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Antioxidant Effect of Methanol Extracts from Lotus Plumule and Blossom (*Nelumbo nucifera* Gertn.)

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ABSTRACT

Lotus (*Nelumbo nucifera* Gertn.) is a major economic aqueous plant in Tainan County. Lotus plumule contains various alkaloids and is used to remove "heat" or as tranquilizer and antihypertensive agent in folk medicine. Lotus blossom contains alkaloids, organic acids, amino acids, and β-carotenoid and is used to "warm" kidney and spleen or used as a cardiotonic in folk medicine. In an attempt to assess the possible antioxidant activities of methanol extracts of lotus plumule and blossom, several *in vitro* assays were conducted. We found that both lotus plumule and blossom possessed strong reducing powers and free radical scavenging abilities. However, only methanol extract of lotus plumule exhibited ferrous ion chelating capabilities, which might contribute to the difference in antioxidant activities between lotus plumule and lotus blossom when analyzing the preventive effects on fatty acid peroxidation and plasmid DNA damage. No mutagenicity in the methanol extract of lotus plumule or blossom was found for *Salmonella typhimurium* TA98 and TA100, either in the presence or absence of S9 mix.

Keywords: Lotus, Nelumbo nucifera Gertn., antioxidant activity

INTRODUCTION

The formation of potentially toxic compounds caused by the oxidative deterioration of lipids in foods, is responsible for the decrease in food quality and safety[(1)]. It is necessary to suppress lipid peroxidation in food in order to preserve flavor, color and nutritional value. The addition of antioxidants to foods is the most effective way for delaying lipid peroxidation which is the reason for the unpleasant flavors. In the food industry, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ), are widely used because they are effective and less expensive than natural antioxidants. Their safety issues, however, are highly debated, thus generating the need to search for substitute materials from natural and safe sources as food antioxidants. Recently, much attention has been focused on vitamins C, E and carotenoids[(2-5)].

Antioxidants are also of interest to biologists and clinicians, because they may help to protect the human body against damages caused by reactive oxygen species (ROS)[(6)]. Various ROS such as singlet oxygen ($^1O_2$), superoxide radical ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) are generated as by-products during aerobic metabolism in cells[(7)]. These highly reactive species have a pontential for bringing about extensive damages, including lipid peroxidation, DNA lesions, and protein fragmentation within the cells of biological macromolecules. It's widely acknowledged that the accumulation of oxidative damages of intracellular macromolecules is an essential element in aging processes and in certain degenerated diseases[(8)].

A lot of studies have analyzed the antioxidant potential of a wide variety of vegetables, including cacao beans[(9)], potato, tomato, spinach, and legumes such as *Phaseolus vulgaris*[(10)]. Seeds, teas, and agricultural residues are also potential antioxidants[(11-13)]. Some of the natural products, especially polyphenolic compounds, have well-proven antioxidant abilities. The protective effects of these compounds have been attributed, in a large part, to their scavenging of free radicals, chelating of transition ions and/or modulating antioxidant proteins within cells[(14)].

Lotus (*Nelumbo nucifera* Gertn.) is a very important crop in Tainan County. Several new products have been developed recently in order to reduce the impact on local farmers after Taiwan joined WTO. In addition to the major edible parts, seeds and rhizomes, lotus blossom and plumule (lian zi xin) have been processed and sold as herbal beverages. Lotus plumule is used to remove "heat" in folk medicine. It is also used as tranquilizing and antihypertensive agents. It contains alkaloids including liensinine, isoliensinine, referine, lotusine, methylcorypalline, and demethylcoclaurine. Among them, referine has been shown to have a vasodilating effect and liensinine has antihypertensive and antiarrhymic abilities[(15)]. Lotus blossom contains alkaloids, organic acids, amino acids, and carotenoid. Lotus blossom is used to 'warm" kidney and spleen or as a cardiotonic agent in folk medicine.

Because there is limited information published to

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prove the biological activities of lotus plumule and blossom, we attempt to assess their possible antioxidant activities in this research.

MATERIALS AND METHODS

I. *Materials*

Lotus plumule and blossom (*Nelumbo nucifera* Gertn.) were purchased from local farms in Bei-Her, Tainan. They were sun dried, sealed in plastic bags and stored at 4˚C until use.

II. *Chemicals*

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), and solvents were from E. Merck (Darmstadt, Germany), unless otherwise indicated. All of the reagents were prepared in deioinized water to eliminate the contamination of metal ions.

III. *Extraction*

Lotus plumule and blossom were extracted with methanol at the ratio of 600 mL per 60 grams at room temperature overnight. The extracts were filtered and concentrated *in vacuo*. The crude extracts were weighed and dissolved in methanol, then packaged in nitrogen and stored at -20˚C until use.

IV. *Antioxidant Activity in a Hemoglobin-Induced Linoleic Acid System*

The antioxidant activities of lotus plumule and blossom were determined by a modified photometry assay[16]. Reaction mixtures (200 µL) containing various amount of extracts, 1 mM linoleic acid emulsion, 40 mM phosphate buffer, pH 6.5, and 0.0016% hemoglobin were incubated at 37˚C for 45 mins. At the end, 2.5 mL of 0.6% HCl in ethanol was added to stop lipid peroxidation. The peroxide value was measured in triplicate using the thiocyanate method by reading the absorbance at 480nm after coloring with 100 µL of 0.02 M $FeCl_2$ and 50 µL of 30% ammonium thiocyanate.

V. *Plasmid Relaxation Assay*

DNA strand damages were measured by converting circular double-stranded supercoiled DNA into nicked circular and linear forms[17]. Reactions were performed in 10 µL of solution containing supercoiled pUC18 plasmid (20 ng), 10 mM Tris-HCl buffer (pH 7.8), 1 mM hydrogen peroxide, 100 µM ferric chloride, 100 µM ascorbic acid and various amounts of the extracts. The mixtures were incubated at 37˚C for 30 mins. and the reactions were stopped by adding 1 µL of 0.5 M EDTA. The samples were separated on 0.7% agarose gel electrophoresis followed by ethidium bromide staining and captured by a CCD camera under UV (UVI, England).

VI. *Reducing Power*

Various concentrations of the extract (50 µL) were mixed with 200 µL of 0.2 M phosphate buffer, pH 6.5 and 200 µL of 1% potassium ferricyanide, then incubated at 50˚C for 20 mins. 10% trichloroacetic acid (250 µL) was added to the mixture and centrifuged at 3000 xg for 10 mins at room temperature. The resulting supernatant was taken and mixed with 500 µL of ddH_2O and 100 µL of 0.1% ferric chloride then incubated at 37˚C for 10 mins. The absorbance at 700 nm was measured. This assay was done in triplicate. Increased absorbance indicated increased reducing power[18].

VII. *1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Effect*[19]

Reactions were performed in 1.25 mL of methanol containing 0.5 mM freshly made DPPH and various amounts of the extract. Reaction mixtures were incubated at 37˚C for 30 mins., and the absorbance at 517 nm was measured. This assay was done in triplicate.

IX. *Scavenging Effect on Hydroxyl Radicals*[20]

Reaction mixtures containing various concentrations of extract, 0.02 M phosphate buffer, pH 7.4, 2 mM H_2O_2, 0.05 mM ferric chloride, 0.05 mM ascorbate, 6 mM deoxyribose and 0.05 mM EDTA were incubated at 37˚C for 30 mins. The degree of deoxyribose oxidation was analyzed as thiobarbituric acid-reactive material.

X. *Ferrous Ion Chelating Effect*[21]

Reaction mixtures containing 100 µL of extract, 200 µL of 0.5 mM ferrous chloride and 200 µL of 5 mM ferrozine were incubated at 37˚C for 10 mins. After adding 1.5 mL of ddH_2O to the mixture, the absorbance at 562 nm was measured. The lower absorbance at 562 nm indicated stronger chelating effect.

XI. *Mutagenicity Assay*

The mutagenecity assay was performed according to the Ames test[22]. The histidine-requiring strains of *Salmonella typhimurium* TA98 and TA100 were purchased from CCRC. The S9 mix was prepared from Sprague-Dawley male rats treated with Aroclor 1254, according to Ames *et al.*[23]. Various amounts of extract were added to overnight-cultured *Salmonella typhimurium* TA98 (0.1 mL) and S9 mix (0.5 mL) or phosphate buffer (0.5 mL) in place of S9 mix. The entire mixture was pre-incubated at 37˚C for 48 hours. Assay of each sample was determined in triplicate plates per run and data presented were means ± SD of

the three determinations. At least two runs of a single experiment were performed to validate reproducibility. The mutagenicity is expressed as the number of revertants per plate, at a given concentration of each sample. In this mutagenicity testing, the result was recognized as positive when the number exceeds twice the number of spontaneous revertants[23].

RESULTS AND DISSCUSSION

The most commonly used method for determining antioxidant activity is to measure the inhibitory degree of autoxidation of linoleic acid, but the analysis takes 5-6 days[24]. Instead, we employed a modified rapid photometric assay as described by Kuo, Yeh, and Pan[16] to evaluate the antioxidant activity of methanol extracts of lotus plumule and blossom. As shown in Figure 1, the antioxidant activities are dose-dependent and reached a plateau (about 85-90% inhibition) when the concentration of lotus plumule exceeded 0.05 mg/mL (Figure 1A) or when the concentration of lotus blossom exceeded 0.6 mg/mL (Figure 1B). This result indicates that the antioxidant activity of lotus plumule is about ten folds higher than that of lotus blossom shown by the peroxide production in the presence of 1 mM linoleic acid.

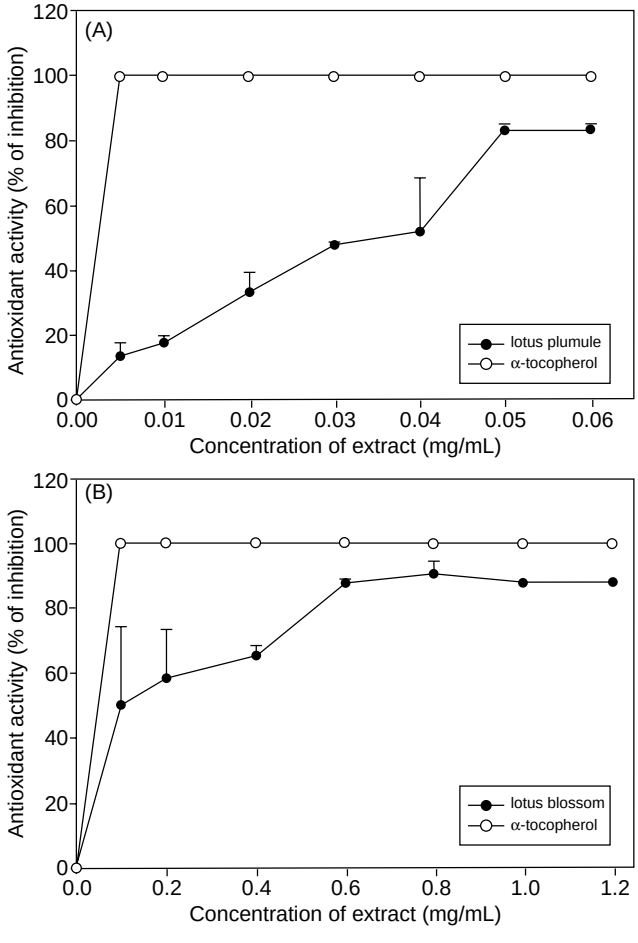

Figure 1. Antioxidant activity of the methanol extracts of lotus plumule and lotus blossom against linoleic acid peroxidaion induced by hemoglobin. The peroxide value was measured in triplicate by thiocyanate method as described in Materials and Methods. α-tocopherol was used as a positive control.

A substantial portion of ROS lethality involves DNA damage by oxidants generated from iron-mediated Fenton reaction[25]. We employed H_2O_2-Fe^{+++}-ascorbate system to induce oxidative damage of DNA. Plasmid relaxation assay was used to semi-quantitatively assess the DNA oxidative damage. In the process of DNA damage, DNA in supercoil was first nicked into open circular form, which was the product of single-stranded cleavage of supercoil DNA. The open circular DNA can be further cleaved by ROS into linear form, which was the result of double-stranded cleavage. Extensive oxidative damage would eventually cause DNA fragmentation and degradation subsequently. As shown in Figure 2, H_2O_2, Fe^{+++}, or ascorbate alone only caused a small portion of supercoil pUC18 DNA, which migrated the fastest, nicked to open circular form, which migrated the slowest (lanes 2, 3 and 4). However, in the presence of all three reagents together, DNA was hydrolyzed to linear DNA band, which migrated between supercoiled and open circular form (lane 5). In the presence of extract, the extent of DNA damage could be significantly reduced and the protective effects were dose-dependent (lanes 6-10). The strongest protective effect shown as open circular plasmid DNA was predominantly produced at 10 mg/ml extracts of lotus plumule and blossom (lane 6). When extract concentration decreased, the occurrence of open circular form of DNA decreased and the linear form of DNA progressively increased (lanes 6-10). We also observed that the linear form DNA was first present at 0.1 mg/mL of plumule extract (Figure 2A, lane 8) and at 1 mg/mL of blossom extract (Figure 2B, lane 7). This result suggested that the antioxidant effect of lotus plumule was stronger than lotus blossom.

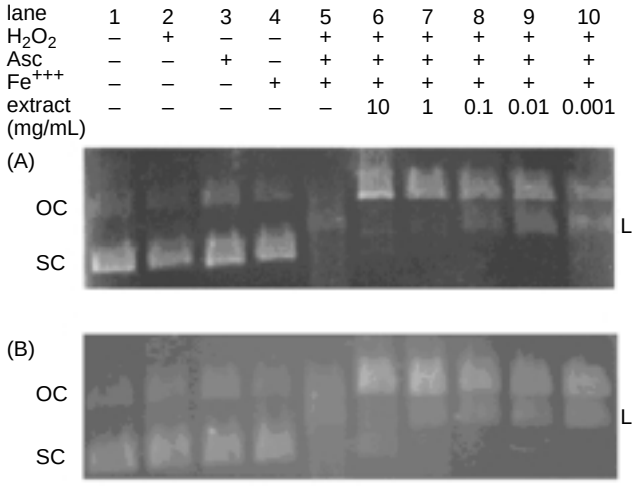

Figure 2. Agarose gel electrophoretic analysis of Fenton-mediated DNA oxidation. (A) inhibitory effect of methanol extracts of lotus plumule (B) inhibitory effect of methanol extract of lotus blossom. SC, supercoil form DNA; OC, open circular form DNA; L, linear form DNA.

The most common method for detecting DNA damage in the cell-free system is enzymatic hydrolysis of DNA to nucleosides and chromatography of the formation of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-OHdG). However, some transition metals can induce DNA strand damage but do not generate 8-OHdG in DNA, thus plasmid relaxation technique has been suggested to be more sensitive[26]. The methanol extracts of lotus plumule and blossom apparently were strong inhibitors of oxidative damage of DNA because they can significantly protect DNA from Fenton-mediated DNA degradation even at concentration as low as 1μg/mL. Oxidative damage in DNA induced by Fenton reaction is thought to arise via a site-specific mechanism, i.e. involving the interaction of a transition metal ion with DNA prior to its reaction with hydrogen peroxide to produce the damaged DNA species[27]. It was suggested that single-strand damage in DNA was formed by hydroxyl radicals generated in free solution[28]. In contrast, the formation of double-strand breaks and 8-OHdG was strongly dependent on the binding of iron (II) to DNA between DNA bases; while the intra-strand cross-links might be formed following metal ion binding to phosphate groups[29]. Therefore, we speculated that part of the antioxidant activity of lotus plumule might arise from the blockage of iron from interacting with DNA so that the formation of more open-circular DNA and fewer linear DNA was observed when higher concentrations of extract was present (lanes 6 and 7 of Figure 2A).

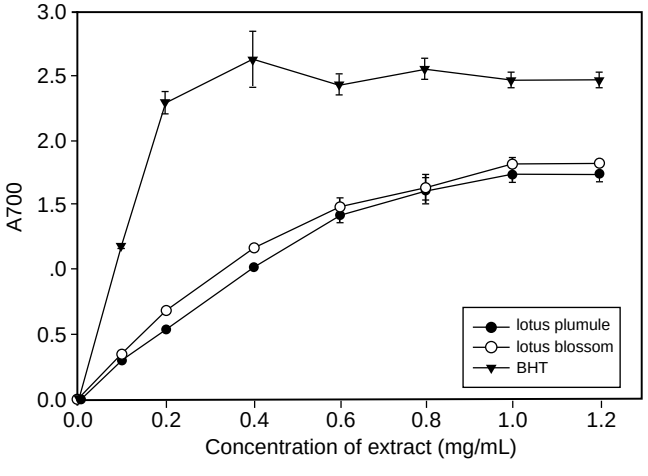

Figure 3. Reducing power of the methanol extracts of lotus plumule and blossom. The absorbance at 700 nm was measured in triplicate. BHT was used as a positive control.

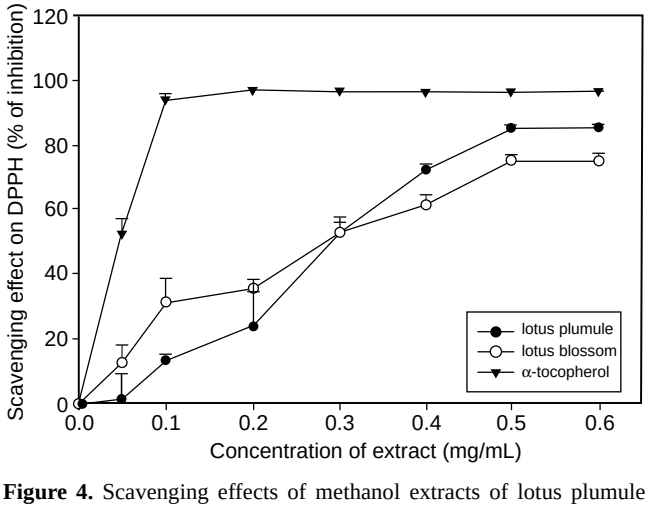

Figure 4. Scavenging effects of methanol extracts of lotus plumule and blossom on 1,1 diphenyl-2-picrylhydrazyl (DPPH) radicals. The absorbance at 517 nm was measured in triplicate.

The antioxidant activities of natural components may have a reciprocal correlation with their reducing powers[30-31]. The reducing powers of the methanol extracts of lotus plumule and blossom are shown in Figure 3. The reducing power increased as the extract concentration increased, indicating some compounds in lotus plumule and blossom were both electron donors and could react with free radicals to convert them into more stable products and to terminate radical chain reactions. The concentrations to attain one absorbance unit at 700 nm were 0.083 ± 0.012 mg/mL for BHT, 0.354 ± 0.054 mg/mL for lotus blossom, and 0.425 ± 0.064 mg/mL for lotus plumule. This result indicated that the reducing power of lotus blossom was slightly higher than lotus plumule but much less than BHT.

It is well known that antioxidants can seize the free-radical chain of oxidation and form stable free radicals, which would not initiate or propagate further oxidation. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) has been used extensively as a free radical to evaluate reducing substances [19,31]. In the current study, the scavenging activities of DPPH exerted by lotus plumule and blossom as well as α-tocopherol were summarized in Figure 4. At lower concentrations, lotus blossom showed slightly higher scavenging activity than lotus plumule, while the opposite effect was observed at higher concentrations. Linear response curves were also obtained and the IC_{50} were estimated as 0.05 ± 0.01 mg/mL for α-tocopherol, 0.296 ± 0.047 mg/mL for lotus blossom and 0.298 ± 0.033 mg/mL for lotus plumule. These results demonstrated that methanol extracts of lotus blossom and plumule had similar free radical scavenging activities.

The deoxyribose method is a simple assay to determine the rate constants for reactions of hydroxyl radicals[20]. When the mixture of $FeCl_3$-EDTA, H_2O_2 and ascorbate were incubated with deoxyribose in phosphate buffer (pH 7.4), the hydroxyl radicals generated attack the deoxyribose and result in a series of reactions that cause the formation of MDA. Any hydroxyl radical scavenger added to the reaction would compete with deoxyribose for the availability of hydroxyl radicals, thus reducing the amount of MDA formation. We herein tested the scavenging activities of lotus blossom and plumule along with positive control, DMSO. In Figure 5, we found that the maximum scavenging capacity on hydroxyl radicals (60%) could be achieved when methanol extract concentrations of lotus plumule were more than 0.02 mg/mL and when methanol extract concentrations of lotus blossom were more than 0.06 mg/mL. Nevertheless, DMSO, a well-known hydroxyl radical scavenger, had 70% scavenging activity at the low-

est concentration tested at 0.01 mg/mL. This result suggested that the scavenging potential on hydroxyl radicals decreased in the order of DMSO > lotus plumule > lotus blossom.

We further tested the ferrous ion chelating activity of methanol extracts of lotus plumule and lotus blossom. We found that only lotus plumule possessed noticeable chelating activity of ferrous ion (Figure 6), while no detectable chelating ability for lotus blossom could be found (data not shown). Linear response curve was also obtained and the IC_{50} was estimated as 4.82 ± 0.39 mg/mL. Although this concentration is much higher than what can be achieved under physiological condition, it may be significant because it minimizes the concentration of metal in lipid peroxidation and DNA oxidation. This may explain why the antioxidant activity of lotus plumule is higher than that of lotus blossom as shown in Figures 1 and 2.

To test the mutagenicity of methanol extracts of lotus plumule and blossom, Ames tests were performed as described in Materials and Methods. For testing doses at 0 to 2.5 mg per plate, no mutagenicity in *Salmonella typhimurium* TA98 or TA100, either with or without S9 mix in the presence of extracts of lotus plumule and lotus blossom, was observed as shown in Table 1. This result indicated that extracts of neither lotus parts were potential mutagens toward *Salmonella typhimurium* TA98 and TA100.

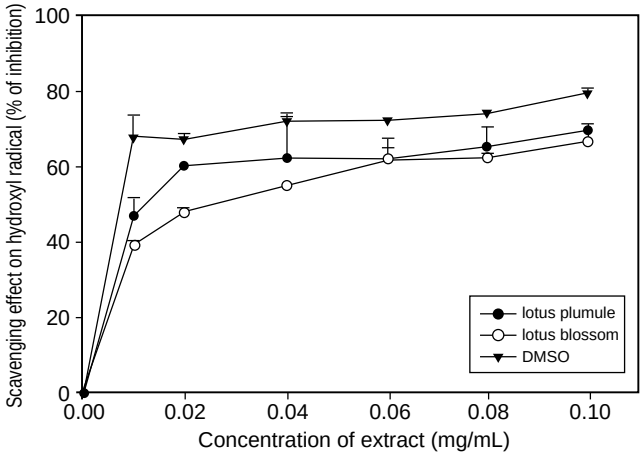

Figure 5. Scavenging effects of methanol extracts of lotus plumule and blossom on hydroxyl radicals. The degree of deoxyribose oxidation was analyzed as thiobarbituric acid-reactivity. DMSO was used as positive control.

Herein, we demonstrated that both lotus plumule and lotus blossom contain antioxidant components while the exact phytochemical characteristics related to their activities are still under investigation.

CONCLUSIONS

The objective of this study was to obtain information on the antioxidant potentials of methanol extracts of lotus plumule and blossom (*Nelumbo nucifera* Gertn.). We employed traditional cell-free assays to investigate the antioxidant activities preventing lipid peroxidation and non-lipid oxidative damages. A modified linoleic acid peroxidation induced by hemoglobin was first performed to evaluate the potential of antioxidant activity of methanol extract of lotus leaves. It has been shown that the products derived from hemoglobin-catalyzed peroxidation of linoleic acid were 9-hydroperoxy-10, 12-octadecadienoic acid (9-HPODE) and 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), which were similar to those obtained from autoxidation[16]. Therefore, this procedure can be used as a rapid screening substitute for traditional autoxidation analysis. The degree of peroxidation was then measured by ferric thiocyanate assay, which is based on the complex of ferric ion with thiocyanate and xylenol orange. It has been shown to be an easy, rapid and sensitive method of measuring LOOHs in lipids[32].

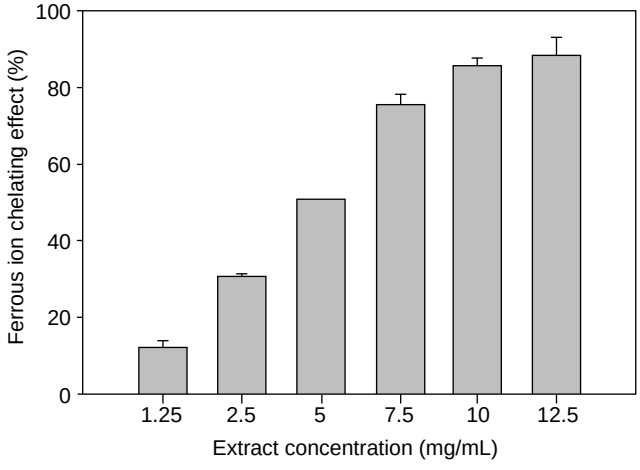

Figure 6. Ferrous ion chelating effect of the methanol extracts of lotus plumule. The absorbance at 562 nm was measured in triplicate.

Table 1. Mutagenicity of methanol extracts of lotus plumule and lotus blossom toward *Salmonella typhimurium* TA98 and TA100 with or without S9 mix

	His$^+$ revertants/plate							
	lotus plumule				lotus blossom			
Concentration	TA98		TA100		TA98		TA100	
(mg/plate)	+ S9	– S9	+ S9	– S9	+ S9	– S9	+ S9	– S9
Control	43 ± 4	15 ± 2	56 ± 11	25 ± 3	28 ± 2	11 ± 3	106 ± 3	76 ± 4
0.5	48 ± 1	18 ± 1	45 ± 15	30 ± 8	40 ± 2	14 ± 3	79 ± 3	76 ± 6
1.0	45 ± 3	17 ± 3	43 ± 7	22 ± 6	37 ± 3	14 ± 7	112 ± 11	60 ± 6
2.5	46 ± 5	17 ± 3	45 ± 7	27 ± 3	34 ± 4	16 ± 2	107 ± 5	53 ± 2

The reducing power increased when the concentrations of methanol extracts of lotus plumule and lotus blossom increased, indicating they were electron donors and could react with free radicals to convert them to more stable products and terminate radical chain reactions. It has been shown that the antioxidant effect exponentially increased as a function of the development of the reducing power, suggesting that the antioxidant properties be associated with the development of the reducing power[33]. Therefore, the antioxidant activities in methanol extracts of lotus plumule and blossom may be related to their reducing powers. In conclusion, reducing power, hydroxyl radical and free radical scavenging effects and/or ferrous ion chelating ability may account for the antioxidant abilities of methanol extracts of lotus plumule and blossom in cell-free systems.

Herbs have played a significant role in maintaining human health and improving the quality of life for thousands of years. They have served as valuable ingredients for seasonings, beverages, cosmetics, dyes, and medicines. Many active phytochemicals, including flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins, and phthalides have been identified[34]. Numerous *in vitro* studies have shown that some of the phytochemicals are potent antioxidants, metal chelators or free radical scavengers, which may account for their health-promoting properties[35].

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