLJ: flowers of Lonicera japonica Thunb.; BLJ: buds of Lonicera japonica Thunb.

**Table 1. Determination of antioxidant activity of BLJ and FLJ using the TBA method.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Absorbance (532 nm)</th>
<th>% Inhibition of TBA formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.3832±0.0100</td>
<td>-</td>
</tr>
<tr>
<td>VC</td>
<td>0.3305±0.0102$^a$</td>
<td>13.6814±2.2130</td>
</tr>
<tr>
<td>BHT</td>
<td>0.0835±0.0020$^a$</td>
<td>78.9226±0.4530</td>
</tr>
<tr>
<td>BLJ</td>
<td>0.1813±0.0112$^a$</td>
<td>51.6217±3.1405</td>
</tr>
<tr>
<td>FLJ</td>
<td>0.2523±0.0030$^a$</td>
<td>32.7243±0.8207</td>
</tr>
</tbody>
</table>

Notes: VC: vitamin C; BHT: butylated hydroxytoluene; FLJ: flowers of Lonicera japonica Thunb.; BLJ: buds of Lonicera japonica Thunb. $^aP<0.01$, $^bP<0.001$, compared to the control group; $^cP<0.01$, compared to the FLJ group.
In conclusion, we found that the extracts from buds and flowers of Lonicera japonica Thunb. have equivalent antioxidant capacities. Further studies are warranted involving the isolation and identification of the components responsible for the antioxidative activity.

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REFERENCES