

Original Article

Antioxidant activities of various extracts of lotus (*Nelumbo nucifera* Gaertn) rhizome

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Lotus rhizome powder was extracted with solvents of different polarities. Antioxidant activities of the extracts were evaluated by a 2, 2'-diphenylpicrylhydrazyl (DPPH) assay and a β -carotene bleaching assay, and compared with that of butylated hydroxyanisole (BHA) and ascorbic acid. Methanol showed the highest extract yield among all of solvents. Although acetone extract had the highest total phenolics content, methanol extract had the highest total phenolics recovery from lotus powder (20.1 mg catechin equivalents/100g lotus powder). Extract of either methanol or acetone demonstrated the highest DPPH scavenging activity at both 66.7 mg/L and 133.3 mg/L. All extracts exhibited higher antioxidant activity coefficient (AAC) than that of ascorbic acid, furthermore, dichloromethane and petroleum extracts had comparable AAC with BHA by the β -carotene bleaching assay. The properties of the extracting solvents significantly affected the yield, total phenolics content and antioxidant activity of lotus rhizome extracts.

Key Words: lotus rhizome extracts, total phenolics content, antioxidant activity, solvent polarity

Introduction

There have been strong evidences indicating that free radicals cause oxidative damage to lipids, proteins, and nucleic acids.¹ Though antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) play an important role in scavenging oxidants and preventing cell injury, these defence mechanisms are not adequate. Consequently, cellular macromolecules are easily subject to oxidative damage.² Several epidemiological studies suggest that a high intake of food rich in natural antioxidants increases the antioxidant capacity of the plasma and reduces the risk of certain, but not all, cancers, heart diseases, and stroke. A great number of aromatic, spicy, medicinal and other plants contain chemical compounds exhibiting antioxidant properties and demonstrate such protective effects.³ In addition to α -tocopherol, ascorbic acid and carotenoids, plant tissues synthesize a wide variety of phenolic compounds.⁴ Several studies have revealed that a major part of the antioxidant activity may be from compounds such as flavonoids, isoflavones, flavones, anthocyanin, catechin and other phenolics⁵ with mechanisms involving both free radical-scavenging and metal chelation.⁶ For instance, the so-called "French paradox" has been attributed in part to the routine consumption of red wine rich in polyphenolics.⁷

Lotus (*Nelumbo nucifera* Gaertn) is a perennial aquatic crop with stout creeping yellowish white colored rhizomes. It is both an ornamental plant and a dietary staple in Eastern Asia, particularly in China.⁸ All parts of *N. nucifera* are used for various medicinal purposes in oriental medicine.⁹ The seed of *N. nucifera* is used in folk remedies as diuretic, cooling agent, antiemetic and antidote in the treatment of

tissue inflammation, cancer,^{10,11} skin disease, leprosy and poisoning.¹⁰ Experimental studies demonstrated that seed of *N. nucifera* has hepatoprotective¹² and antifertility activities¹³ as well as free radical scavenging activity. Liu et al. (2004)¹¹ reported that seed of *N. nucifera* could suppress cell cycle progression, cytokine genes expression, and cell proliferation in human peripheral blood mononuclear cells. The leaf of *N. nucifera* is considered best for 'overcoming body heat', and stopping bleeding.¹⁴ It is used as a drug for hematemesis, epistaxis, hemoptysis, hematuria and metrorrhagia¹⁵ in traditional Chinese medicine. The leaf of *N. nucifera* contains several flavonoids and alkaloids, and is effective in the treatment of hyperlipidaemia in rodents.^{16,17} The 95% ethanol extract of the leaf of *N. nucifera* was found to display significant anti-HIV activity.⁹ The stalk extract of *N. nucifera* showed anti-pyretic effect,¹⁸ while leaf and stamen extracts showed anti-oxidant effect and strong radical scavenging activity.^{19,21}

In China, lotus rhizome is a common vegetable, it can be cooked into different dishes or eaten raw. Especially, it has been applied in Chinese herbal prescriptions to alleviate tissue inflammation, cancer, and liver cirrhosis for a long time.²² Compared with leaf and seed of *N. nucifera*, phytochemicals in lotus rhizome and their functional properties were still little studied, though it was noticed for rich antioxidants content and strong antioxidant activity.^{8,22}

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Many factors can affect the extract recovery of antioxidant phytochemicals from natural materials. Previous studies have reported the influence of some variables (e.g., temperature, time, solvent-to-solid ratio, etc.) on the phenolic yields of diverse plant materials ranging from almond hulls to pine sawdust.²³ Solvents such as water, ethanol, methanol, acetone or their mixture are commonly used to extract phytochemicals from plants, and they are another important factor affecting both extraction yield and antioxidant activity of extracts. Due to the complexities of both the chemical characteristics of solvents and the diverse structure and composition of the plant materials, the behaviors of material-solvent systems were different from each other and can hardly be predicted. No single solvent could extract all the antioxidants of different polarity and solubility in a single plant.

A number of *in vitro* methods have been developed to measure the efficiency of dietary antioxidants either as pure compounds or in food mixtures. These methods can be classified into two major types based on the chemistry mechanisms involved: assays involving hydrogen atom transfer reactions, including ORAC (oxygen radical absorbance capacity), TRAP (total radical trapping antioxidant parameter), Crocin bleaching assay, IOU (inhibited oxygen uptake), inhibition of linoleic acid oxidation, inhibition of LDL oxidation; and assays by electron-transfer reaction, including TEAC (Trolox equivalent antioxidant capacity), FRAP (ferric ion reducing antioxidant parameter), DPPH (2, 2'-diphenylpicrylhydrazyl) scavenging activity, copper (II) reduction capacity, total phenols assay by Folin-Ciocalteu reagent.²⁴ Frankel and Meyer pointed out that it is problematic to use one-dimensional methods to evaluate multifunctional food and biological antioxidants.²⁵

In the current study, we evaluated the antioxidant activity of various extracts of lotus rhizome with two methods based on different mechanisms. The objective of this study was to investigate the effect of extracting solvents on the yield, total phenolics and antioxidant activity of lotus. Antioxidant activity of lotus extract was compared with that of ascorbic acid and BHA.

Materials and methods

Materials

Lotus rhizome was purchased from a local market. It was washed by tap water, chopped in pieces and dried in 60°C for 72h, and then ground to pass a 1 mm sieve. The sample powder was stored at -20°C before experiment. 2, 2'-diphenylpicrylhydrazyl (DPPH) and β -carotene were obtained from Sigma and all other chemicals used were of analytical grade.

Extraction

Five grams of the lotus rhizome powder were weighed and put into 150 mL flasks. Each flask was added with 50 mL of either methanol, ethanol, acetone, dichloromethane or petroleum ether. After one week of storage at room temperature, the supernatant and the sediment were separated by vacuum-filtration. The solvent in the supernatant was evaporated by vacuum-evaporator at 35°C. The dried extract was then weighed.

Determination of total phenolic content

Total phenolic compounds were determined with Folin-Ciocalteu reagent using gallic acid as standard according to Slinkard and Singleton.²⁶ 1 mL of extracts in a volumetric flask was diluted with 46 mL of distilled water. 1 mL of Folin-Ciocalteu reagent was added and the content in the flask was mixed thoroughly. After 3min, 3 mL of Na₂CO₃ (2%) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 765 nm. The concentration of total phenolic compounds was calculated with a linear regression equation obtained from gallic acid standard graph: Absorbance=8.3886×gallic acid (μ g)-0.0114. And the results were expressed as milligrams of gallic acid equivalents (GAE) per 100g of dry extract or per 100g of dry lotus powder.

Determination of DPPH· radical scavenging capacity

The antioxidant activity of lotus extracts were measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH· method as modified by Sanchez-Moreno et al.²⁷ 0.1 mL of the sample at various concentrations was added to 2.9 mL of DPPH· (60 μ M) solution. When DPPH· reacts with an antioxidant compound donating hydrogen, it is reduced, resulting in a decrease in absorbance at 517 nm. The absorbance was recorded at 10 min intervals up to 30 min using a UV-Vis spectrophotometer. The remaining concentration of DPPH· in the reaction medium was calculated with linear regression equation from a calibration curve: $A_{517\text{ nm}} = 0.123 [\text{DPPH}\cdot] + 0.0455$ ($r = 0.9865$). The percentage of remaining DPPH· ($[\text{DPPH}\cdot]_R$) was expressed as $[\text{DPPH}\cdot]_R (\%) = 100 \times [\text{DPPH}\cdot]_T / [\text{DPPH}\cdot]_{T=0}$, where $[\text{DPPH}\cdot]_T$ is the concentration of DPPH· at 30 min time and $[\text{DPPH}\cdot]_{T=0}$ is the initial concentration. The percentages of remaining DPPH· were plotted against the sample or standard concentrations to obtain the amount of antioxidant necessary to reduce the initial concentration of DPPH·. A kinetic study was also conducted to evaluate the free radical scavenging properties of lotus rhizome extract using stable DPPH·.

Determination of antioxidant activity by β -carotene bleaching method

The prevention of oxidative loss of β -carotene in a β -carotene/linoleic acid emulsion was used to assess the antioxidant ability of the lotus rhizome extracts.²⁸ Two milligrams of β -carotene were dissolved in 10 mL chloroform and 1 mL of the β -carotene solution was mixed with 20 mg of purified linoleic acid and 200 mg of Tween 40 emulsifier in a round-bottom flask, chloroform was then removed in a rotary vacuum evaporator. Distilled water (50 mL) was added to the flask and the mixture was stirred in a sonicator. Lotus rhizome extract or ascorbic acid or BHA was added to 5 mL of the β -carotene/linoleic acid emulsion to a final concentration of 40 mg/L. The control contained 0.2 mL water and 5 mL β -carotene/linoleic acid emulsion. An absorbance at 470 nm was immediately recorded after addition of sample. The vials were capped and placed in a water bath at 50°C. The absorbance of the emulsion at 470 nm was determined every 20 min until 120 min. A second emulsion

(B), consisting of 20 mg of linoleic acid, 100 mg of Tween 40 and 50 mL of water, was also prepared. Water (200 μ L) with 5 mL emulsion B was used to zero the spectrophotometer. Antioxidant activity coefficient (AAC) was calculated with the following equation: $AAC = (A_{A(120)} - A_{C(120)}) \times 1000 / (A_{C(0)} - A_{C(120)})$; where $A_{A(120)}$ is the absorbance of the antioxidant at 120 min, $A_{C(120)}$ is the absorbance of the control at 120 min, and $A_{C(0)}$ is the absorbance of the control at 0 min.

Statistical analyses

All analyses were run in triplicates and results averaged. Statistical analyses were performed with the Excel and SPSS softwares package.

Results

Yield

The extraction yields with different solvents varied from 4.6 g to 0.41 g per 100 g lotus powder (4.6% to 0.41%) (Fig 1), and can be ranked from high to low in the following order: Methanol extract > ethanol extract > ethyl acetate extract > acetone extract > petroleum ether extract > dichloromethane extract. The yield of methanol extract was 11.1 times of that of dichloromethane extract. Our result agreed with the yields from *Gevuina avellana* hulls and the yields from buckwheat, which all showed the following order: methanol extract > ethanol extract > acetone extract.^{28,29}

Total phenol content

Total phenolics contents (mg catechin equivalent/100 g extracts), as affected by the extracting solvents, were ranked from high to low: acetone extract > ethyl acetate extract > dichloromethane extract > methanol extract > petroleum ether extract > ethanol extract (Table 1). The total phenolics content of acetone extract was 3.9 times and 3.3 times of those of ethanol extract and petroleum ether extract, respectively. Although total phenolics in acetone extract was the highest one when expressed against the weight of extracts, methanol extract had the highest recovery rate against lotus rhizome powder (20.1 mg catechin equivalent/100 g lotus rhizome powder). The order of recovery from high to low was: methanol extract > acetone extract > ethyl acetate extract > ethanol extract > dichloromethane extract > petroleum ether extract.

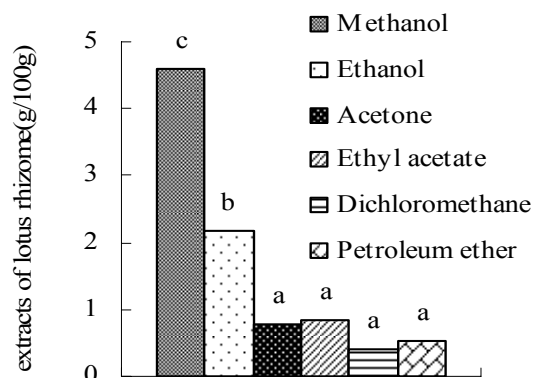


Figure 1. Yield of lotus rhizome extracts (N=3, values with different superscripts were significantly different at $p=0.05$, a=the lowest yield)

Table 1. Total phenolics in lotus extracts (N=3)

Sample	Total phenolics (mg catechin equivalents/100g extracts)	Total phenolics (mg catechin equivalents/100g dry lotus)
Methanol	437.5 ^a	20.1 ^c
Ethanol	324.2 ^a	7.3 ^b
Acetone	1266.0 ^b	9.6 ^b
Ethyl acetate	1081.2 ^b	9.5 ^b
Dichloromethane	580 ^{ab}	2.4 ^a
Petroleum ether	383.8 ^a	2.2 ^a

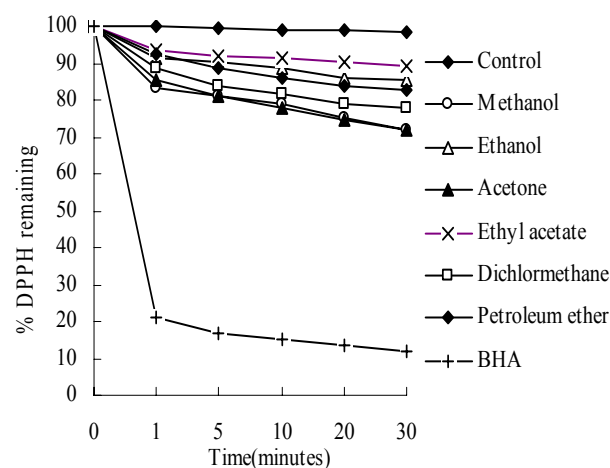


Figure 2. Reaction kinetics of lotus extracts with DPPH radical. The DPPH concentration was 60 μ M in all reaction mixtures

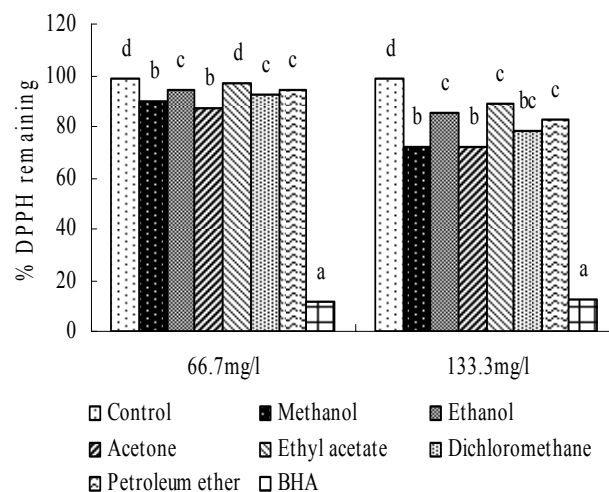


Figure 3. Free radical scavenging capacity of lotus extracts as determined by the DPPH method

DPPH radical scavenging activity

DPPH \cdot is used as a free radical to evaluate antioxidant activity of some natural compounds. The degree of its discoloration is attributed to hydrogen donating ability of test compounds.³⁰ Fig 2 showed the decrease in concentration of DPPH \cdot with time when the concentrations of all extracts and BHA were 133.3 mg/L. Fig 3 illustrated the relative effectiveness of different extracts of lotus rhizome in reducing concentration of DPPH \cdot . Significant DPPH \cdot radical scavenging activity was evident in all of the tested extracts at 133.3 mg/L. Extracts of methanol and acetone demonstrated higher scavenging activities than other extracts. However, all extracts showed lower

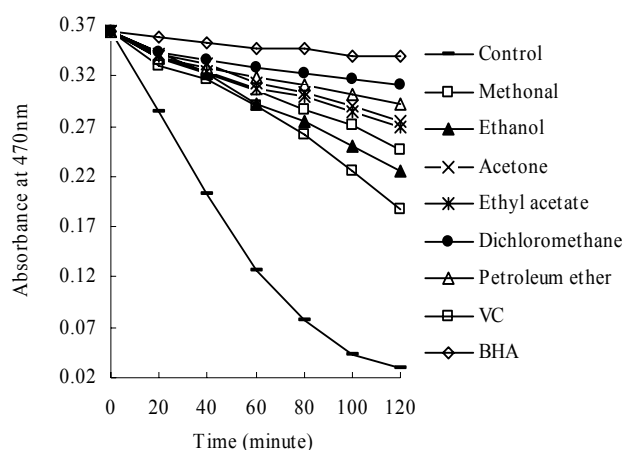


Figure 4. Changes of absorbance at 470nm with time in β -carotene/linoleic acid emulsions added with lotus rhizome extracts (40 mg/L).

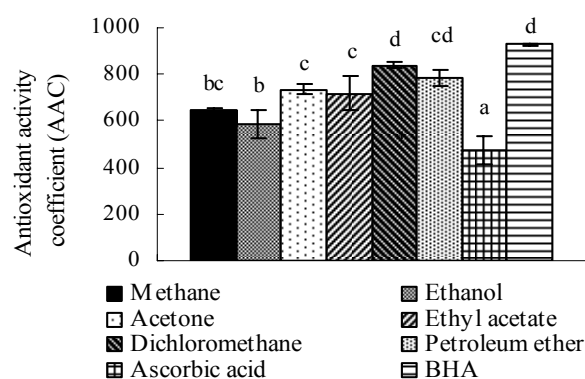


Figure 5. Antioxidant activity of lotus extracts analyzed by β -carotene bleaching method

activity than BHA and ascorbic acid (data not shown) at the same concentration. The order of antioxidant activity of lotus rhizome extracts was: methanol extract = acetone extract > dichloromethane extract > petroleum ether extract > ethanol extract > ethyl acetate extract. At 66.7 mg/L, lotus rhizome extracts showed similar scavenging activity trends to 133.3 mg/L except that the DPPH scavenging activity of ethyl acetate extract at 66.7 mg/L was not statistically significant.

Antioxidant activity determined by β -carotene bleaching method

In the β -carotene bleaching assay, the control sample without addition of antioxidant oxidized most rapidly, while lotus extracts, ascorbic acid or BHA showed varied antioxidant activities (Fig 4). The absorbance decrease in emulsion sample added with dichloromethane extract was significantly slower than that in samples added with other extracts. In this assay, all extracts of lotus rhizome as well as BHA exhibited better antioxidant activities than ascorbic acid. Fig.5 showed the antioxidant activities of the lotus extracts in comparison with that of ascorbic acid and BHA at the concentration of 40 mg/L. Their antioxidant powers were ranked in the order: BHA > dichloromethane extract > petroleum ether extract > acetone extract > ethyl acetate extract > methanol extract > ethanol extract > ascorbic acid. Dichloromethane and petroleum ether extracts demonstrated comparable AAC with BHA at the same concentration.

Discussion

The antioxidative phytochemicals in grains, vegetables and fruits have received increasing attention recently for their potential role in prevention of human diseases as well as in food quality improvement.³¹ Lotus rhizome has been used both as vegetables and medicine in eastern Asia, particularly in China. It was regarded to be one of the many vegetables rich in antioxidant components,³² and many of its functional effects were related to the antioxidants it contains.¹ Despite this, lotus rhizome has not been explored extensively for its phytochemical attributes. Several researches using different methods have proved that lotus exhibited effective antioxidant capacity.^{8,32} In our experiment we checked the effects of solvents on antioxidant activity and phenolic content of lotus rhizome extracts.

Extraction is critical to the recovery of antioxidant phytochemicals. The extraction yield depends on solvent, time and temperature of extraction as well as the chemical nature of the sample. Under the same time and temperature conditions, the solvent used and the chemical property of sample are the two most important factors.³⁰ The extract yield of lotus rhizome, ranged from 0.4% to 4.6% (w/w) was in general higher than that of potato peel, which was 0.5%, comparable with that of buckwheat, which was 1.5% to 4%²⁹ depending upon different solvents, but was lower than that of lentil seeds and grape pomace which were 23.3% and 42% respectively.²⁸ In our experiment, methanol was proved to be the best solvent for extracting antioxidant from lotus rhizome, this agrees with reports by Bensky et al¹⁴ and Azizah et al.³³

Among the different groups of naturally occurring antioxidants from plants, polyphenolics are perhaps one of the most important types. Our results showed that solvent significantly affected the phenolic content from lotus rhizome extract. The acetone extract of lotus rhizome showed the highest phenolic content among all solvents studied. Acetone was superior to methanol in selective extraction of total phenolics, this agrees with research results by Bensky et al.¹⁴ However, methanol extract gives the highest total phenolic recovery from the lotus rhizome because its extract yield is much higher than acetone extract.

The antioxidant activity of plant extracts vary with assay methods.²⁹ Therefore, a single assay may be inadequate.³⁴ For this reason, we cross checked antioxidant activities of various extracts of lotus rhizome with two antioxidant activity assays based on different mechanisms, namely DPPH assay based on electron-transfer reaction and β -carotene bleaching assay based on hydrogen atom transfer reaction. Many studies indicated that only polar extracts of plants showed effective antioxidant activity, and some researches further proved that moderate polarity extracts are more potent, even if their total antioxidant recovery from the plant is not high.³⁵ Methanol appears to perform best in extracting polar compounds such as phenolics, flavonoids and other polar material in cereals.^{36,37} In the present study, methanol extract of lotus rhizome showed the highest antioxidant activity by DPPH assay. However, it was the dichloromethane extract which showed the strongest antioxidant activity by β -carotene bleaching assay. This discrepancy is not

uncommon. For example, it was reported that though lipophilic extract of coriander demonstrated strong activity in the 15-LO assay (a method to test the activity of antioxidant against linoleic acid peroxidation), it showed low antioxidant activity in DPPH assay.³⁶ The author believed the reason was that the involvement of proton donation by the active compounds may be of less importance for 15-LO inhibition than for DPPH radical scavenging.

There have been two opinions on the correlation between phenolics and total antioxidant activity. Some reports demonstrated positive correlation between them,^{35,38,39} and the others showed no correlation.^{29,38} Our results agree with the latter, it seems that no correlation exists either between the percentage inhibition (% scavenging effect) assayed by DPPH and total phenolics ($R^2=0.0544$), or between the antioxidant activity (AAC) assayed by β -carotene bleaching method and total phenolics ($R^2=0.0552$). Kaur and Kapoor (2002) thought this phenomenon can be explained on the basis of high antioxidant activity of some individual phenolic units, which may act as efficient antioxidants rather than contributing to high total phenolics,³² while Kähkönen et al.³⁹ thought that total phenolics content did not include all the antioxidants, such as ascorbic acid, carotenoid and tocopherol. Vinson et al. (2001)⁴⁰ held the idea that the synergism among the antioxidants in the mixture made the antioxidant activity not only dependent on the concentration of antioxidant, but also on the structure and interaction among the antioxidants. This probably is the reason why samples with similar concentrations of total phenolics may vary remarkably in their antioxidant activity. Different methods based on different mechanisms for measurement of antioxidant activity may lead to different results even from the same sample. For example, the antioxidant activity of berry extract was mainly related to anthocyanins in the method based on low density lipoproteins (LDL) oxidation, but mainly correlated with hydroxycinnamates in the assay of liposome oxidation.³⁹

In conclusion, the extracting solvent significantly affected the yield, total phenolic content and antioxidant activity of lotus rhizome extracts. Methanol extract had the highest extract yield and total phenolic recovery, as well as the highest antioxidant activity when determined by the DPPH assay, while dichloromethane extract showed the highest antioxidant activity when evaluated by the β -carotene assay. Thus, our results indicate that selective extraction from natural materials, by an appropriate solvent, is important for obtaining fractions with high antioxidant activity. Methanol could be the optimal solvent for extraction of lotus antioxidant.

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