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Research Article

Antioxidant and cytoprotective properties of seeds and seed by-products from lotus (*Nelumbo nucifera*)

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Abstract

The seed of *Nelumbo nucifera* Gaertn., commonly known as Chinese lotus, has been used as an indigenous medicine in Taiwan. The present study aims to evaluate the variable antioxidant activity of *Nelumbo nucifera* ethanolic extracts (NNEE) collected from different parts of the lotus. The seed epicarp had significantly higher extractable total phenolic content (TPC) and flavonoid content (TFC) than the seed and seed pod. Compared to the seed and seed pod, the seed epicarp also exhibited lower IC_{50} values of 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), EC50 for reducing power, and hydrogen peroxide (H_2O_2) scavenging activity. Moreover, DPPH and ABTS radicals, reducing power activity, and H_2O_2 scavenging activity by NNEE were highly and significantly associated with TPC and TFC. Seed epicarp NNEE at a concentration of 25 μg/mL gave significantly higher protection to Raw 264.7 mouse macrophages against H_2O_2 induced damage compared with blanks. Seed epicarp NNEE was also found to have the most significant protection effect on oxidative DNA damage. Therefore, the NNEE of seed epicarp exhibited higher *in vitro* and *ex vivo* antioxidant activity, and can be used as a functional ingredient representing a potential source of natural antioxidants to prevent and treat oxidative stress-related disorders.

Keywords: antioxidant activity; ethanolic extract; oxidative DNA damage; seed pod; seed epicarp

Introduction

Lotus (*Nelumbo nucifera* Gaertn.) is an aquatic perennial belonging to the family Nelumbonaceae, and is an ornamental plant and dietary staple in Eastern Asia (Hu and Skibsted, 2002). It is estimated that the annual harvest of lotus seeds in China has reached 15,000 tons (Guo, 2009). Virtually, all parts of the lotus plant are used: the root is used as food, seed as medicine, thalamus as fruit, stalks as pickle, and leaves as a food vegetable (Pall and Dey, 2015; Yang *et al.*, 2017). The seed of *N. nucifera* has been treated as a diuretic and cooling agent, antiemetic, and antidote in the treatment of tissue inflammation and cancer (Liu *et al.*, 2004).

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The lotus leaf contains flavonoids, which have antioxidant (Lee *et al.*, 2015), anti-diabetic (Liu *et al.*, 2013), and anti-cancer (Yoon *et al.*, 2013) activity. The pharmacological potential of ethanol-soluble extracts of the lotus root, including as antioxidants and in anti-obesity treatment (You *et al.*, 2014), are proposed to be closely related to phenolic compounds. In addition, increasing attention has been paid to analyzing bioactive components from lotus seed embryos (Zhu *et al.*, 2017). However, in Taiwan, thousands of tons of the inedible parts of lotus seed pods and epicarps are discarded during the processing of lotus seeds. Systematic studies on antioxidant properties of lotus seedpods and epicarps are very scarce, although the lotus seedpod has been reported for its procyanidins possibly having the potential to stimulate antioxidant enzyme activity and scavenge oxygen free radicals (Duan *et al.,* 2013), and polyphenols have been identified in the lotus seed epicarp at different ripening stages (Liu *et al.*, 2015). The industrial production of food products from lotus seed pods and epicarps is not occurring or popular. Therefore, it is worthy to study practical applications of lotus seedpods and epicarp extracts in the future for food to enhance its nutritional quality and therapeutic value, and also provide income for lotus producers and the regional economy.

There has been increasing interest in using antioxidants from plants as functional foods and nutraceutical products with antioxidant properties (Brewer, 2011), and there is great interest in the use of potent dietary antioxidants in preventive strategies with applications ranging from oxidative reactions in foods and pharmaceuticals to the role of reactive oxygen species (ROS) in chronic degenerative diseases (Farombi *et al.*, 2004). Lotus seeds are traditional and seasonal food, but seed pods and epicarps are inedible parts and by-products. Studies have shown that those inedible parts of seeds are rich in procyanidins and polyphenols from lotus seedpod (Duan *et al.*, 2013) and seed epicarp (Liu *et al.*, 2015), respectively, and also have biological activities as well, such as antioxidants (Duan *et al.*, 2013; Liu *et al.*, 2015). The development and utilization of these by-products will improve the economic benefits of a whole lotus plant. Although a variety of traditional herbal medicines are known to be potent sources of antioxidant compounds, relationships between antioxidant activities and the prevention of cell oxidative damage by *Nelumbo nucifera* ethanolic extracts (NNEE) are not well researched. Different parts of lotus seed NNEE might display variable antioxidant levels. No literature on the antioxidant properties of lotus seed components against free radical-induced oxidative stress *in vitro* cell experiments is presently available. In view of these deficiencies, we aimed to evaluate the total phenols and flavonoids and antioxidant activities of NNEE from the different parts of the lotus seed that can protect from and inhibit oxidative damage to rat Raw 264.7 macrophages induced by hydrogen peroxide (H_2O_2). In addition, the DNA protective effect of NNEE was also checked against Fenton's induced DNA damage of calf thymus DNA.

Materials and Methods

Source of plants and preparation of plant extracts

The largest area under cultivation of lotus (*Nelumbo nucifera* Gaertn. ssp. *nucifera*) is located in the regions surrounding Tainan City, Taiwan. The most common and consumed lotus seed cultivar in Tainan City is white lotus, which was chosen for sampling in this study. All samples were obtained after being fully harvested, and mature lotus seeds with seedpods and epicarps were collected from a local Lotus Farm commercial entity, the Tainan Agricultural cooperative (Bai-He District, Tainan City, Taiwan). No specific permission was required for collection of samples from these locations and activities. A voucher specimen was deposited in Taiwan District Agricultural Research and Extension Station, Taiwan. Collected samples were washed with tap water and, after being peeled, the seeds, seed pods (wraps the seeds), and epicarps (rind or hulls of the seeds) were carefully re-washed, air-dried, weighed, and lyophilized using a Freeze Dryer (FD-5060, Panchum Scientific, Taipei, Taiwan). Dried samples were then ground into powder, screened through a 40-mesh sieve, and stored at -80 °C. Twenty grams of powder from each sample were extracted with 200 mL of

95% ethanol, stirred on a stirring plate at 25 °C overnight (Lin *et al.*, 2017), and then filtered through #1 filter paper (Whatman, Hillsboro, OR, USA). The filtrate was concentrated in vacuo and lyophilized to dryness to obtain the 95% ethanolic extracts. The dried filtrates were weighed to determine the extracted yield of soluble constituents. The extraction yield (%) was calculated as the dried filtrate weight (g) divided by the sample weight (g, dry mass) × 100%. The ethanolic extract was then stored at -20 °C until being used for antioxidant activity assays.

Analysis of the total phenol content (TPC) and total flavonoid content (TFC)

Total phenolic compounds were extracted from different parts of the lotus with Folin-Ciocalteau (FC) reagent, using gallic acid as a standard (Pourmorad *et al.*, 2006). Three hundred microliters of FC reagent (diluted 10-fold in distilled-deionized water, ddH_2O) and 240 µL 1 M sodium bicarbonate solution was added to 30 µL NNEE. After incubation for 15 min at 20 °C, the absorbance at 765 nm was read. TPC was expressed as mg gallic acid equivalents (GAE)/g dry extract. The linearity range of the calibration curve was 1 to 9 µg/ml ($r^2 = 0.99$). TFCs were determined according to the method described by Kashiwada *et al.* (2005). Six hundred microliters of an NNEE were mixed with an equal volume of 10% $AlCl_3$. After incubation for 15 min, the absorbance 510 nm was read. The TFC, using rutin as a standard, was expressed as mg rutin equivalents (RE)/g dry extract. The linearity range of the calibration curve was 10 to 200 µg/ml ($r^2 = 0.99$).

Measurement of the scavenging activity against 1,1-diphenyl-2- picrylhydrazyl (DPPH) and 2,2-azino-bis-(3-ethyl benzothiazoline-6-sulfonicacid) (ABTS) radicals

The scavenging activity against the DPPH radical by different parts of NNEE was determined by the method of Gyamfi *et al.* (1999) with modifications. Briefly, an aliquot of 70 µL of NNEE with serial dilutions was added to 140 µL of 150 µM DPPH freshly prepared in methanol, mixed well, and left to stand for 60 min at room temperature in the dark to allow the reaction to proceed. DPPH radicals are scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH-H. The color changes from purple to yellow after reduction, which can be quantified by the decrease in absorbance at a wavelength of 517 nm.

The scavenging effect (%) = [1 – (absorbance of a sample) / (absorbance of a blank DPPH solution)] × 100. (1)

The blank was treated in the same manner, except that methanol was used instead of a sample, and ascorbic acid was used as the control. The half-inhibition concentration (IC_{50}) of DPPH radicals was calculated as the antioxidant concentration required for providing 50% of the antioxidant activity. Each sample was measured with at least three different concentrations in the DPPH test. Total antioxidant capacities of hydrophilic and lipophilic antioxidants were determined using the horseradish peroxidase (HRP)-catalyzed oxidation of ABTS. The reaction mixture contained 0.1 mL of 1 mM ABTS, 100 µL of 44 U/mL peroxidase, 0.1 mL of 0.5 mM H_2O_2, and 600 µL of ddH_2O. After 1 h, 0.01 mL of an NNEE was added to the mixture, and after 10 min, absorbance was measured at 734 nm. Trolox (TR) was used as a standard. The IC_{50} value was obtained by interpolation from a linear regression analysis.

Measurement of reducing power

The reducing power of the extracts was determined according to the method of Yen and Chen (1995) with modifications. Briefly, an aliquot of 75 µL of NNEE was mixed with an equal volume of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferrocyanide. The mixture was incubated at 50 °C for 20 min. After cooling down on ice, 300 µL of ddH_2O, 75 µL of 10% trichloroacetic acid, and 300 µL of 0.1% ferric chloride were added to the mixture. After 15 min, the absorbance at 700 nm was measured against a blank. A blank was prepared using 95% ethanol without extract. Increased absorbance of the reaction mixture indicated a higher reducing power. Ascorbic acid was used as a standard. EC_{50} (effective concentration at which the absorbance is 0.5) was used for the reducing power.

H_2O_2-scavenging activity

The determination of H_2O_2-scavenging activity was done according to the method of Johnston *et al.* (2008) with modifications. Briefly, a phenol red-HRP detection reagent was prepared by mixing equal volumes of 600 μg/mL phenol red and 108 U/mL HRP in phosphate-buffered saline (PBS; pH 7.2). A 60 μL volume of sample or vitamin C was mixed with 60 μL of 300 μL H_2O_2 in wells. After 15 min at ambient temperature, 60 μL of the phenol red-HRP detection reagent was added for another 45 min incubation. Finally, the assay was terminated by adding 30 μL of 1 N NaOH and then measuring absorbance at 610 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader.

The scavenging effect (%) = [1 – (absorbance of a sample) / (absorbance of a blank H_2O_2 solution)] × 100. (2)

A blank was prepared using distilled water without extract.

Cell culture and a 3-(4,5-dimethylthiazol)-2-yl-2,5-diphenyltetrazolium bromide (MTT)-based cytotoxicity test

The cytotoxic effects of the NNEE against Raw 264.7 cells were determined by an MTT assay. It is a standard and rapid colorimetric method used to determine the effects of potential agents or compounds on the proliferation of cells. Mouse macrophage Raw 264.7 cells were obtained from the Bioresource Collection and Research Center (Hsinchu City, Taiwan). Raw 264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and a 1% penicillin/ streptomycin mix (Invitrogen, Camarillo, CA, USA) in 10 cm Petri dishes at 37 °C in a 5% CO_2 environment. Before NNEE treatment, 100 μL of Raw 264.7 cells (10^5 cells/well) were seeded in 96-well plates and incubated overnight. Lyophilized powders of NNEE were dissolved in DMEM containing 0.3% dimethyl sulfoxide for the preparation of 25, 50, and 100 μg/mL, and 50 μL of each NNEE was added to each well. Cells were incubated for 24 h, then 50 μL of MTT (0.5 mg/mL) was added and allowed to react for 2 h at 37 °C. After draining, cells were again washed with PBS, dissolved in 120 μL of dimethyl sulfoxide, and monitored for absorbance at 570 nm (A_{570}) using an ELISA reader for cell viability:

Cell viability (%) = [(A_{570} of the extract-treated group) / (A_{570} of the control group)] × 100 (%). (3)

Cells without added extract were used as controls.

Protective effect of NNEE against H_2O_2-induced oxidative stress

The above-mentioned Raw 264.7 cells were incubated overnight in plates containing NNEE (25 μg/mL), followed by the addition of 50 μL of 0.8 mM H_2O_2 to each well, which was then allowed to react for 24 h at 37 °C. After that, 50 μL of MTT (0.5 mg/mL) was added and allowed to react for 2 h at 37 °C. After draining, cells were washed with PBS, dissolved in 120 μL DMSO, and monitored for absorbance at 570 nm using an ELISA reader for cell viability:

Cell viability (%) = [(A_{570} of the extract-treated group) / (A_{570} of the control group)] × 100 (%). (4)

The control was DMEM without added H_2O_2 and sample extract.

Analysis of the protection effect of NNEE from DNA damage

The protection effect of the extracts was determined according to the method of Hsieh *et al.* (2015) with modifications. Each 45 μL aliquot of a reaction mixture, which was a blend of various NNEE (0.1, 0.3, and 0.5 mg/mL of NNEE), 1 μL of calf thymus DNA solution (25.0 A_{260} unit/ml) (Amersham Biosciences, Piscataway, NJ, USA), 0.9 μL of 1.8 mM $FeSO_4$, and 3.6 μL of 24 mM H_2O_2, was incubated at room temperature for 15 min. After incubation, 10 μL of 1 mM EDTA was added to stop the reaction. The control and blank were the calf thymus DNA respectively treated and untreated with H_2O_2 solution in the absence of NNEE. Each 5 μL aliquot of the reaction mixture was applied on 1% agarose gel containing 0.1% ethidium bromide. One-kb DNA ladder (GeneMark, Taipei, Taiwan) was used as a marker. The electrophoresis was

conducted at 100 V in TBE buffer (10 mM Tris-boric acid-EDTA, pH 7.4) for 10 min, and the gel then visualized by UV light using a GLUBIO Gel Catcher-1500 image analysis system (Medclub Scientific Co., Tainan, Taiwan).

Statistical analysis

All analyses were determined in triplicate, and results are expressed as means and standard deviations (SD). An analysis of variance (ANOVA) with the least significant difference (LSD) test at 0.05 was performed using SAS vers. 9 (SAS Institute, Cary, NC, USA). Pearson's correlation coefficients of total phenols, flavonoids, DPPH, ABTS, reducing power, and H_2O_2-scavenging activity treated with NNEE were also determined.

Results and Discussion

Antioxidant activity

The extraction yield of different parts of NNEE varied from 1.19±0.07% (seed epicarp) to 3.33±0.31% (seed pod). Accordingly, the yield of the NNEE fractions showed obvious variation due to the variable water content. Table 1 shows that TPC and TFC contents in NNEE samples exhibited significant differences among the different parts of the lotus seed.

Table 1. Contents of total phenols (TPC) and flavonoids (TFC) from ethanolic extracts from different parts of the lotus seed

Sample	TPC (mg GAE/ g extract)	TFC (mg RE/ g extract)
Seed	42.13±1.69^{c}	56.35±1.30^{c}
Seed pod	357.11±2.59^{b}	514.12±6.39^{b}
Seed epicarp	580.45±17.58^{a}	784.94±45.33^{a}

Each value is expressed as the mean ± SD (n = 3). Means with different letters significantly differ (p < 0.05). GAE, gallic acid equivalents; RE: rutin equivalents.

Seed epicarp and seed had the highest (580.45±17.58 mg GAE/g extract) and lowest (42.13±1.69 mg GAE/g extract) TPC values, respectively. The TFC of all tested samples ranged from 56.35±1.30 (seed) to 784.94±45.33 (seed epicarp) mg RE/g extract. Furthermore, NNEE from different parts of the lotus seed also showed antioxidant activities, proving that each has the capacity to scavenge DPPH, ABTS radicals, reducing power, and H_2O_2 (Table 2).

Table 2. Antioxidation and reducing power from ethanolic extracts from different parts of the lotus seed

Sample	DPPH (IC_{50}) (μg/mL)	ABTS (IC_{50}) (μg/mL)	Reducing power (EC_{50}) (mg/mL)	H_2O_2-scavenging activity (IC_{50}) (mg/mL)
Seed	117.75±10.02^{a}	598.22±26.54^{a}	1.59±0.03^{a}	3.80±0.53^{a}
Seed pod	17.63±0.92^{b}	48.70±1.57^{b}	0.12±0.01^{b}	0.39±0.05^{b}
Seed epicarp	12.11±0.40^{c}	38.25±2.10^{c}	0.08±0.01^{c}	0.30±0.01^{b}
Control	Ascorbic acid 11.67±0.41^{c}	Trolox 51.83±4.70^{b}	Ascorbic acid 0.09±0.02^{bc}	Ascorbic acid 0.05±0.01^{c}

The half-inhibition concentration (IC_{50}) was calculated as the antioxidant concentration required for providing 50% of the antioxidant activity. EC_{50} (effective concentration at which the absorbance is 0.5) was used for the reducing power. Each value is expressed as the mean ± SD (n = 3). Means with different letters significantly differ (p < 0.05).

NNEE tested for *in vitro* free radical scavenging potential using the DPPH method showed potent activity as evidenced by low IC_{50} values, and exhibited significant differences in the DPPH-radical scavenging activity by exhibiting a wide range of IC_{50} values from 12.11±0.40 (seed epicarp) to 117.75±10.02 μg/mL (seed). Notably, the IC_{50} value of seed epicarp at 12.11±0.40 μg/mL was comparable to that of the ascorbic acid control at 11.67±0.41 μg/mL. In addition, seed epicarp NNEE had significantly higher ABTS radical-scavenging activity (38.25±2.10 μg/mL) than the other tested samples (48.70±1.57 and 598.22±26.54 μg/mL). Seed epicarp also had significantly higher reducing power activity with the lowest EC_{50} (0.08±0.01 mg/mL of NNEE) compared to the other tested samples (0.12±0.01 and 1.59±0.03 mg/mL), which was close to the ascorbic acid control that had an EC_{50} value of 0.09±0.02 mg/mL. Moreover, seed NNEE had significantly higher IC_{50} values (3.80±0.53 mg/mL) for H_2O_2-scavenging activity compared to the seed pod (0.39±0.05 mg/mL), seed epicarp (0.30±0.01 mg/mL), and ascorbic acid (0.05±0.01 mg/mL) control. These results indicate that the seed epicarp NNEE had a significantly higher scavenging efficacy for DPPH and ABTS radical cations, and reducing power activity than did seed pod and seed NNEE, reflecting the relevance of evaluating the major mechanisms of antioxidant actions on cell protection. Moreover, NNEE are electron donors and can react with free radicals, convert them to more-stable products, and terminate radical chain reactions.

Many researchers reported that influence of different extraction solvents, such as methanol, ethanol, acetone, propanol, and ethyl acetate have been commonly used for the extraction of phenolics from fresh product (Durling *et al.*, 2007; Alothman *et al.*, 2009; Lezoul *et al.*, 2020). A previous report indicated that 70% alcohol solvents exhibit a higher extraction efficiency for flavonoids from lotus leaves than 100% alcohol solvents (Chen *et al.*, 2012). The ethanol extract of the *N. nucifera* seed has been evaluated for its antioxidant activity using DPPH free radical scavenging effects with a median inhibition concentration (IC_{50}) of 6.49 mg/ml (Sohn *et al.*, 2003). DPPH and ABTS scavenging capacities of the ethyl acetate fraction of lotus seed were 94.6 and 91.9% and those of the water fraction of lotus seedpod were 94.5 and 95.2% at 0.8 mg/mL, respectively. Moreover, the ethyl acetate fraction of seed and the water fraction of seedpod also showed high ferric reducing antioxidant power as well from their flavonoid and proanthocyanidin contents (Kim and Shin, 2012). The antioxidant activity of hydro alcoholic extract of *N. nucifera* seeds exhibited strong free radical scavenging activity as evidenced by the lower IC_{50} value in DPPH at 16.12±0.41 μg/mL compared to that of rutin, the standard used (Rai *et al.,* 2006). In our study, the IC_{50} values of DPPH and ABTS radicals, reducing power activity, and H_2O_2-scavenging activity by NNEE were highly and significantly associated with TPC and TFC ($r = -0.9078 \sim -0.9946$), indicating a positive relationship between DPPH•- and ABTS•-scavenging activities, reducing power value, H_2O_2-scavenging activity, and TPC and TFC (Table 3).

Table 3. Correlation coefficients of total phenols, flavonoids, DPPH, ABTS, reducing power activity, and H_2O_2 treated with ethanolic extracts of lotus

	TPC	TFC	IC_{50} of DPPH	IC_{50} of ABTS	Reducing power	IC_{50} of H_2O_2
TPC	-	0.9526*	-0.9110*	-0.9151*	-0.9195*	-0.9078*
TFC		-	-0.9927*	-0.9928*	-0.9946*	-0.9825*
IC_{50} of DPPH			-	0.9990*	0.9997*	0.9890*
IC_{50} of ABTS				-	0.9996*	0.9938*
Reducing power					-	0.9910*

* $p < 0.05$ for each correlation coefficient.

These results suggest that phenolic compounds were effective hydrogen donors. Therefore, the excellent antioxidant activity of NNEE indicates high TPC and TFC. Yan *et al.* (2011) reported that TPC in lotus extracts significantly correlate with DPPH and ABTS radical scavenging activity. Hong *et al.* (2008) reported

that ferric reducing ability exhibits a good linear correlation between antioxidant activity and TFC or TPC. There is also a strong correlation between the antioxidant activity and TPC of Chinese bayberry. Using other antioxidant evaluation methods based on different mechanisms to evaluate antioxidant activity may bring different results even from the same sample. Furthermore, different extraction solvents result in differences in extract composition, and consequently in antioxidant activity (Pinelo *et al.,* 2004). In general, antioxidant activity in functional compounds is highly associated with the analytical method, and it is difficult to directly compare and interpret the patterns of antioxidant activity from various studies. In our study, the polarities of NNEE differ due to the extracts from the three different parts of lotus with different antioxidant activities, which could influence their uptake and cellular distributions (Miranda-Vilela *et al.*, 2008; Tomsone *et al.*, 2012).

The total flavonoid intake of an average American is approximately 20 mg/day, and for Japanese it is around 63 mg/day in food originating from plants (Beecher, 2003). The content and composition of phenolic compounds have been well investigated in various lotus tissues (Chen *et al.*, 2012) except the seed pod and epicarp. In our study, both TPC and TFC were highest in the seed epicarp. Therefore, the antioxidant effect of NNEE in the epicarp has value, particularly for finding an inexpensive source for natural antioxidants and functional food. The seed epicarp reveals its potential to be developed as an active ingredient or food additive, and thus increase the economic value of Taiwanese white seed lotus for the food industry. Lotus can be found abundantly in wetlands, ponds, lakes, canals etc., and lotus seeds marketed in Taiwan vary widely in their antioxidant potentials and thus may impart different health benefits to consumers. The inedible epicarps of lotus seeds having high antioxidant value from 95% ethanolic extraction can be used to develop products with high nutraceutical value, which can play a significant role in providing good nutrition and improving human health. People can consume lotus seed as a low-cost nutritious food and use it as a low-cost medicine for the treatment of diseases. The obvious antioxidant activity in seed epicarp confirms its important role in the bioactivity of whole lotus, and this suggests that seed epicarp removal may induce a more significant nutrient loss. The determination of TPC and TFC in the various parts of the lotus plant is valuable for increasing the bio-availability of lotus products. Therefore, effective dose *in vivo* studies are worthy of further investigation.

Cytotoxicity test and protective and inhibitory effects of NNEE on Raw 264.7 cells

The Raw 264.7 murine macrophage cell line was used in this study because it has been widely applied to the antioxidant research of traditional herbs (Tuntipopipat *et al.*, 2009). The effects of NNEE from different parts of the lotus on the viability of Raw 264.7 cells are shown in Table 4. The cell viability test showed that none of the NNEE constituents in concentrations ranging from 25 ~ 100 μg/mL had any cell toxicity, nor could they significantly impair cell proliferation *in vitro*. No cytotoxic effects were observed after treatment with 25 μg/mL of NNEE from all parts of the lotus against Raw 264.7 cells (104.54±2.60 ~ 111.51±9.76 μg/mL); thus, the concentration of 25 μg/mL was used for further study.

The protective effects of NNEE from different parts of the lotus against H_2O_2-induced oxidative stress in Raw 264.7 cells are given in Figure 1. The survival rates of treatments of 25 μg/mL NNEE from each part of the seed were significantly higher (ranged from 93.27±2.40% ~ 100.32±3.07%) than those of blanks (60.00±1.98%), indicating that all three NNEE extracts can protect Raw 264.7 cells from oxidative stress damage caused by H_2O_2.

Table 4. Cell toxicities from NNEE from different parts of the lotus seed on the viability of Raw 264.7 cells

Sample	25 μg/mL	50 μg/mL	100 μg/mL
Seed	109.15±3.17^{a}	108.13±5.59^{a}	110.13±2.30^{a}
Seed pod	111.51±9.76^{a}	111.65±11.52^{a}	113.56±9.12^{a}
Seed epicarp	104.54±2.60^{a}	101.45±1.66^{ab}	98.78±2.44^{b}

Values are expressed as percentage relative to control value (100%). Each value is expressed as the mean ± SD (n = 3). Means with different letters significantly differ ($p < 0.05$).

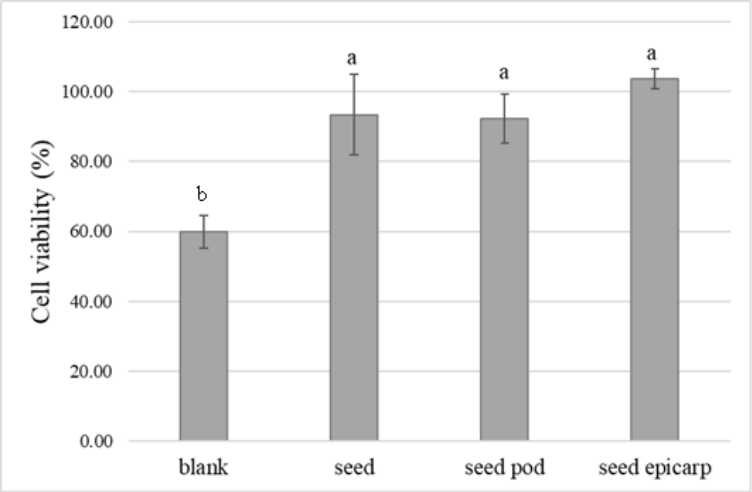

Figure 1. Protective effects of NNEE from different parts of the lotus seed against H_2O_2-induced oxidative stress in Raw 264.7 cells

Cell viability (%) = [(A_{570} of extract treated group) / (A_{570} of control group)] x 100. The blank was H_2O_2 without sample extract. Each value is expressed as the mean ± SD (n = 3). Means with different letters significantly differ (p < 0.05)

Analysis of the protection effect of NNEE from DNA damage

By assaying the retention of intact DNA molecules, the protection effect of various NNEE on DNA damage induced by hydroxyl radicals was evaluated. Figure 2 demonstrates that the protection offered against DNA damage by NNEE is concentration-dependent, and the inhibition activities of NNEE on DNA damage were increased as NNEE concentrations increased. The oxidative stress of calf thymus DNA was induced by Fenton reaction, and Figure 2 shows a large number of small molecular damaged DNA fragments in the control lane. However, after incubating calf thymus DNA with 0.1, 0.3, and 0.5 mg/mL NNEE from three parts of the lotus, only seed epicarp and seed pod showed significantly inhibitory effects. The best DNA protection effect was observed when the NNEE extract of seed epicarp was more than 0.3 mg/mL. Seed epicarp NNEE revealed a more significant effect on DNA cleavage protection compared to the seed and seed pod under the same concentration of NNEE. Oxidative damage to DNA is one of the most important mechanisms in the initiation of carcinogenesis, mutagenesis, and cytotoxicity (Prieto *et al.*, 1999). Such damage is usually caused by hydroxyl radicals, the most reactive among ROS. The Fenton reaction is complex and capable of generating both hydroxyl radicals and higher oxidation states of the ferric (Winterbourn, 1995). Since seed epicarp NNEE possess good antioxidant activity, its protective effects on DNA may have contributions from its rich content of total phenols and flavonoids. Apparently, seed epicarp NNEE had a higher antioxidant content compared to the other parts, suggesting that some antioxidants ingredients in NNEE samples play important roles in antioxidant activity.

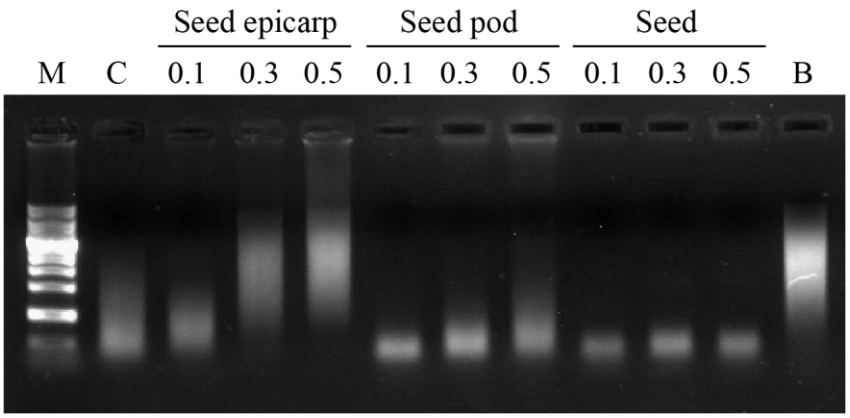

Figure 2. Electrophoretic pattern at concentrations of 0.1, 0.3, and 0.5 mg/mL NNEE from different parts of the lotus seed

The control (C) and blank (B) was calf thymus DNA respectively treated and untreated with hydrogen peroxide solution in the absence of NNEE. A one-kb DNA ladder is shown as a marker (M). Electrophoresis was performed using 1% agarose in the presence of ethidium bromide

Fenton reaction is the main cause of intracellular hydroxyl radicals generation. Hydrogen peroxide and hydroxyl radicals can cause toxicity to cells and damage nucleus DNA, so scavenging these ROS can protect cells from oxidative stress damage. Wu *et al.* (2003) and Yen *et al.* (2006) used lotus leaf methanol extract and lotus seed extract, respectively, for the protections from hydrogen peroxide-induced cytotoxicity and DNA damage. In our study, it is the first to find that lotus epicarp and seedpod ethanol extract are superior to lotus seed extract in terms of antioxidant capacity and ability to protect cells and DNA from hydrogen peroxide and hydroxyl radicals damage, and lotus epicarp is the best. Although lotus epicarp is a by-product of lotus, it still has the value of developing into an antioxidant. Hwang et al. (2016) reported that the antioxidant activity of *Cornus officinalis* in RAW 264.7 cells was achieved by increasing the performance of intracellular antioxidant enzymes, including Cu/ZnSOD, MnSOD, catalase, and glutathione peroxidase, suggesting the therapeutic potential of *C. officinalis* extract as an antioxidant agent. Thus, we assume that lotus epicarp and seedpod may have a similar intracellular protective effect against hydrogen peroxide protection machinery. Furthermore, significant growth inhibition effects of procyanidins from the lotus (*N. nucifera* Gaertn) seed pod on mouse melanoma B16 were found both *in vivo* and *in vitro* (Duan *et al.*, 2010). Chao *et al.* (2015) also reported that the ethanolic extract of red bean (RBE) can diminish H_2O_2-induced oxidative damage in the RAW 264.7 macrophage. Phenolic compounds and cyanidin-3-*O*-glucoside from BRE may have efficacy as overall *in vitro* anti-inflammatory and antioxidant agents. In the present study, total phenols and flavonoids from seed epicarp NNEE offered protection against H_2O_2-induced damage in Raw 264.7 cells, and this protective effect is considered, in large part, to be related to the various antioxidants they contain. Total phenolic compounds in seed epicarp NNEE possessing potent *in vitro* antioxidant activity may be a major contributor to inhibiting the formation of ROS, and shows an anticancer potential in Raw 264.7 cells. Therefore, our seed epicarp NNEE results show that it can be used to prevent and treat oxidative stress-related disorders, which should stimulate research into the contents, capacities, and functions of antioxidant systems in herbal plants. Further studies should focus on the valuable outcomes of its effects in animal models.

Conclusions

The Taiwanese lotus seed pod and epicarp were identified as containing potent polar antioxidants, and the distribution of total phenols and flavonoids in the lotus seed was not uniform. NNEE from different parts of the seed displayed variations in antioxidant substances. The seed epicarp possessed higher amounts of total phenols and flavonoids and was high in free radical-scavenging activities as evaluated using DPPH and ABTS. The most significant effect on DNA cleavage protection was also found to be seed epicarp NNEE at a concentration of 25 μg/mL, and this strong oxidative DNA damage preventive activity and radical scavenging activity of NNEE may be associated with its rich content of total phenols and flavonoids. The seed epicarp exhibited higher *in vitro* and *ex vivo* antioxidant activity and possessed a protective capability for the biological membrane system. Therefore, seed epicarp NNEE can be used to prevent and treat oxidative stress-related disorders. Our observations may enhance the potential application of the agricultural waste seed epicarp as a novel and inexpensive source of natural antioxidants, especially for the currently fast-growing functional food industry.

Authors' Contributions

CPL and YPY contributed to the study conception, design and project administration. Plant seeds were collected and prepared by CCW and MCH. Data collection and analysis were performed by CCW and WTC. The manuscript was written by KHL, and all authors commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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