Phytocomponents and antioxidant activity in the methanol extract of seed lotus (*Nelumbo Nucifera* Gaernt.) from Viet Nam

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Abstract
In this study, we determined the phytocomponents using GC-MS and evaluated antioxidant activity by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay of the methanolic extract of six lotus varieties seed in Viet Nam (N. Nucifera). GC-MS chromatogram of the methanolic extract of six lotus varieties seed in Viet Nam showed 27 peaks which indicate the presence of 27 phytochemical constituents. The major phytochemical constituent is methyl-alpha-d-galactopyranoside (66,86-78,69%) in all lotus varieties. Antioxidant activity of liquid extract and condensed extract was found as 8.20-15.67 mg/mL and 0.850-1.286 mg/mL in comparison with IC_{50} of ascorbic acid (3.2 µg/mL).

Moreover, 50 % of the DPPH scavenging ability of liquid and condensed extract differed from each other. From obtained results, the seeds of six lotus varieties in Viet Nam were found to be potential for bioactive compounds and antioxidant activity.

Keywords: Antioxidant activity, DPPH assay, Extract, GC-MS analysis, Lotus seed, *Nelumbo nucifera*, Phytocomponents.

Introduction
Lotus (*Nelumbo nucifera* Gaertn.) is an ornamental aquatic plant[26] commonly grown in Australia, China, India and Japan which belongs to the family Nelumbonaceae[11,22]. The lotus provides people with many benefits from beautiful flowers to food[31] and also medicinal herbs[29]. Almost every part of this plant can be used and seeds are the main consumption part[12]. Lotus seeds are edible, rich in nutrients and can be dried for storage. Not only these seeds contain a rich variety of essential amino acids, unsaturated fatty acids, carbohydrates, vitamins, calcium, iron, zinc, phosphorus and other trace elements[23], they also have water-soluble polysaccharides, alkaloids, flavonoids and other bioactive components[39].

Therefore, lotus seed is used as medicine and food[37]. The seeds or their extracts have been reported to possess anti-proliferative[36], anti-fibrosis[33], antidepressant, anti-inflammation[1], astringent[15], hepatoprotective and free radical scavenging[18], anti-obesity and hypolipidemic effects[35], anti-inflammatory[9,13,34] immunomodulatory[14] and antiviral activities[7,8]. Besides, seed lotus is also used in diet and treatment of cancer[38], arrhythmia and skin diseases.[3,37]

Several bioactive compounds have been derived from *N. nucifera* parts belonging to different chemical groups, including alkaloids, flavonoids, glycosides, triterpenoids, vitamins etc. which have their therapeutic impact[12].

Several studies have reported the antioxidant activities of different extracts of *N. nucifera* seeds and photo components isolated from them.

Abdelhamid et al[1] determined the phytocomponents of the ethanol extract of *N. nucifera* seed using the GC-MS method. This result showed that the ethanolic extract of *N.nucifera* seed had thirty-eight phytochemical constituents. Among thirty-eight compounds identified, only twelve were reported to have biological activities[1]. Kim et al[4] showed that 80% methanol extract of *Nelumbo nucifera* seed's embryo decreases in ROS production in mouse hippocampal HT_{22} cells and scavenges DPPH free radical (IC_{50} value as 240.51 μg/mL) and H_2O_2 (IC_{50} value as 1769.01 μg/mL)[4,20].

Sugimoto et al[28] showed that methanol extract of *N. nucifera* seed contains neferine which has various therapeutic effects such as induction of sedation, hypothermia, anti-fever effects and anxiolytic effects comparable with those of diazepam but with a different mechanism[30]. The free radical scavenging activity potential of the lotus seed using DPPH assay has been also reported by Sohn et al[26] and Rai et al.[21]

There are six different lotus varieties in Thua Thien Hue province of Viet Nam including four pink lotus varieties and two white lotus varieties. These lotus varieties are the GiaLong pink lotus, the PhuMong pink lotus, the VinhThanh red lotus (also called the light red or dark pink lotus), the High-yield lotus, the Concave white lotus and the Convex white lotus[16].

There have been not many reports on the phytochemical screening and the antioxidant activities of the methanol extract of lotus seeds cultivated in Hue province of Viet Nam. Therefore, the present study was aimed to investigate the chemical constituents and the antioxidant activities of the methanolic extract of *N. nucifera* seeds in Viet Nam.

Material and Methods

Collection and processing of plant material: Lotus mature seeds (23 to 25 days old) were collected from lotus flowers of six lotus varieties including GiaLong pink lotus, PhuMong pink lotus, VinhThanh red lotus, High-yield lotus, Concave white lotus and Convex white lotus, all of which are cultivated in Thua Thien Hue Province of Vietnam. Seeds lotus were washed under tap water to remove dust particles, then they were dried and the seed coat was removed and discarded. Finally, seeds without coats were pulverized to powder by using a mechanical grinder.

Preparation of the extract: 100 g of the powder of seeds was soaked in 500ml of 70% methanol for 24h. The extracts were then filtered through Whatmann filter paper no. 41 along with 2 gm sodium sulfate to remove the sediments and traces of water in the filtrate. The filtrate was then concentrated by a rotator evaporator. 0.1 g of condensed extract was used for GC-MS analysis[1].

GC-MS analysis: GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer system comprising an AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column TG 5MS composed of 5% phenyl methyl polysiloxane operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 1 µl was employed at (split ratio of 10:1) injector temperature 200°C; ion source temperature 200 °C. The oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10 °C/min to 200 °C, then 5 °C/min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV a scanning interval of 0.5 s and fragments from 40 to 650 Da. MS transfer line temperature 280 °C[1].

Identification of phytocomponents: Identification of compounds was conducted using the database of the Wiley library combined with the National Institute of Standards and Technology (NIST) library. The name and area under the peak of the components of the test materials were ascertained.

Determination of antioxidant activity: The antioxidant activities of liquid extract and condensed extract of the six lotus varieties were measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method as described in Vuong et al.[3] An amount of 1 ml sample was added to the test tube followed by 1 mL of DPPH of 0.2 mM and 30-minute incubation in the darkness. Absorbance was measured at 517 nm. The ascorbic acid was used as control. Liquid extract solution was diluted with ratios of 30; 15; 7.5; 3.75 and 1.875 mg/mL; Condensed extract was prepared by serial dilutions of 2; 1; 0.5; 0.25 and 0.0125 mg/mL. DPPH solution 0.2 mM was prepared in 70 % methanol and ascorbic acid was prepared as of 1, 0.5, 0.25; 0.125 and 0.1 mg/mL.

DPPH free radical scavenging abilities were calculated as:

$$SC\% = \frac{OD_c - OD_m}{OD_c} x100$$

where ODm is optical density of sample after deducting blank (without DPPH) and ODc is optical density of blank sample after deducting blank (without DPPH). The standard curve was developed with percentages of DPPH inhibitions at different concentrations. From there, calculate the value of IC_{50} based on the standard curve equation y = ax + b with y = 50 % to find x (x is the value of IC_{50} which needs to be found)[0].

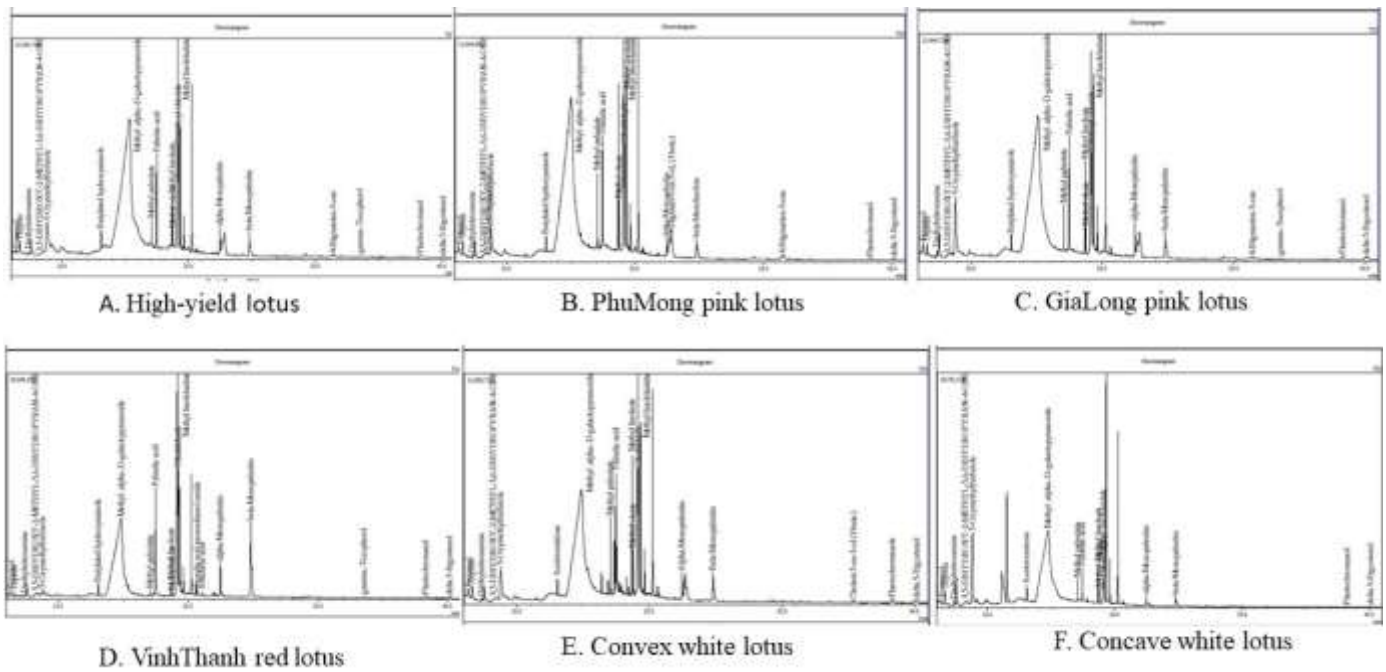

Fig. 1: GC-MS chromatogram of the methanolic extract of six lotus varieties seed in Viet Nam

Results and Discussion

Phytochemicals and Constituents: GC-MS chromatogram analysis of the methanol condensed extract of *N. nucifera* seed showed twenty-seven peaks indicating the presence of twenty-seven phytochemical constituents in the six lotus varieties (Fig. 1). Among the six lotus varieties, the PhuMong pink lotus and the VinhThanh red lotus have twenty phytochemical constituents, the GiaLong pink lotus and Highyield lotus have nineteen phytochemical constituents, the White lotus varieties have eighteen phytochemical constituents, the Convex white lotus has seventeen phytochemical constituents (Table 1).

On comparison of the mass spectra of the constituents with Wiley and NIST library, the twenty-seven phytocompounds were characterized and identified and their retention time (rt), molecular weight (mw), molecular formula and concentration (peak area %) are presented in table 1.

Among the compounds, fourteen compounds were the most prevailing in all seed lotus varieties with relatively high ratio including methyl-alpha-D-galactopyranoside (66,86-78,69%), furaneol (0,20-0,53%), thymin (0,21-1,44), diethylnitroamine (0,11-0,42%), 3,5dihydroxyl-2-methyl-5,6-dihydropyran-4-one (0,42-1,06%), 5-oxymethyl furfurole (2,60-11,04%), methyl palmitate (0,17-1,81%), palmitic acid (2,22-7,41%), methyl linoleate (1,23-3,16%), methyl oleate (0,13-1,05%), methyl linolelaidate (2,69-13,44%), alpha-monopalmitin (0,80-1,55%), plastochro manot (0,24-0,53%) and delta-5-esgosterol (0,29-0,57%).

In addition, each different lotus variety will present some different compounds, for example, VinhThanh red lotus variety has six more compounds: butylated hydroxyl, beta-monopalmitin, gamma-tocophenol, olealdehyde, caprylic acid and arachidic acid in which two compounds caprylic acid and arachidic acid are absent in the remaining lotus varieties.

Table 1
Phytocomponents identified in methanolic extract of six lotus varieties *(N. nucifera)* seed by GC-MS

S.N.	Name	High-yield lotus		PhuMong pink lotus		GiaLong pink lotus		VinhThanh red lotus		Convex white lotus		Concave white lotus	
		R. Time (Minute)	Area %	R. Time (Minute)	Area %	R. Time (Minute)	Area %	R. Time (Minute)	Area %	R. Time (Minute)	Area %	R. Time (Minute)	Area%
1	Furaneol	6.308	0.20	6.287	0.24	6.361	0.44	6.335	0.27	6.311	0.43	6.294	0.53
2	Thymin	6.648	0.72	6.623	0.46	6.657	0.39	6.614	0.21	6.633	0.62	6.638	1.44
3	Diethylnitroamine	7.374	0.13	7.357	0.11	7.411	0.23	7.396	0.15	7.366	0.15	7.370	0.42
4	3.5dihydroxyl-2-methyl-5.6-dihydropyran-4-one	7.550	0.87	7.539	0.56	7.579	1.02	7.568	1.06	7.545	0.66	7.554	0.42
5	5-oxymethylfurfurole	8.810	3.56	8.771	2.60	8.827	4.75	8.794	0.78	8.779	3.50	8.805	11.04
6	butylated hydroxylanisole	13.080	0.49	13.081	0.29	13.082	0.31	13.079	0.41	-	-	-	-
7	methyl-alpha-D-galactopyranoside	15.281	75.27	15.030	78.69	15.112	77.17	14.839	66.86	14.865	71.60	14.765	71.57
8	methyl palmitate	17.076	0.59	17.082	1.18	17.077	0.80	17.075	0.17	17.080	1.81	17.072	1.09
9	palmitic acid	17.486	3.76	17.483	2.92	17.489	3.41	17.504	7.41	17.493	4.58	17.451	2.22
10	methyl linoleate	18.695	1.23	18.702	2.69	18.697	1.61	18.693	0.49	18.700	3.16	18.692	1.97
11	methyl oleate	18.757	0.42	18.764	0.84	18.759	0.44	18.758	0.13	18.763	1.05	18.757	0.67
12	methyl linolelaidate	19.144	8.53	19.129	5.20	19.136	5.80	19.175	13.44	19.143	7.69	19.083	2.69
13	lyoleoyl Chloride	19.185	1.15	-	-	19.179	0.76	-	-	-	-	-	-
14	alpha-monopalmitin	22.476	0.80	22.478	0.37	22.478	0.79	22.485	1.55	22.480	0.83	22.470	0.75
15	beta-monopalmitin	24.795	0.89	-	-	24.796	0.71	24.842	4.26	24.802	0.97	24.784	0.68
16	4-stingmasten-3-one	31.430	0.44	31.513	0.69	31.478	0.38	-	-	-	-	-	-
17	gamma-tocophenol	33.569	0.09	-	-	33.586	0.07	33.593	0.12	-	-	-	-
18	Plastochromanol	38.316	0.40	38.336	0.25	38.333	0.47	38.340	0.41	38.336	0.53	38.291	0.24
19	delta-5-esgosterol	40.108	0.46	40.150	0.29	40.148	0.45	40.154	0.48	40.156	0.57	40.100	0.35
20	2.3-Diethylnitrosamine	-	-	8.619	0.04	-	-	-	-	-	-	-	-
21	Olealdehyde	-	-	19.173	0.74	-	-	19.217	1.62	19.186	1.29	19.133	0.69
22	stigmast-5en-3ol (3beta)	-	-	22.865	1.40	-	-	-	-	-	-	19.133	0.69
23	beta-monolinolein	-	-	24.799	0.44	-	-	-	-	-	-	-	-
24	caprylic acid monoethanol amide	-	-	-	-	-	-	20.701	0.09	-	-	-	-
25	arachidic acid	-	-	-	-	-	-	21.087	0.07	-	-	-	-
26	aceto veratrone	-	-	-	-	-	-	-	-	13.081	0.37	13.075	0.87
27	cholest-5-en-3-ol	-	-	-	-	-	-	-	-	35.399	0.19	-	-

Note: "-": Absent

Two white lotus varieties have some new compound as aceto veratrone (0,37-0,87%) and cholest-5-en-3-ol (0,19% only in the Concave white lotus).

The chemical constituents identified in the methanol condensed extract of *N.nucifera* seed belong to different chemical groups. The most prevailing component in the condensed extract was methyl-alpha-d-galactopyranoside. Its peak area accounts for 66,86-78,69% in all lotus varieties, The PhuMong pinks lotus has the highest result (78,69%) followed by the GiaLong pinks lotus (77,17%) and the High-yield lotus (75,21%). The smallest peak area % is found in Vinh Thanh red lotus (66,86%). The concave white lotus and the convex white lotus have similar results (71,57- 71,60%). This is a derivative of monosaccharide with anomalous methyl substituents. It has many roles in the structure and function of living organisms. Besides, this compound also contributes to the sweetness, aroma and flexibility of food.

Among the compounds, most of the compounds were reported to have biological activities. 4-stigmastadiene-3-one is an antibacterial steroid, Stigmast-5-en-3-ol is a phytosterol that has an antidiabetic property[1] and palmitic acid is a fatty acid that has many important roles in the human body [22]. Also, there are many new phytochemical constituents present in the seeds of the six lotus varieties in Viet Nam such as furaneol, diethylnitroamine etc. The results of this study contribute to confirm the medicinal role of lotus seeds in the field of medicine.

Antioxidant activities of methanol liquid and condensed extract seed from six lotus varieties in Viet Nam

Antioxidant activities of methanol liquid extract from lotus seeds: The antioxidant activity of plant extracts cannot be evaluated by only one single method due to the complex nature of phytochemicals and antioxidant activity determination is highly reaction-mechanism dependent. Multiple chemical or biological assays have been developed to evaluate the antioxidant activity and explain the antioxidant mechanism of action of plant extracts. Of those, the DPPH assay, ABTS assay and reducing power assay are the most commonly used assays to evaluate the antioxidant activities of plant extracts[40].

In this study, assays of DPPH scavenging activity were used for the determination of the antioxidant activity of the lotus seed. The results of these evaluations were expressed as SC% and IC_{50} values which are shown in table 2 and table 3. Table 2 indicated that all the tested lotus seed extracts exhibited appreciable DPPH radical scavenging activity ranging from 24.31-89.15%.

The result showed that the antioxidant activity was proportional to the increasing of lotus seed content. Inline concentration of extraction 30 mg/mL showed the highest radical scavenging mounting to 58,42-89,15 % while concentration 1,785 had the lowest 24.31-39.95% potential (Table 2).

Concave white lotus has the highest result, the lowest result is VinhThanh red lotus. Linear regression equations obtained from lotus seed extract were performed as previously described (Table 3) with correlations coefficient from 0.952-0.998. The amount exhibiting 50% of DPPH in methanol liquid extract of six lotus seed varieties was 8.20-15.67 mg/mL which was many times lower than that of ascorbic acid 3,2 µg/mL.

Table 2
DPPH free radical scavenging activity of lotus seed extracts in methanolic

Concentration of extraction (mg/mL)	% Free radical scavenging activity SC (%SC)					
	High-yield lotus	PhuMong pink lotus	GiaLong pink lotus	VinhThanh red lotus	Convex white lotus	Concave white lotus
30	87.32[a]	85.75[a]	88.51[a]	58.42[a]	85.98[a]	89.15[a]
15	66.53[b]	66.36[b]	63.79[b]	50.06[b]	62.21[b]	62.82[b]
7,5	42.24[c]	41.76[c]	46.60[c]	44.51[c]	38.93[c]	47.73[c]
3,75	31.59[d]	29.37[d]	30.95[d]	43.16[d]	30.76[d]	41.12[d]
1,875	26.69[e]	24.31[e]	28.99[e]	41.79[e]	30.23[e]	39.95[e]

Table 3
IC_{50} values of lotus seed liquid extract and ascorbic acid control

Lotus variety	Equation	Unit	R^2	IC_{50}
High-yield lotus	y = 2.1902x + 25.411	mg liquid extract/mL	0.964	11.23[c]
PhuMong pink lotus	y = 2.216x + 23.748		0.952	11.85[b]
GiaLong pink lotus	y = 2.1469x + 26.811		0.975	10.80[d]
VinhThanh red lotus	y = 0.5956x + 40.664		0.996	15.67[a]
Convex white lotus	y = 2.0878x + 25.351		0.980	11.81[b]
Concave white lotus	y = 1.7969x + 35.264		0.998	8.20[e]
Ascorbic acid	y = 6762x + 28.324	µg/mL	0.963	3.20[f]

Concave white lotus has the lowest IC_{50} with 8.20 mg/mL, so DPPH radical scavenging activity of this lotus variety is the highest, next to GiaLong pink lotus (10.80 mg/mL) and high-yield lotus (11.23 mg/mL), PhuMong pink lotus and convex white lotus have similar antioxidant activity with 15,67 mg/mL.

Antioxidant activities of methanol condensed extract from six varieties lotus seeds: Liquid extract was concentrated by the vacuum evaporation system (EI-VAP Advantage G3, Heidolph - Germany). Antioxidant activities were performed as previously described (Table 4 and 5). Methanol dried extract of N. nucifera showed significant dose-dependent antioxidant activity at concentrations ranging from 0.125 mg/mL to 2mg/mL. Maximum antioxidant activity increase was observed with methanol condensed extract of six lotus varieties seed at a concentration of 2 mg/mL which exhibited appreciable DPPH radical scavenging activity ranging from 62.97-77.97%.

Minimum antioxidant activity decrease was observed with methanol dried extract of six lotus varieties at a concentration of 0.125 mg/mL which exhibited appreciable DPPH radical scavenging activity ranging from 9.86-26.91%. Thus, the antioxidant capacity is proportional to the content of lotus seed extract. Methanol dried extract of *N. nucifera* exhibited a dose-dependent DPPH inhibitory effect with an IC_{50} value from 0.850-1.286 mg/mL.

However, it was not more effective than ascorbic acid which showed an IC_{50} value of 3.2 µg/mL (265-401 fold less potent as compared to ascorbic acid) (Table 5) in which the concave white lotus has the highest DPPH radical scavenging activity and the lowest in the VinhThanh red lotus variety. High-yield lotus has medium DPPH radical scavenging activity with an IC_{50} value of 11.23 mg/mL.

The antioxidant activity of the dried extract was higher than that of liquid extract from lotus seed. Because the dried extract undergoes high concentration and removes some impurities, so the ability of impurities to affect the free radical scavenging activity decreases. The antioxidant activity of lotus seeds has been studied and confirmed by many authors around the world. Lotus seeds extracted in methanolic, ethanolic and hydroalcoholic have strong free radical scavenging activity. Sohn et al[26] showed that ethanol extracts from *Nelumbo nucifera* seed showed potent free radical scavenging effects with a median inhibition concentration of 6.49 g/ml.

The antioxidant activity of the hydroalcoholic extract of lotus seed has been reported by Rai et al[21] using the DPPH and nitric oxide methods. The hydroalcoholic extract exhibited strong free radical scavenging activity with IC_{50} values of 6.12 ± 0.41 mg/ml in the DPPH assay and 84.86 ± 3.56 mg/ml in the nitric oxide assay.

The antioxidant activity in these studies was determined by secondary compounds isolated from lotus seeds such as polyphenols, alkaloids and flavonoids so that IC_{50} values were much lower (that means antioxidant activity is higher) than our results.

According to our results, the IC_{50} value of methanolic extract of lotus seed is lower than previously reported because we tested methanol liquid and condensed extract seed without the extraction and isolation of antioxidant compounds. This is very important in real life.

Table 4
DPPH free radical scavenging activity of lotus seed dried extracts in methanolic

Concentration of dried extraction (mg/mL)	% free radical scavenging activity SC (%SC)					
	High-yield lotus	PhuMong pink lotus	GiaLong pink lotus	VinhThanh red lotus	Convex white lotus	Concave white lotus
2	77.59[a]	77.97[a]	76.30[a]	62.97[a]	76.43[a]	77.97[a]
1	45.99[b]	51.44[b]	54.20[b]	47.50[b]	51.34[b]	61.74[b]
0.5	30.23[c]	38.32[c]	36.39[c]	33.97[c]	37.30[c]	41.61[c]
0.25	19.95[d]	22.15[d]	27.74[d]	30.25[d]	27.84[d]	31.52[d]
0.125	9.86[e]	19.61[e]	22.34[e]	22.59[e]	25.67[e]	26.91[e]

Table 5
IC_{50} values of lotus seed dried extract and ascorbic acid control.

Lotus variety	Unit	R^2	IC_{50}
High-yield lotus	mg condensed extract /mL	0.988	1.160[b]
PhuMong pink lotus		0.978	1.036[c]
GiaLong pink lotus		0.988	1.006[d]
VinhThanh red lotus		0.971	1.286[a]
Convex white lotus		0.996	1.005[d]
Concave white lotus		0.953	0.850[e]
Acid ascorbic	µg/mL	0.963	3.200[f]

Conclusion

In conclusion, the seeds of six lotus varieties in Viet Nam have twenty-seven various bioactive compounds. Antioxidant activity of liquid extract and the condensed extract was found as 8.20-15.67 mg/mL and 0.850-1.286 mg/mL, many times lower than that of ascorbic acid with IC50 3.2 µg/mL.

From obtained results, the seeds of six lotus varieties in Vietnam were found to be potential for bioactive compounds antioxidant activity. Further experiments should be carried out in the future to detect bioactive compounds and investigate their biological activities. Therefore, seed lotus is recommended as a plant seed of pharmaceutical importance.

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