

การวิเคราะห์สารออกฤทธิ์ทางชีววิทยาที่มีคุณสมบัติการต้านอนุมูลอิสระและการยับยั้งไทโรซิเนสของบัวหลวง

Quantitative Analysis of Bioactive Compounds with Antioxidant and Tyrosinase Inhibitory Effects of *Nelumbo nucifera*

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บทคัดย่อ

บัวเป็นพืชสมุนไพรที่นิยมใช้อย่างกว้างขวางโดยเฉพาะเอเชียซึ่งมีสารออกฤทธิ์ทางชีวภาพที่น่าสนใจและมีคุณสมบัติส่งเสริมสุขภาพ สารประกอบเหล่านี้ได้แก่สารต้านอนุมูลอิสระที่เป็นสารประกอบฟีนอลและฟลาโวนอยด์สามารถจับกับอนุมูลอิสระและยับยั้งกลไกที่ทำให้เกิดการควบคุมการเสื่อมสลายและโรคต่างๆ ในการศึกษาครั้งนี้ได้ทำการศึกษาคงสมบัติการต้านอนุมูลอิสระและการยับยั้งกิจกรรมไทโรซิเนสของสารสกัดจากส่วนต่างๆของบัวเพื่อเปรียบเทียบและการนำไปประยุกต์ใช้ทางการแพทย์ต่อไป ในการศึกษาได้สกัดส่วนของเปลือกบัว เนื้อบัว ผักบัว และเมล็ดบัว ทำการตรวจวิเคราะห์คุณสมบัติการรีดิวซ์เฟอริก/การต้านอนุมูลอิสระเพื่อใช้ในการศึกษาคงสมบัติการต้านอนุมูลอิสระ นอกจากนี้ได้ตรวจวิเคราะห์สารประกอบฟีนอลิก สารประกอบฟลาโวนอยด์ ทั้งหมดและสารประกอบแทนนินของสารสกัด ตรวจวิเคราะห์กิจกรรมการยับยั้งไทโรซิเนสโดยมีกรดโคจิกเป็นสารควบคุมจากการศึกษาพบคุณสมบัติของสารสกัด แสดงว่ามีสารประกอบฟีนอลิก ฟลาโวนอยด์ และแทนนิน ส่วนสกัดต่างๆมีคุณสมบัติต้านอนุมูลอิสระ อย่างไรก็ตามเมล็ดมีคุณสมบัติการต้านอนุมูลอิสระสูงสุดจากการวิเคราะห์โดยการรีดิวซ์เฟอริก/การต้านอนุมูลอิสระโดยมีคาร์รีดิวซ์เฟอริก/การต้านอนุมูลอิสระใกล้เคียงกับสารมาตรฐานกรดแอสคอร์บิก คุณสมบัติการรีดิวซ์เฟอริก/การต้านอนุมูลอิสระมีค่าเพิ่มขึ้นตามปริมาณสารสกัดที่สูงขึ้น สารประกอบฟีนอลิกทั้งหมด (61.43 ± 1.52 มิลลิกรัม กรดแกลลิก/กรัมสารสกัด) และสารประกอบฟลาโวนอยด์ทั้งหมด (3.34 ± 0.65 มิลลิกรัม คลอซีทิน/กรัมสารสกัด) ของผักบัวมีปริมาณสูงกว่าสารสกัดจากส่วนอื่นได้แก่เปลือกเมล็ด เนื้อเมล็ดและเมล็ด การศึกษานี้แสดงให้เห็นว่าเมล็ดและผักบัวมีคุณสมบัติการต้านอนุมูลอิสระและการยับยั้งกิจกรรมไทโรซิเนสสูง ผลการศึกษานี้แสดงว่าส่วนประกอบดังกล่าวมีศักยภาพในการสามารถใช้เป็นสารต้านอนุมูลอิสระและยับยั้งไทโรซิเนสที่ได้จากธรรมชาติได้

คำสำคัญ: บัว การต้านอนุมูลอิสระ ไทโรซิเนส

Abstract

Nelumbo nucifera is a well known and widely used herb, especially in Asia, which contains several remarkable bioactive constituents and possesses health promoting properties. Antioxidant compounds like

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phenols and flavonoids scavenge free radicals and thus inhibit the oxidative mechanisms that lead to control degenerative and other diseases. In this study, the antioxidant and tyrosinase-inhibition activities of extracts from different parts of *N. nucifera* were assessed in an effort to compare and validate the medicinal potential. *N. nucifera* pericarp, endosperm, torus and embryo were extracted. Ferric-reducing/antioxidant power (FRAP) assay was used to detect the antioxidant activity. Further, the total yield, total phenolic (TPC), total flavonoid contents (TFC) and tannins of all the fractions were determined. Tyrosinase enzyme inhibition was investigated with kojic acid as positive control. Phytochemical screening of crude extract revealed the presence of phenolics, flavonoids, and tannins. All the fractions exhibited antioxidant activities, however, embryo showing the highest antioxidant activity based on the results of FRAP assay tests. The above fraction has shown the significant FRAP when compared with standard ascorbic acid. The FRAP activities increased with increasing crude extract/fractions content. The TPC (61.43 ± 1.52 mg GAE/g extract) and TFC (3.34 ± 0.65 mg QE/g extract) of torus were found significantly higher as compared to other parts for pericarp, endosperm and embryo. The present study showed that the embryo and torus exhibited strong antioxidant and tyrosinase-inhibition activities. These results suggest that these parts could be exploited as a potential source of natural antioxidant agents as well as tyrosinase inhibitors.

Keywords: *Nelumbo nucifera*, antioxidant, tyrosinase

Introduction

Nelumbo nucifera, commonly known as *N. nucifera* or sacred *N. nucifera* is an aquatic perennial plant belonging to family *Nelumbonaceae*.¹ *N. nucifera* is an important aquatic economic plant, not only as a dainty and ornamental flower but also as a source of herbal medicine with strong antipyretic, cooling, astringent, and demulcent properties. The seeds and leaves are also eaten.² All parts of the *N. nucifera* plant are used: the rhizome is used as food, seed as medicine, thalamus as fruit, leaves as plate (thali), stalks as pickle, petals for colour extraction, and tender leaves as food after being blended with vegetables.³

Tyrosinase is catalytic enzyme in browning of fruits and vegetables due to catalysis of phenolic compounds oxidation to active quinones which lead to browning formation of polymeric pigments. These quinones may

irreversibly react with amino or sulfhydryl groups of amino acid in proteins, lead to decrease essential amino acids and reduce proteins digestibility and their nutritive value. To prevent the browning and preserve the nutritional value of food, development of high effective tyrosinase inhibitors has great importance for the agricultural field and the food industry.⁴ Most of the inhibitors so far characterized are synthetic compounds or derived from higher plants.⁵ Among synthetic compounds, the specificity and mechanism of inhibition are very variable and characteristic of the chemical nature of the compounds.⁶ Some inhibitors affect both mono- and diphenolase activities of tyrosinase by chelating the active site of the enzyme. Since plants are a rich source of bioactive chemicals that are mostly no side effects, interest in finding tyrosinase inhibitors from natural sources is increasing.^{7,8} The effects of plant materials could result from isolated substances but typically derive from the synergy



of different bioactive compounds present in the plant.

In the present study, the antibrowning activity in term of inhibit tyrosinase activity and antioxidant activity of extracts from embryo, torus, pericarp and endosperm of *N. nucifera* was evaluated.

Materials and Methods

Chemicals and Samples

N. nucifera was purchased from Talat Tai local market, Pathom Thani province. Flavonoids (quercetin), phenolic acids (tannic acid and gallic acid) and ascorbic acid were all from Sigma Chemical Co. (St. Louis, MO). TPTZ (2,4,6-tripyridyl-s-triazine) was purchased from Fluka Chemicals (Madrid, Spain). All other reagents were of analytical grade.

Extraction of *N. nucifera* samples

Five grams of each of the *N. nucifera* constituents, pericarp, endosperm, embryo and torus were cut into small pieces and homogenized with blender. The samples were extracted with 75% methanol at room temperature for 12 h (repeated three times) and then was filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). The residue was evaporated at 50°C reduced pressure.

Determination of total Phenolic contents

The amount of total phenolics in extracts was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). Concentration of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL of gallic acid were prepared in methanol. Concentration of 0.1 and

1mg/mL of plant extract were also prepared in methanol and 0.5mL of each sample were introduced into test tubes and mixed with 2.5mL of a 10 fold dilute Folin-Ciocalteu reagent and 2mL of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature before the absorbance was read at 760 nm spectrometrically. All determinations were performed in triplicate. The Folin-Ciocalteu reagent being sensitive to reducing compounds including polyphenols is producing a blue color upon reaction which is measured spectrophotometrically.⁹

Total flavonoid content

The total flavonoid content was determined using a modified version of the method described by Zhishen et al.¹⁰ Each test sample (250 μ L) and 1.25 mL of distilled water were added then 75 μ L of 5% NaNO₂, 150 μ L of 10% AlCl₃ was added. After 6 min 0.5 mL of 1 M NaOH was added. The absorbance of the solution was measured against a blank at 510 nm using a spectrophotometer.

Determination of tannin contents:

Tannin content in each extract sample was determined using insoluble polyvinyl-pyrrolidone (PVPP), which binds tannins as described by Makkar et al.¹¹ One milliliter of extract dissolved in methanol (1 mg/mL), in which the total phenolics were determined, was mixed with 100 mg PVPP, vortexed, kept for 15 min at 4°C and then centrifuged for 10 min at 3,000 rpm. In the clear supernatant the non-tannin phenolics were determined the same way as the total phenolics.⁹ Tannin content was calculated as a difference between total and non-tannin phenolic content.

Ferric – reducing / antioxidant power (FRAP) assay

The antioxidant capacity was estimated according to the procedure described by Benzie and Strain¹² with some modifications. The FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L FeCl₃·6H₂O and 25 mL of 0.3 mol/L acetate buffer, pH 3.6. Nine hundred microliter of FRAP reagent, prepared freshly and warmed at 37 °C, was mixed with 90 µL of distilled water and 30 µL of test sample, water, or methanol as appropriate for the reagent blank. Readings at the absorption maximum (595 nm) were taken using a Beckman DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Temperature was maintained at 37 °C and the reaction monitored for up to 30 min. Aqueous and methanolic solutions of known Fe(II) concentrations in the range of 100-2000 µmol/L (FeSO₄·7H₂O) were used for calibration.

Assay for tyrosinase inhibitory activity

Tyrosinase inhibitory activity was measured spectrophotometrically according to the method of Masamoto et al.¹³ with some modifications. Ten microliter of extract solution in DMSO was added to a 96-well microplate and mixed with 60 µL 50 mmol/L phosphate buffer (pH 6.8) on ice. Twenty microliter of 0.9 mg/mL L-DOPA in phosphate buffer was added. Ten microliter of mushroom tyrosinase (500 U/mL in phosphate buffer) was added and the assay mixture was then incubated at 30°C for 10 min. Following incubation, the amount of dopachrome production in the reaction mixture was determined spectrophotometrically at 450 nm in a microplate reader. Kojic acid (2.9–77 mmol/L) dissolved in 50

mmol/L phosphate buffer was used as a positive control. The concentration for 50% inhibition (IC₅₀) was determined. Each measurement was performed at least in triplicate.

Results and Discussions

Total phenolics and tannins contents

The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent. The values obtained for the concentration of total phenols are expressed as mg of GAE/g of extract (Table 1). The total phenolic contents in the examined extracts ranged from 11.66 ± 0.42 to 61.43 ± 1.52 mg GAE/g. The highest concentration of phenols was measured in torus extracts.

The non-tannins contents was examined in plant extracts and expressed in terms of quercetin equivalent. The highest concentration of non-tannins was measured 14.12 ± 0.92 in torus extract (Table 1).

Tannin contents of the extract from different part of *N. nucifera* are expressed in terms of mg GAE per liter, and shown in Table 1. In 1 gram extract, 9.21-47.30 mg GAE of tannins were detected. The tannins contents were examined in *N. nucifera* extracts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent. The values obtained for the concentration of tannin contents are expressed as mg of GAE/g of extract. The highest concentration of tannins was measured 47.30 ± 1.53 in torus extract (Table 1).

Among the extracts, torus had the highest contents of phenolics and tannin, followed by embryo, pericarp and endosperm.



Table 1 The total phenolic, total flavonoid, tannin and non-tannin contents (mg/g extract) present in *N. nucifera* constituents.

Constituent	Embryo (mg/g)	Endo-sperm (mg/g)	Peri-carp (mg/g)	Torus (mg/g)
Phenolics	24.78 ±0.26a	11.66 ±0.42b	21.06 ±1.46c	61.43 ±1.52d
Flavonoids	2.35 ±0.35a	1.28 ±0.42b	1.57 ±0.48b	3.34 ±0.65c
Non-tannins	4.95 ±0.25 a	2.45 ±0.36b	5.074 ±0.26c	14.12 ±0.92d
Tannins	19.82 ±1.27a	9.21 ±0.68b	15.99 ±1.86c	47.30 ±1.53d

Mean values and standard deviations with different letters (a, b, c, d) in the same row indicate significant differences ($P < 0.05$) among different *N. nucifera* constituents.

Flavonoids

The concentration of flavonoids in plant extracts of *N. nucifera* was determined using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalent, mg of QE/g of extract (Table 1). Torus contains the highest flavonoid concentration as 3.34 ± 0.65 mg QE/g. The lowest flavonoid concentration was measured in endosperm extract of 1.28 ± 0.42 mg of QE/g.

The flavonoids are secondary metabolites that may possess biological activity and have beneficial effects on human health as antimicrobial, anti-inflammatory, anti-cholesterolemic, antioxidant and anti-cancer agents.¹⁴⁻¹⁶ Flavonoids are reported to possess strong free radical scavenging activities based on their ability to act as hydrogen or electron donors and chelate transition metals.¹⁷ Total flavonoid contents of *N. nucifera* were expressed as mg quercetin equivalents/L. The results, shown in Table 1, varied from 1.28 to 3.34 quercetin equivalents/L.

Among constituents of *N. nucifera*, torus contained the highest amount of flavonoid content, followed by embryo, pericarp and endosperm. These results indicated that a higher flavonoid content was associated with a higher total phenolic content of the extract.

The data showed a considerable variation in *N. nucifera* content from different part of *N. nucifera*. Plant phenolics are a major class of bioactive constituents. It possesses the ideal chemistry for free radical scavenging activity due to the presence of high reactivity as hydrogen or electron donors and metal chelating activity. So the regular consumption of phenolics rich fruits and vegetables along with foods will inhibit carcinogenesis and mutagenesis in humans.¹⁸

According to our results, these extracts from *N. nucifera* contained significant amount of total phenolics that may increase antioxidant intake in human diet.

FRAP assay

FRAP was conducted on extracts of *N. nucifera* fractions to investigate their antioxidant potentials. In this assay, reduction of ferric tripyridyl triazine (Fe^{3+} -TPTZ) complex to ferrous form which has an intense blue colour can be monitored by measuring the change in absorption at 595 nm. Antioxidation effect of *N. nucifera*'s embryo, endosperm, pericarp and torus extracts was measured by FRAP assay as shown in Figure 1. As concentration of extracts was increased, absorbance at 595 nm represented as FeCl_2 concentration was generally increased. The highest FRAP was embryo extract while the lowest FRAP was endosperm.

EC (50% inhibition concentration) of *N. nucifera*'s extracts is summarized in Table 2 with vitamin C (typical anti-oxidation reference

ingredient). EC concentration of endosperm, pericarp, torus and embryo extracts showed 443.41 ± 12.63 mg/L, 316.27 ± 9.25 mg/L, 232.64 ± 7.34 mg/L and 144.75 ± 4.07 mg/L, respectively. This showed that embryo extract had higher anti-oxidation power than the other extracts, while the torus extract had two folds lesser anti-oxidation power than that of embryo.

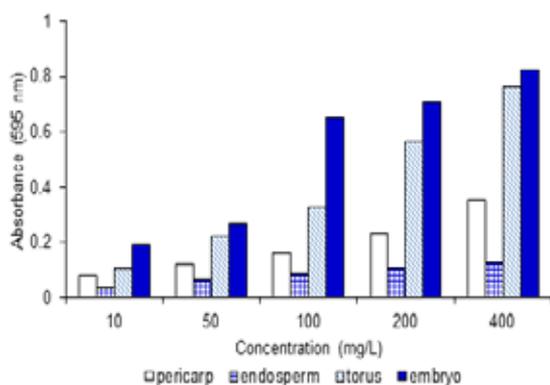


Figure 1 Antioxidation effect of various concentration of *N. nucifera*'s embryo, endosperm, pericarp and torus extracts measured by FRAP assay.

Table 2 shows the EC values at 30 min of the *N. nucifera* extracts. EC values and therefore ferric reducing ability, were lower at longer reaction times as expected from the kinetic behavior of the samples.

Table 2 EC Values of extract at 30 min of reaction with the FRAP assay.

Extract	EC (mg/L)
Endosperm	$443.41 \pm 12.63a$
Pericarp	$316.27 \pm 9.25b$
Torus	$232.64 \pm 7.34c$
Embryo	$144.75 \pm 4.07d$
Ascorbic acid	$82.43 \pm 5.32e$

Mean values and standard deviations with different letters (a, b, c) in the same column indicate significant differences ($P < 0.05$) among different *N. nucifera* constituents.

EC concentration of extract having a ferric reducing ability equivalent to that of 1 mmol/L $FeSO_4 \cdot 7H_2O$. Data are the mean values of three different determinations.

FRAP assay is a generally used method for determining the antioxidant capacity¹⁹ due to not complication and cheap. The antioxidant capacity of extracts is measured by the ability of the antioxidants in these extracts to reduce ferric to ferrous iron of FRAP reagent. The reducing properties are usually associated with the reductones which are present in the extract and they donate electron to free radicals to form more stable compounds. Furthermore, reductones also react with certain precursors of peroxide, and reduce the oxidized intermediates produced from reaction of lipid peroxidation.²⁰

Extracts from four parts of *N. nucifera* were examined for their antioxidant potential by using FRAP assay (Table 2). The antioxidant potentials were of varying levels in all the extracts (EC value of 144.75 ± 4.07 – 443.41 ± 12.63 mg/L). The highest antioxidant potential was found in embryo extract (EC value of 144.75 ± 4.07 mg/L), followed by torus (EC value of 232.64 ± 7.34 mg/L), pericarp (EC value of 316.27 ± 9.25 mg/L) and endosperm (EC value of 443.41 ± 12.63 mg/L). Toh et al.²¹ reported the FRAP value of peel and pulp of different pomelo fruit varieties (0.51–1.01 mmol Fe(II)/100 g FW) and showed lower antioxidant potential than those in this study. The embryo extract shows the highest antioxidant value. It might be the embryo contains alkaloids that are not present in the other constituents of



lotus. The aporphine-type alkaloids, such as nuciferine, O-nornuciferine, N-nornuciferine, anonaine, roemerine, dehydronuciferine, and pronuciferine, and bisbenzylisoquinoline-type alkaloids, including liensinine, isoliensinine, and neferine, predominantly accumulate in the embryo of the seeds. These alkaloids could inhibit lipogenesis.²² So, these alkaloid might be involved in antioxidant activity

Tyrosinase inhibitory activity

Anti-browning effect of *N. nucifera* 's endosperm, pericarp, torus and embryo extracts was measured by tyrosinase inhibition assay (Table 3). Tyrosinase inhibition effect of all *N. nucifera* 's extracts was lower than that of kojic acid ($IC_{50} = 77.23$ mg/L). IC_{50} of *N. nucifera* 's embryo extract was 126.14 mg/L and IC_{50} values of endosperm, pericarp and torus extracts were 2,246.82, 871.34, and 362.43 mg/L, respectively.

Tyrosinase is a polyphenol oxidase with a dinuclear copper active site and is involved in enzymatic browning of fruits and vegetables. Over-activity of this enzyme leads to hyperpigmentation of fruits and vegetables. Chemical agents that demonstrated tyrosinase inhibitory activity have been used to suppress melanogenesis and can be clinically useful for the treatment of browning in fruits and vegetables. Kojic acid is one of the popular chemicals used in anti-browning agent but cytotoxic and mutagenic effects have been proved.²³

To avoid this problem, more attention has been paid to the use of plant-based natural products instead of chemical agents as anti-tyrosinase substances. The tyrosinase inhibition activity of lotus composition are shown in Table 3. All the lotus composition revealed significant tyrosinase inhibitory activity of the concentration of

2,500 mg/L ranged between $62.47 \pm 2.43\%$ and $88.43 \pm 2.26\%$. Embryo and torus extract showed slightly lower activity than the reference compound kojic acid ($91.24 \pm 3.16\%$). Tyrosinase inhibition activity of lotus constituents was in the order of embryo>torus>pericarp> endosperm.

Table 3 The percentage of inhibitory activity (% inhibition) of tyrosinase with *N. nucifera* composition extract.

Concentration (mg/L)	Endo-sperm	Peri-carp	Torus	Emb-ryo	Kojic acid
10	16.36± 1.53a	21.88± 1.52b	27.25± 1.24c	33.41± 1.76d	37.22± 1.62e
100	19.17± 1.74a	35.44± 1.63b	39.14± 2.25c	48.09± 2.92d	54.03± 2.73e
500	38.05± 2.23a	42.94± 2.41b	54.98± 2.54c	70.36± 2.53d	73.42± 1.45c
1,000	45.84± 2.27a	59.02± 1.53b	71.93± 2.62c	79.82± 1.47d	84.83± 2.24e
2,500	62.47± 2.43a	72.63± 1.46b	79.43± 3.76c	88.43± 2.26d	91.24± 3.16e

Mean values and standard deviations with different letters (a, b, c, d) in the same row indicate significant differences ($P < 0.05$) among different *N. nucifera* constituents.

Previously, tyrosinase inhibitory potential of flavonoids such as naringin, hesperidin and nobiletin from citrus fruits were reported.²⁴⁻²⁶ Juices from *C. hystrix* and *C. maxima* (Red & White) fruits can be used as a preventive agent against enzymatic oxidation in food and living systems.

Conclusions

In the present study, samples from different parts of *N. nucifera* demonstrated that they not only possesses antioxidant and radical scavenging activities but also exhibits inhibitory potential

against tyrosinase *in vitro*. Consequently, *N. nucifera* constituents that are high contents in phenolics and flavonoids are suitable and promising for the development of safe food products, natural additives and anti-browning additives. Hence, it may be concluded that the antioxidant and inhibitory potentials against tyrosinase action of *N. nucifera* constituents can be used for the future health food additives and anti-browning additives in food products. Further studies will be conducted on identification of bioactive compounds involved in antioxidant activity and tyrosinase inhibitory activity and determination of their efficacy by *in vivo* studies.

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