

Phytochemicals, and Anti-oxidative DNA Damage Activity Against H_2O_2 of *Nymphaea lotus* Linn. Flowers Ethanolic Extracts

Ubon Rerk-am¹, Janram Saenkhum¹, Bantika Kongsombat¹, Prapaipat Klungsunya¹, Chuleratana Banchonglikitkul¹

Abstract

Introduction: *Nymphaea lotus* Linn. is a tender aquatic plants in the Nymphaeaceae family. The common name in Thailand is called “BuaSai” and has been used a part of stem as vegetable. For the part of the flowers used as one kind of ingredients in a Thai traditional recipe called Yahom.

Methods: The phytochemical contents of *N. lotus* flowers were analysed using HPLC technique comparing with authentic standard. The evaluation of antioxidant activity in ethanolic extract of *N. lotus* flowers using the 2,2-Diphenyl-1-picrylhydrazin (DPPH) radical scavenging assay and lipid peroxidation (b-carotene bleaching model). The anti-oxidative DNA damage was performed following pre-treatment TK6 cells with extracts at concentration of 37.5, 75 and 150 $\mu\text{g}/\text{mL}$ for 24 hr follow by H_2O_2 induction.

Results: The ethanolic extracts of *N. lotus* flowers were composed of phenolic and flavonoid compounds such as gallic acid, corilagin, geraniin, ellagic acid, kaempferol-3- β -D-glucopyranoside and quercetin. Anti-oxidant activity value (EC_{50}) was 1.56 $\mu\text{g}/\text{mL}$ and 2.8 $\mu\text{g}/\text{mL}$ that determined using DPPH and β -carotene bleaching assay, respectively. The range of those concentration was showed as the dose response of DNA protective effect at the percentage of 56.55 ± 4.87 , 90.96 ± 0.97 and 93.53 ± 0.78 .

Conclusion: *N. lotus* flowers extract showed high potent of anti oxidative DNA-damage activity due to phenolic and flavonoid compounds in crude extracts. Thus, the *N. lotus* flowers extract could be used as anti-aging agent in nutraceutical and cosmetic applications.

Keywords: *Nymphaea lotus* Linn, anti-oxidative DNA damage activity, phytochemical compounds, flavonoid compounds, phenolic compounds

¹ Researcher, Pharmaceuticals and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR),

* **Corresponding author:** Ubon Rerk-am, Pharmaceuticals and Natural Products Department Thailand Institute of Scientific and Technological Research (TISTR), 35 Moo 3, Techno Polis, KlongLuang, PathumThani 12120, Thailand, E-mail: Ubon@tistr.or.th

1. Introduction

Nymphaea lotus Linn., the white water lily belongs to the Nymphaeaceae family. The common name in Thailand is called BuaSai. For centuries it has been cultivated in Southeast Asia, especially in ponds and tanks around the temple. Thai peoples used apart of stem as vegetable. The chemical component in the leaf have shown the presence of alkaloids, tannin, and flavonoids compounds such as myricetin, nympholide A and B (Sowemimoet *al.*, 2007). The ethanolic extract of *N. lotus leaves* are displayed anti-radical scavenging activity of DPPH, ABTS, nitric oxide and hydroxyl radical (H_2O_2). (Afolayanet *al.*, 2013). All the parts of white water lily (*N. alba*) extract were acted as anxiolytic and muscle relaxant properties (Thippeswamyet *al.*, 2011). The phenolic compounds such as gallic acid, ellagic acid have been identified in the flowers. The ethanolic extracts exhibited high anti-radical scavenging activity in DPPH, nitric oxide and hydroxyl radical (Madhusudhananet *al.*, 2011).

Oxidative stress is defined in general as excess formation of highly reactive molecules such as reactive oxygen species (ROS). The excess production of free radicals and reactive oxygen species are thought to cause oxidative damages including lipid peroxidation, DNA lesions and protein fragmentation within the cell of biological macromolecules and certain degenerated diseases like cancer, inflammation, cardiovascular and neurodegenerative diseases (Emenet *al.*, 2009). Hydrogen peroxide (H_2O_2) is one of highly reactive oxygen species byproduct of normal aerobic metabolism. Generation of H_2O_2 in

biological system results in immediately damage to DNA molecules and the sequent DNA lesions (Klungsupyaet *al.*, 2012).



Figure 1: *Nymphaea lotus* Linn flower (water lily)

2. Materials and Methods

Preparation of plant extracts:

The fresh flowers of *N. lotus* were collected from Lad LumKaew, Pathumthani Province. The whole of flower were dried at 40 °C, ground into powder and extracted with 2 liters 95% ethanol for 10 times at room temperature. The combined filtrates of ethanol solution were evaporated under reduced pressure at room temperature to yield 8.9 % w/w of crude ethanolic extract.

HPLC analysis

Exactly 1 mg of gallic acid, corilagin, geraniin, ellagic acid, kaempferol-3- β -D-glucopyranoside, quercetin, kaempferol authentic standard (99.29%) and 20 mg *N. lotus* extract were accurately weighed and transferred to a 5 mL volumetric flask and dissolved firstly in methanol. The samples were then sonicated at room

temperature for 20 min. The solution so obtained was filtered through 0.45 µm pore size filter before use.

HPLC instruments: Waters 2695 separation module equipped with waters 2998 photodiode array detector

Column: pHedure C-18 column; 250 mm x 4.6 mm with 5 µm (VertiseptTM).

Mobile phase: solvent A (0.005 % TFA/H₂O), solvent B (0.001 % TFA/ACN) Elution system; 0 - 10 min : 14 % solvent B 10 -30 min : 18 % solvent B 30 - 50 min : 25 % solvent B 50 - 60 min : 35 % solvent B 60 - 70 min : 70 % solvent B 70 - 80 min : 100 % solvent B flow rate 1.0 mL/min at 30 °C. The wavelength was monitored at 254 nm.

Scavenging of Diphenyl-picrylhydrazyl (DPPH) Radicals Assay

The free radical scavenging activity of ethanolic extracts was analyzed by the DPPH assay (Duan *et al.*, 2006). The amount of 100 µl of various concentration samples were reacted with 100 µl of 6 mM DPPH ethanolic solution in a 96-well plate, incubated at 37 °C for 30 min. The absorbance was measured at 517 nm using a UV-VIS microplate reader. All experiments were carried out in triplicates.

Lipid Peroxidation (b-Carotene Bleaching Model)

The antioxidant activity of crude extract was measured by b-carotene bleaching model system with slight modification (Kawareet *et al.*, 2008).. Emulsion I was prepared by dissolving 10 mg of b-carotene in 10 mL of chloroform. Four

milliliters of b-carotene solution, 40 mg of linoleic acid and 400 mg of Tween 40 were mixed and removed chloroform at 50 °C under vacuum by rotary evaporator. The emulsion was further made up to 100 mL with MillQ water. Emulsion II was prepared the same as emulsion I without b-carotene. Test sample, 50 µl of varied concentration samples were mixed with emulsion I. Blank sample, 50 µl of varied concentration samples were mixed with emulsion II. Absorbance was measured at 450 nm after incubated reaction mixture in oven at 50 °C and kinetic reading was monitored from zero time (t=0) till 60 min.

Hydrogen peroxide-induced DNA damage in TK6 cells.

TK6 human lymphoblast cell line (ATCC CRL-8015) was cultured as cell suspension and maintained as exponentially growth phase in RPMI 1640 medium supplemented with 10 % heat inactivated horse serum and 1 % penicillin-streptomycin and incubated at 37 °C in humidified atmosphere containing 5 % CO₂. The cell was maintained by addition of fresh medium every 2-3 days. At the density of 2 x10⁵ cell/mL were seeded onto 12 well-plate and separately exposed to medium containing various concentration of *N. lotus* extract. The plate incubated for 24 h at 37 °C in 5 % CO₂ incubator. By the end of treatment time, *N. lotus* extract was removed by centrifugation at 3,000 rpm for 3 min. TK6 human lymphoblast cell was treated with 50 µM H₂O₂ and performed Comet assay (single cell gel electrophoresis) (Klangsupyaet *et al.*, 2012). Trolox was used as positive antioxidant compound.

3. Results

Phytochemical analysis

The chemical constituents of *N.lotus* flower were analyzed using HPLC technique. The chromatogram composed of gallic acid (RT:9.122),

corilagin (RT :15989), geraniin (RT:17.845), ellagic acid (RT:32.043), kaempferol-3-β-D-glucopyranoside (RT:41.628), quercetin (RT:57.869), comparison to authentic standard as shown in Fig 2.

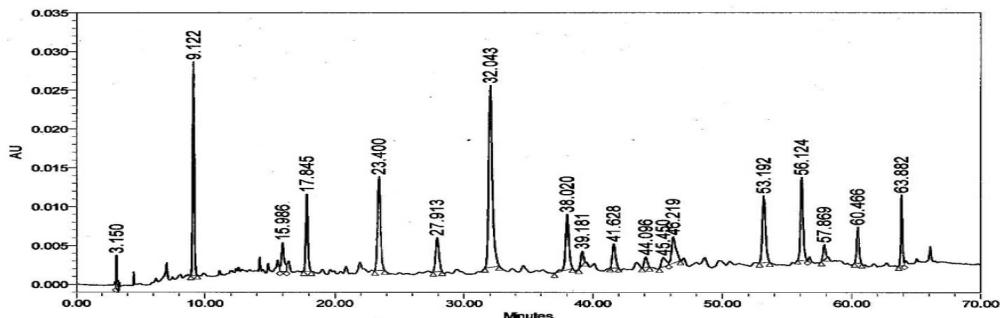


Figure 2: HPLC chromatogram of ethanolic extract of *N. lotus* flower at 254 nm RT; 9.122:gallic acid, 15.989:corilagin, 17.845:geraniin, 32.043:ellagic acid, 41.628:kaempferol-3-β-D-glucopyranoside, 57.869:quercetin

Radical scavenging activity

The concentration of *N. lotus* flowers ethanolic extracts in antioxidants properties to quench DPPH radical (EC₅₀) was showed in Table 1. The EC₅₀ value of crude extract was 1.58 µg/mL, slightly lower activity than Trolox (1.9 µg/mL) and gallic acid (0.98 µg/mL). Lipid peroxidation was estimated of capacity of crude extract to

protect b-carotene color change from yellow to colorless. The activity (EC₅₀) of crude extract, gallic acid and Trolox were 2.80 µg/m, 1.1 µg/mL and 0.9 µg/m, respectively. These results show that the ethanolic extracts of *N. lotus* flowers are strong antioxidant agent comparing to well-known anti-oxidant activity standard compounds (Trolox and gallic acid).

Table1. Antioxidant activity (EC50) of ethanolic extract of *N.lotus* flowers, compared with standard

Sample	DPPH assay (µg/mL)	Lipid peroxidation (µg/mL)
Ethanolic extract of <i>N.lotus</i> flowers	1.58	2.80
Gallic acid	0.98	1.10
Trolox	1.90	0.90

Anti-oxidative DNA damage activity

Anti-oxidative DNA damage activity against H₂O₂ using comet assay was demonstrated, after TK6 lymphoblast cell suspended in vary concentration of *N. lotus* extract and then treated with 50 µM H₂O₂ for 5 min producing oxidative DNA damage. The result was about 10-fold greater than control. This was indicated

by highly increased comet tail length (TL) and tail movement (TM) values in treated cells. Anti-oxidative DNA of *N. lotus* extract was indicated by reduction in TL and TM damage parameters in comparison with TK6 cell received H₂O₂ alone (Fig.2). The DNA protective effect was found in all concentration test as showed in Table 2.

Table 2 : The inhibition DNA damage against H₂O₂ using comet assay of ethanolic extract of *N. lotus* flower

Sample	Tail Length			Tail Moment			%inhibition					
	Ave	SD	SD	Ave	SD	SD	Ave	SD	SD			
	(µm)			(%)								
Control	15.79	13.04	14.42	1.95	0.43	0.45	0.44	0.02	95.69	95.29	95.49	0.29
50 µM H ₂ O ₂	54.44	57.40	55.92	2.09	9.97	9.63	9.80	0.24	0.00	0.00	0.00	0.00
75 µg/mL Trolox	45.46	54.94	50.20	6.70	7.94	7.03	7.49	0.64	20.38	26.99	23.69	4.67
<i>N. lotus</i> extract 37.5 µg/mL	38.05	43.72	40.89	4.01	3.99	4.52	4.25	0.37	59.99	53.10	56.55	4.87
<i>N. lotus</i> extract 75 µg/mL	23.70	19.30	21.50	3.11	0.97	0.81	0.89	0.12	90.27	91.64	90.96	0.97
<i>N. lotus</i> extract 150 µg/mL	18.76	17.97	18.36	0.56	0.59	0.68	0.63	0.06	94.09	92.98	93.53	0.78

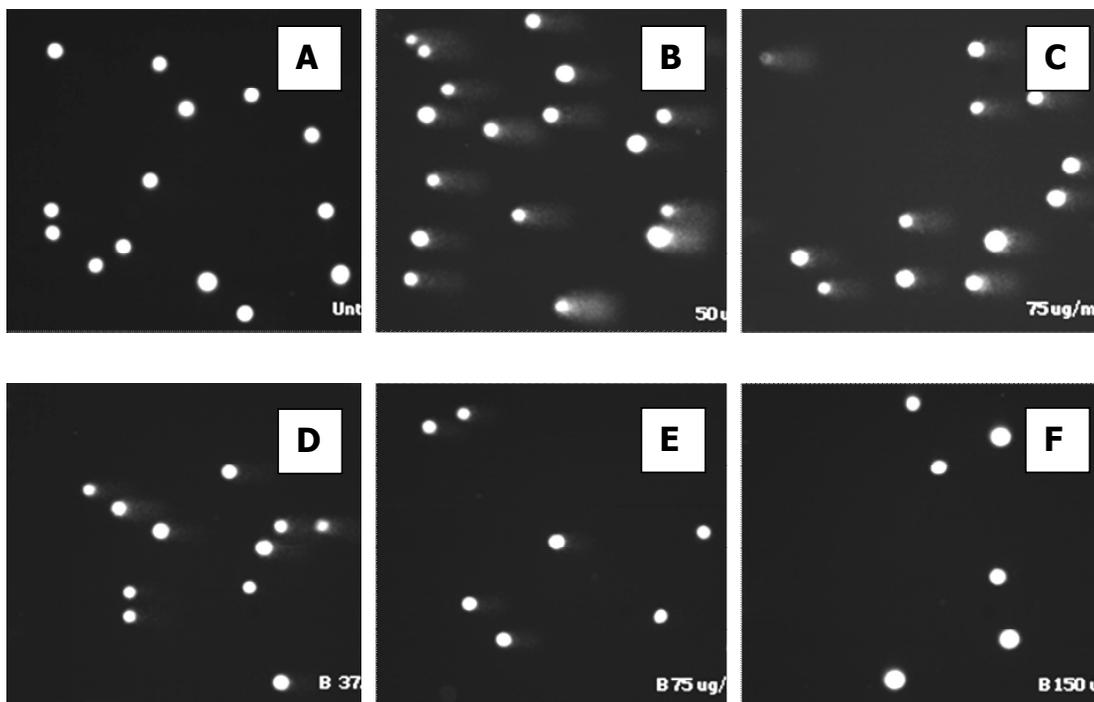


Figure 3: DNA comet image:[A]: control cells, [B]: 50 μM H_2O_2 -treated cells, [C]:50 μM H_2O_2 -treated cells+75 $\mu\text{g}/\text{mL}$ Trolox (positive control), [D],[E],[F] : 50 μM H_2O_2 -treated cells + *N. lotus* extract at 37.5, 75.0 and 150 $\mu\text{g}/\text{mL}$, respectively.

4. Conclusion

The ethanolic extract of *N. lotus* flowers showed high potent of radical scavenging and antioxidative DNA-damage activity. The active components are mediated the biological property against reactive oxygen species corresponding to high concentration of bioflavonoid (kaempferol-3- β -D-glucopyranoside and quercetin) and phenolic compounds (gallic acid, ellagic acid, corilagin and geraniin). Thus the ethanolic extracts of *N. lotus* flowers possess potential antioxidant property. It would be promising to do further studies as anti-wrinkle agents. Future biological investigations on human dermal fibroblast need to be done in order to confirm these activities and safety evaluation.

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