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Antioxidant activity and protective effect against oxidative stress induced-hemolysis of *Nymphaea lotus* L. extracts

Yoswaris Semaming[1, *], Sudawan Chunpricha[1], Atchariya Suriya[2]

[1] Program in Veterinary Technology, Faculty of Technology, Udon Thani Rajabhat University, Udon Thani, Thailand
[2] Program in Food and Services, Faculty of Technology, Udon Thani Rajabhat University, Udon Thani, Thailand
*Correspondent author: mai_jap@hotmail.com

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Abstract

Reactive oxygen species have been shown to play a role in degenerative diseases. Water lily or *Nymphaea lotus* L. is an aquatic plant. It has been used as medicinal plant to treat chronic diseases, especially cardiovascular disease, diabetes mellitus and aging. This aim of this study was to determine the phytochemical composition, antioxidant activities, ferric reducing antioxidant power and protective effect against oxidative stress induced-hemolysis of *Nymphaea lotus* L. petal extracts in rats red blood cells. *Nymphaea lotus* L. petal was extracted with distilled water, 50% ethanol and 95% ethanol. The extracts contained total phenol contents of 485.77 ± 15.70 mg/g, 660.43 ± 16.65 mg/g and 652.77 ± 18.66 mg/g respectively. Their total flavonoid contents were 17.40 ± 4.67 mg/g, 48.39 ± 6.21 mg/g and 42.10 ± 5.07 mg/g, respectively. DPPH scavenging activity was represented by IC_{50} values of 15.42±0.78 µg/ml, 8.35 ± 0.44 µg/ml and 8.96 ± 0.78 µg/ml, respectively. The ferric antioxidant activity of the distilled water extract was 1.31g. In 50% ethanol, it was 1.24 g and in 95% ethanol, it was 1.36g. This is similar to vitamin C at 1 g. The hemolysis protection value presented by the IC_{50} was 114.66 ± 5.73µg/ml, 45.37 ± 3.40 µg/ml and 69.45 ± 5.16µg/ml, respectively. These studies revealed the antioxidant activity and protective effects afforded cell membranes from free radicals by *Nymphae lotus* L. petal extracts. This was due to their rich composition of phenolic compounds.

Keywords: *Nymhaea lotus* L., Oxidative stress, Antioxidant, Ferric reducing antioxidant power, Hemolysis

1. Introduction

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. Examples include peroxides, superoxides, hydroxyl radicals, and singlet oxygen. In a biological context, ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis [1]. They move around in the body and have deleterious effect on cells. This may result in significant damage to cell structures. An antioxidant is a molecule that inhibits the oxidation of other molecules. To balance the oxidative state, plants and animals maintain complex systems of overlapping antioxidants, such as glutathione and enzymes (e.g., catalase and superoxide dismutase) produced internally or vitamins A, C and E obtained by ingestion. Phytochemicals such as phenolic acids, polyphenols and flavonoids can scavenge free radicals and result in inhibition of oxidative mechanisms that are responsible for many diseases in humans [2]. Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer or coronary heart disease. A polyphenol is a type of antioxidant containing a polyphenolic substructure. Numbering over 4,000 distinct species, many of these compounds have antioxidant activity *in vitro*, but are unlikely to have antioxidant roles *in vivo* [3].

Nymphaea lotus L. is an aquatic plant commonly known as water lily. The water lily is grown in various parts of Southeast Asia. The leaves of *Nymphaea lotus* L. have antioxidant activity and a rich phytochemical

composition [4]. The flowers of some species of the Nymphaea family such as *Nymphaea alba* are used in traditional medicines as cardiotonic, sedative, analgesic and anti-inflammatory agents [5]. The quantitative chemical composition and antioxidant potential of *Nymphaea lotus* L. flowers remains unknown. The present study investigated the phytochemical composition and evaluated antioxidant properties of extracts from these petals. The aims of this study were to evaluate total phenolic and total flavonoid contents of *Nymphaea lotus* L. flower extracts, to determine their level of 1,1-dihydroxy-1,2-picrylhydrazyl (DPPH) scavenging and to evaluate their protective effects against 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) induced-hemolysis. *Nymphaea lotus* L. petal extracts were made using distilled water, 50% ethanol and 95% ethanol.

2. Materials and Method

2.1 Plant collection and extraction

The flowers of water lilies were collected from Nong Han Kumphawapi Lake Kumphawapi District, Udon Thani Province in May, 2015. Their identity was authenticated and classified as *Nymphaea lotus* L. var. pubescencs Hook. f. & Th. by Assist. Prof. Dr. Maliwan Nakkuntod and Mr. Tasanai Punjansing, Naresuan University. The petals were rinsed under running water, air dried in the laboratory and later pulverized before extraction. The one hundred grams of the resulting plant powder was soaked in distilled water for 3 h at 60 °C, in 50% ethanol for 7 days and in 95% for 3 days on orbital shaker at room temperature. The extract was filtered using a Buchner funnel through Whatman No. 2 filter paper. The filtrate was concentrated to dryness under a reduced pressure at 50 °C using a rotary evaporator and later was dried by lyophilization.

2.2 Determination of total phenols

The total phenolic content of *Nymphaea. lotus* L. petal extracts was determined spectrophotometrically [6]. Five milliliters of the extract (1 mg/ml) was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water 1:9 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were mixed for 15 sec and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using a spectrophotometer. The results were expressed as mg/g of tannic acid equivalent.

2.3 Determination of total flavonoids

The amount of total flavonoids in petal extracts was determined using an aluminium colorimetric assay method [6]. Briefly, 0.5 ml of a 2% $AlCl_3$ ethanol solution was added to 0.5 ml of the sample solution. After 60 min at room temperature, its absorbance was measured at 420 nm. A yellow color indicates the presence of flavonoids. Extracts samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid contents were expressed as mg/g of quercetin.

2.4 DPPH radical scavenging assay

A DPPH assay was performed using the method of Mascato et al. (2015) [7]. About 1,000 µl of a 0.004% of DPPH in a methanol solution was added to 1,000 µl of extracts at various concentrations (0.025 - 0.5 mg/ml), with vitamin C and gallic acid used as standards. The mixture was vortexed thoroughly and left under dark conditions at room temperature for 30 min. The absorbance was measured at 517 nm. Radical scavenging activity was determined as percent inhibition using the following equation:

$$\text{Inhibition (\%)} = \frac{(Abs517_{control} - Abs517_{sample}) \times 100}{Abs517_{control}}$$

where, $Abs517_{control}$ is the absorbance of $DPPH^+$ and methanol, and $Abs517_{sample}$ is the absorbance of DPPH radical and sample extract or standards.

2.5 Ferric reducing power assay

The reducing power of extracts of *Nymphaea. lotus* L. was evaluated according to the method of Li et al. (2011) [8]. Various concentrations (0.025 - 0.5 mg/ml) of the extracts and standards were mixed with 2.5 ml of a 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium hexacyanoferrate II. The mixture was incubated at 50° C for 20 min, and then 2.5 ml of 10% trichloroacetic acid was added to the mixture and it was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of a 0.1% $FeCl_3$ solution. Its absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated stronger reducing power.

2.6 Investigation of protective effects of Nymphaea lotus L. extracts on AAPH induced-hemolysis

2.6.1 Preparation of red blood cells

Rats were anesthetized with Zoletil® 100 mg/kg BW i.p. and red blood cells (RBCs) were collected from the abdominal aorta in a heparinized tube. The RBCs were separated from plasma by centrifugation at 1,500 *g* for 10 min and washed three times with five volumes of phosphate buffer saline (PBS, pH 7.4) and diluted to a 20% cell suspension.

2.6.2 Induction of hemolysis

Red blood cells lysis was induced by a peroxyl radical initiator [9]. AAPH caused an oxidation of the lipids and proteins of cell membranes resulting in RBC break down. Five hundred microliters of a RBC suspension were mixed with 500 µl of *Nymphaea lotus* L. extracts at various concentrations (200 - 1000 µg/ml), then 250µl of 400 mM AAPH was added. The mixture was incubated at 37 °C for 3 h in a water bath. After incubation, 2 ml of PBS was added into the reaction mixture followed by centrifugation at 2000 *g* for 10 min. The absorbance of supernatant at 540 nm was measured using a spectrophotometer. The percentage of inhibition was calculated with the following equation:

$$\text{Inhibition (\%)} = \frac{(Abs540_{control} - Abs540_{sample}) \times 100}{Abs540_{control}}$$

where, $Abs540_{control}$ is the absorbance of the sample without extract, and $Abs540_{sample}$ is the absorbance of the sample with the extract or standards.

2.7 Chemicals

Ascorbic acid (vitamin C) and ferric chloride hexahydrate were obtained from Sigma Chemical Co., (St Louis, MO, USA). 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Alrich Chemical Company Inc., (Milwaukee, WI, USA). 1,1-dihydroxy-1,2-picrylhydrazyl (DPPH) was obtained from Fluoka Chemika (AG, Switzerland).

2.8 Statistical analysis

All data are presented as a mean value ± SD of at least 3 experiments and analyzed using One-Way ANOVA followed by LSD test to show specific group differences. The level of significance was uniformly set at a p-value less than 0.05.

3. Results and Discussions

3.1 Phytochemical components in Nymphaea lotus L. petal extracts

In this study, the 100 g of dried *Nymphaea lotus* L. petal yielded 4.10 g, 22.5 g and 8.07 g in distilled water, 50% ethanol and in 95% ethanol, respectively. Almost all the phytochemicals had antioxidant activity that protected the cells from ROS and therefore, reduced the risk of developing of degenerative diseases [10]. The amounts of phenols and flavonoids from *Nymphaea lotus* L. extract were considerable. The results of quantitative phytochemical analysis are shown in Table 1. The quantity of total phenols was significantly higher ($p<0.05$) in 50% ethanol (660.43 ± 16.65 mg/g) than in 95% ethanol (652.77 ± 18.66 mg/g) and in distilled water (485.77 ± 15.70 mg/g). The quantity of total flavonoids was significantly higher ($p<0.05$) in 50% ethanol (48.39 ± 4.91 mg/g) than in 95% ethanol (42.10 ± 5.07 mg/g) and in distilled water (17.40 ± 4.67 mg/g). This is in agreement with Anthony et al. (2013), who reported the composition of phytochemicals in *Nymphaea lotus* linn. leaf extracts [11]. Interestingly, total phenol and total flavonoid contents from petal extracts is higher than from leaf extracts.

Table 1 Phytochemical analysis of *Nymphaea lotus* Lextracts (total phenolic content expressed as tannic acid equivalents per g of extract and total flavonoid express as quercetin equivalents per g of extract)

Solvents	Total phenol (mg/g)	Total flavonoid (mg/g)
DW	485.77 ± 15.70	17.40 ± 4.67
50%ETH	660.43 ± 16.65[a]	48.39 ± 4.91[a]
95%ETH	652.77 ± 18.66[a]	42.10 ± 5.07[a]

Data are mean ± SD (n= 3), DW: petal extract by distilled water; 50% ETH: petal extract by 50% ethanol; 95%ETH: petal extract by 95% ethanol. [a] Significantly different in comparison with DW ($p<0.05$)

This study showed that the total phenolic contents of 50% and 95% ethanolic extracts were significantly higher than that of the aqueous extracts. And the total phenol contents were highest in the 50% ethanol extract. This is important because natural phenolic compounds play an important role in prevention and treatment of cancer [12]. A previous study reported a positive correlation between free radical scavenging activity and the level of total phenolic compounds [13]. In the future, a study should determine and classify the phenolic compounds in the 50% ethanol petal extract.

The total flavonoids content was the highest in the 50% ethanol petal extract. Flavonoids are involved in scavenging reactive oxygen species [14]. Previous studies reported that flavonoids had hypolipidemic activity [15]. It has also been established that flavonoids from medicinal plants possess high antioxidant potential due to their hydroxyl groups. They can protect humans better against free radical related diseases [16]. It has been confirmed experimentally that flavonoids enhance the vasorelaxant process [17], and prevent platelet activity-related thrombosis [18], thereby reducing the risk of cardiovascular mortality.

3.2 DPPH scavenging activity of Nymphaea lotus L. petal extracts

The DPPH assay has been widely used for the determination of antioxidant activity of pure compounds as well as of various plant extracts. A lower DPPH IC_{50} represents a stronger antioxidant capacity [19]. The results of the IC_{50} measurements generated for inhibition of DPPH radical are shown in Table 2. The concentration required to scavenge 50% of the DPPH radical (IC_{50}) was determined from a series of concentration tests. Lower the IC_{50} revealed the higher DPPH scavenging activity. The magnitude of the IC_{50} values of test samples was distilled water (15.42 ± 0.78 µg/ml) > 95% ethanol (8.96 ±0.78 µg/ml) > 50% ethanol (8.35 ± 0.44 µg/ml) > vitamin C (5.06 ± 0.12µg/ml) > gallic acid (2.20 ± 0.03 µg/ml).

Table 2 DPPH scavenging activity of *Nymphaea lotus* L extracts

Group	IC_{50} (µg/ml)
VC	5.06 ± 0.12
GA	2.20 ± 0.03[a]
DW	15.42 ± 0.78[a,b]
50%ETH	8.35 ± 0.44[a,b,c]
95%ETH	8.96 ± 0.78[a,b,c]

Data are mean ± SD (n= 3), VC: vitamin C; GA: gallic acid; DW: petal extract by distilled water; 50% ETH: petal extract by 50% ethanol; 95% ETH: petal extract by 95% ethanol. [a] Significantly different in comparison with VC ($p<0.05$), [b] Significantly different in comparison with GA ($p<0.05$), [c] Significantly different in comparison with DW ($p<0.05$)

DPPH is characterized as stable free radical by the delocalization of the spare electron, where the molecule do not dimerise, as happens in the case of other free radicals [20]. The DPPH scavenging activity of the 50% ethanol extract was strongest among the extracts. The phenolic compounds present in the *Nymphaea lotus* L. extracts could be responsible for the observed DPPH radical scavenging activity, since phenols can readily donate hydrogen atoms to radicals [21]. This is because of the quantity of phenolic compounds in an extract is directly proportional to its free radical scavenging capacity [13]. A previous study reported a positive correlation between DPPH radical scavenging activity and total phenolic content [22]. Moreover, the study reported that phenolic compounds have chemopreventive properties such as antioxidant, anticarcinogenic and anti-inflammatory effects [23]. Future study should determine antioxidant activity in animal models.

3.3 Ferric reducing antioxidant power of Nymphaea lotus L. petal extracts

Ferric reducing ability of plasma (FRAP, also Ferric ion reducing antioxidant power) is an antioxidation capacity assay. It is often used to measure the antioxidant capacity of herbs, foods, beverages and nutritional supplements containing polyphenols [24&25]. The results showed that the capability of *Nymphaea lotus* L. extract to reduce ferric cyanide was not different among the group of extracts when comparing the concentration of each extract at the same absorbance (OD=0.3) (Table 3). The ferric reducing activity of extracts is reported as vitamin C equivalents per g of extract. The result showed this value for 0.80 in 50% ethanol, 0.76 in distilled water and 0.73 in 95% ethanol. The higher values revealed higher ferric reducing activity.

Table 3 Ferric reducing antioxidant power of *Nymphaea lotus* L petal extracts

Group	Concentration (µg/ml) (at OD=0.3)
VC	5.96 ± 0.31
GA	1.93 ± 0.25[a]
DW	7.81 ± 0.60[a,b]
50%ETH	7.44 ± 0.69[a,b]
95%ETH	8.12 ± 0.32[a,b]

Data are mean ± SD (n= 3), VC: vitamin C; GA: gallic acid; DW: petal extract by distilled water; 50% ETH: petal extract by 50% ethanol; 95% ETH: petal extract by 95% ethanol. [a] Significantly different in comparison with VC ($p<0.05$) , [b] Significantly different incomparison with GA ($p<0.05$) , [c] Significantly different in comparison with DW ($p<0.05$)

The Fenton reaction (Iron or Ferrous) is believed to be responsible for the creation of free radicals. This is because iron exists at the highest concentration of any transition metal in most living organisms. It generates two types of oxidants which can cause oxidative damage [26]. In this study, the FRAP activity of the 50% ethanol petal extract was higher than for other extracts. According to Anthony et al. (2013), *Nymphaea lotus* L. leaf extract has ferric reducing antioxidant activity [11]. This activity results from its rich total phenolic and flavonoid contents. Future study should determine this activity for the 50% ethanol petal extract in an animal model.

3.4 Protective effect of Nymphaea lotus L. petal extracts on AAPH-induced hemolysis

Cell membranes are most susceptible to free radical attack due to their high content of polyunsaturated fatty acids. Lipid oxidation of RBC membranes, mediated by AAPH, induces membrane damage and subsequent hemolysis [27]. The protective ability of the extracts against AAPH-induced hemolysis is presented in Table 4. AAPH caused more than a 95% erythrocyte lysis after incubation for 3 h. The protective effects of vitamin C, gallic acid and petal extract are reflected by the percentage of hemolysis inhibition at various concentrations of phytochemicals. The results showed that IC_{50} values were significantly the lowest for gallic acid (34.65 ± 1.73 µg/ml), followed by the 50% ethanol extract (45.47 ± 3.40 µg/ml), vitamin C (57.04 ± 3.72 µg/ml), the 95% ethanol extract (69.45 ± 5.16 µg/ml) and the distilled water extract (114.66 ± 5.73 µg/ml), respectively. Lower IC_{50} values represent higher protective ability against oxidative stress-induced hemolysis.

Table 4 Protective effect of *Nymphaea lotus* L petal extracts on AAPH-induced hemolysis

Groups	IC_{50} (µg/ml)
VC	57.04 ± 3.72
GA	34.65 ± 1.73[a]
DW	114.66 ± 5.73[a]
50%ETH	45.37 ± 3.40[a,b,c]
95%ETH	69.45 ± 5.16[a,b,c,d]

Data are mean ± SD (n=3), VC: vitamin C; GA: gallic acid; DW: petal extract by distilled water; 50% ETH: petal extract by 50% ethanol; 95% ETH: petal extract by 95% ethanol. [a] Significantly different in comparison with VC ($p<0.05$) , [b] Significantly different in comparison with GA ($p<0.05$) , [c] Significantly different in comparison with DW ($p<0.05$) and [d] Significantly different in comparison with 50%ETH ($p<0.05$)

Interestingly, the present study revealed that *Nymphaea lotus* L. petal extracted using 50% ethanol gave a greater protective effect to cell membranes from the oxidation of RBC incubated with AAPH higher than vitamin C. This is the first evidence to of an antioxidant activity of *Nymphaea lotus* L. petal extract for cell membrane protection from reactive oxygen species. This activity must be further investigated in an animal model.

4. Conclusion

This study revealed that the petals of *Nymphaea lotus* L. are very rich in phenols and flavonoids. These results also showed that extracts of *Nymphaea lotus* L. petals have antioxidant power. It can be suggested that their phenolic compounds may be responsible for the high antioxidant power of this plant. Further study should investigate in animal model.

5. Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

6. Acknowledgements

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