SUPPLEMENTARY MATERIAL

Antioxidant, cholinesterase inhibition activities and essential oil analysis of *Nelumbo nucifera* seeds.

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Abstract:

N. nucifera seeds essential oil, crude extract and subsequent fractions were evaluated for their DPPH, ABTS and superoxide anion free radical scavenging and cholinesterase inhibitory activities. The ethyl acetate fraction and essential oil showed outstanding antioxidant activities with IC₅₀ values of 191 µg/ml, 450 µg/ml (DPPH), 123 µg/ml, 221 µg/ml (ABTS) and 69 µg/ml, 370 µg/ml (Super oxide anion) respectively. The ethyl acetate fraction and essential oil also caused significant inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) with IC₅₀ values of 70 ± 0.6, 64 ± 0.8 and 75 ± 0.3, 58 ± 0.2, in dose dependent manner. The first ever gas chromatography-mass spectrometry (GC-MS) analysis of the essential oil obtained from *N. nucifera* seeds resulted in identification of 19 constituents mainly comprised of oxygenated sesquiterpenes responsible for their promising bioactivity. The crude and fractions revealed the presence of saponins, flavonoids, steroids, alkaloids, terpenoids and cardiac glycosides in phytochemical investigation.

Key words: *Nelumbo nucifera*, antioxidant, cholinesterase inhibition, Essential oil, GC-MS analysis

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Experimental

Plant material, extraction and fractionation

Mature and fully dried seeds of *Nelumbo nucifera* were collected in Bannu, KP, Pakistan. Further taxonomic identification was made by Prof. Abdur Rehman, Govt. Post Graduate College Bannu, KPK, Pakistan. A voucher specimen (BG-201) has been submitted to the herbarium of University Science and Technology, Bannu, KPK Pakistan. The shade dried seeds were milled into a fine powder (5 Kg), soaked in methanol (10 L) at room temperature twice for 7 days, filtered and concentrated under vacuum at low temperature (40 °C) using a rotary evaporator. A combined blackish crude extract (250 g) was obtained which was sequentially portioned into the *n*-hexane (N₁, 25 g), chloroform (N₂, 45 g), ethyl acetate (N₃, 55 g), *n*-butanol (N₄, 50 g) and aqueous (N₅, 30 g) fractions respectively.

Essential oil (EO) extraction

N. nucifera seeds (100 g) were suspended in 5 L water and subjected to hydro-distillation for 4 hours using a cleverger-type apparatus for essential oil extraction. The resulted extracted oil (1 %) was separated from water by extracting three times with EtOAc (300 ml) and was dried by filtration over anhydrous sodium sulphate. The oil was placed in sealed tubes and kept at 4 °C. (Shojaaddin et al, 2008)

Chemicals

An antioxidant assay kit (Cat. No CS0790) and chemical reagents dimethyl sulfoxide (DMSO), Nitro blue tetrazolium (NBT), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Germany. The solvents: methanol, *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and ascorbic acid were purchase from Merck. Acetylcholinesterase (Electric-eel EC 3.1.1.7), butyrylcholinesterase (horse-serum E.C 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5, 5'-dithiobis [2-nitrobenzoic-acid] and galanthamine were purchased from Sigma. All other chemicals were of analytical grade.

In vitro antioxidant assay

2.3.1 DPPH (1, 1-diphenyl-2-picryl-hydrazyl) free radical scavenging activity

DPPH free radical scavenging assay was performed according to the procedure (Kulisic, Radonic, katalinic and Milos, 2004). Briefly, in a reaction mixture, 900 µl of DPPH solution was added and mixed with 100 µL of test samples, leading to the concentration of 100, 250, 500 and 1000 µg. The reaction mixtures were mixed well and kept in dark for one hour at a controlled temperature (37 °C). Spectrophotometric absorbance of reaction mixture was

measured at 517 nm. 3% methanol was used as a blank while mixture of 100 µl 3% methanol sample and 900 µL of DPPH were taken as negative control. Ascorbic acid was used as positive control.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) method

Assays were performed by using the antioxidant Assay Kit (Cat. no CS0790). The ABTS substrate and working solution were prepared by adding 25 µl of 3% hydrogen peroxide solution (cat. no 323381) to 10 ml of ABTS substrate solution for a standard curve and used within 20–30 minutes. Trolox (2-10 µg) was used as a standard inhibitor.. 10 µl of a trolox standard and 20 µl of myoglobin working solution were added to the wells to obtain trolox standard curve followed by the addition of 10 µl test samples and 20 µl of myoglobin working solution to the wells for the test samples. In step 2, 150 µl of ABTS substrate working solution was added to each well and incubated for 5 minutes at room temperature proceeded by the addition of 100 µl of stop Solution (Cat. no S3446) to each well. The endpoint was measured as absorbance at 405 nm using a plate reader (Huang et al, 2010).

Superoxide anion scavenging assay

Nitro blue tetrazolium reagent (NBT) method was used to measure the activity, as described by Sabu and Ramadasan (Sabu & Ramadasan, 2002). The reaction mixture consisted of 0.5 ml of NBT solution (1M NBT in 100 mM phosphate buffer, pH 7.4). 0.3 ml NADH solution 1M NADH in 100 mM phosphate buffer, pH 7.4 and 0.1 ml of different fractions and ascorbic acid (50 mM phosphate buffer pH 7.4) was mixed. The reaction was started by adding 100 µl of PMS (phasphomethazone sulphate) solution (60 µM PMS in 100 mM phosphate buffer pH 7.4) in the mixture. The tubes were uniformly illuminated with an in candescent visible light for 15 minutes and the optical density was measured at 530 nm before and after the illumination. The percentage inhibition of superoxide generation values of the control and samples were measured. Ascorbic acid was used as positive control.

Acetylcholinesterase and butyrylcholinesterase inhibition assay

Acetylcholinesterase and butyrylcholinesterase inhibiting activities were measured according to a modified spectrophotometric method (Ellman et al, 1961). 0.2mM of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 62 mM sodium phosphate buffer (pH 8.0, 880 µl), test compound solution (40 µl) and acetylcholinesterase or butyrylcholinesterase solution (40 µl) were mixed and incubated for 15 minutes (25° C). The reaction was then initiated by the addition of acetylthiocholine or butyrylthiocholine (40 µl), respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine were monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the

enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, at a wavelength of 412 nm (15 min). All the reactions were performed in triplicate using BMS spectrophotometer (BMS Spec 5000, Breukhoven, USA).

GC and GC-MS analysis:

GC analysis of the essential oil was executed on Shimadzu-GC-9A gas chromatograph (Shimadzu Corporation, Japan) with installed SPB-5 capillary column (30m× 0.53 mm ID; 0.3 µm). Signals corresponded to elution were recorded by FID detector at 220 eV. Nitrogen gas was used as carrier gas at 1.0 ml/min flow rate, split ratio was set as 1:30 and injector temperature was fixed at 240°C. The column temperature was maintained at 50°C for the first 5 min and then ascended up to 235°C at rate of 5°C/min. GC-MS spectra were recorded on Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, USA), coupled with a Jeol, JMS-HX 110 mass spectrometer (Jeol Limited, Japan) with injector at 270 °C while splitting ratio set at 1:30. GC-MS analyses were carried out on an equivalent column HP-5 (25m × 0.22mm and 0.25 µm) with identical gradient thermal ramping and temperature parameters as mentioned above for GC analysis

Phytochemical Screening

Chemical tests were carried out on all the fractions (**N₁ - N₅**) of the *N. nucifera* seeds using standard procedures to identify the phyto-constituents (Masood et al, 2013).

Statistical analysis

All assays were carried out in triplicates, and results are expressed as mean ± SEM. ANOVA test was used to analyze the differences among IC₅₀ of various fractions for different assays. The IC₅₀ values were calculated using graph pad prism software.

Table S1: Phytochemical profile of crude and fractions (N₁-N₅) of *Nelumba nucifera*

Phytochemicals	Crude extract	N ₁	N ₂	N ₃	N ₄	N ₅
Tannins	+	-	-	+	-	-
Phlobatannins	+	+	-	-	+	-
Saponins	-	-	+	+	-	-
Flavonoids	+	-	+	+	-	-
Steroids	+	-	+	-	+	-
Alkaloids	-	-	-	+	+	+
Quinone	+	-	+	+	-	-
Coumerin	+	-	-	+	-	-
Terpenoids	-	+	-	-	-	-
Glycosides	-	-	-	+	+	+

Key: - = absent, + = present

Table S2: GC/MS analysis of essential oil from *Nelumbo nucifera* seeds

Scan Number	Retention Time (in minutes)	Compound	Rel. Abundance (%)
8	3.15	<i>3-carene</i>	0.85
42	3.9	<i>Camphene</i>	5.13
71-77	4.59	<i>α-pinene</i>	4.27
115-122	5.47	<i>1-8-cineole</i>	25.64
134	5.88	<i>Borneol</i>	7.69
166	6.58	<i>α-terpeneol</i>	11.11
181	6.9	<i>Linalol</i>	1.71
290	9.27	<i>Geraniol</i>	4.27
312	9.75	<i>Thujol</i>	3.42
603	16.05	<i>Fernesene</i>	1.71
652	17.1	<i>γ-Gurjunene</i>	6.84
680	17.72	<i>γ-cadinene</i>	3.42
733	18.87	<i>Bicycle-Germacrene</i>	5.13
834	21.05	<i>τ-Cadinol</i>	1.71
875	21.93	<i>α-asarone</i>	10.26
883	22.12	<i>α-Eudesmol</i>	1.71
891	22.28	<i>α-bisabolol</i>	0.85
916	22.83	<i>β-Bisabolol</i>	0.85
1058	25.9	<i>Fernasonic acid</i>	3.42

Figure S1: GC (Total Ion) Chromatogram of *N. nucifera* seeds essential oil.

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