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Antioxidant and Acetylcholinesterase Inhibitory Properties of the Indian Medicinal Plant "Shankhapushpi" Used for Enhancing Memory Function

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Abstract

Objective - At least seven plants known as Shankhapushpi are used in Indian system of medicine for improving memory function. The present study was aimed to analyze the antioxidant and acetylcholinesterase inhibitory properties of the methanolic extracts of five of these plants, e.g. *Clitorea ternatea, Canscora decussata, C. diffusa, Evolvulus alsinoides, E. nummularius*.

Methods – Acetylcholinesterase activity was assayed modifying the method of Ellman et al. (1961) using mice brain homogenates as the enzyme source. Antioxidant activity of the extracts were determined by measuring DPPH radical and superoxide radical scavenging activity, metal chelation effect and total antioxidant capacity.

Results - All the plants (except *C. ternatea*) inhibited acetylcholinesterase in a dose dependant manner, significantly scavenged DPPH radical and superoxide radical and chelated metal ions. Total antioxidant capacity (equivalent to ascorbic acid) of the plant extracts were also good. It was found that *C. decussata* has the highest acetylcholinesterase inhibitory activity. Antioxidant activity in all systems (except metal chelation property) was highest in *C. decussata*.

KEYWORDS: Shankhapushpi, *Canscora decussata*, *Canscora diffusa*, *Evolvulus alsinoides*, *Evolvulus nummularius*, *acetylcholinesterase*, antioxidant

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Introduction:

"*Shankhapushpi*" is the medicinal plant used in Indian system of medicine for improving memory function. At least seven plants are known as *Shankhapushpi*. These are *Clitorea ternatea* L., *Canscora decussata* Schult., *C. diffusa* R. Br., *Evolvulus alsinoides* L., *Convolvulus microphyllus* Sieber ex Spreng, *C. pleuricaulis* Choisy (Mitra, 1985). *Evolvulus nummularius* is also locally known as *Shankhapushpi*. The roots of *C. ternatea* are intellect promoting (Jain et al., 2003).

Acetylcholine (Ach) stimulation of the nicotinic receptors appears to be associated with cognitive process and memory. The Ach has a very short life due to the presence of acetylcholinesterase (AchE) which hydrolyses the ester bond of the molecule, thus leading to loss of stimulatory activity (Silman and Sussman, 2005; Houghton et al., 2006). The neuropathological occurrence associated with memory loss is a cholinergic deficit which has been correlated with the severity of Alzheimer's disease (AD) (Bierer et al., 1995; Collerton, 1986; Perry et al, 1978). Approaches to enhance cholinergic function in AD have included simulation of cholinergic receptors or prolonging the availability of acetylcholine (Ach) released into the neuronal synaptic cleft by inhibiting Ach hydrolysis by acetylcholinesterase (AchE); the latter may be achieved through the use of AchE inhibitors (Howes and Houghton, 2003). Among the possible strategies for enhancing brain cholinergic activity, acetylcholinesterase inhibitors have been the most extensively used for the symptomatic treatment of AD (Siqueira et al., 2003). Neurodegenerative diseases such as AD are free radical reactions, which are reported to initiate cell injury. So the use of antioxidants has also been used to slow AD progression and neuronal degeneration (Howes and Houghton, 2003; Maxwell, 1995; Slater, 1984; Spiteller, 1993).

In the present paper we report a comparative study of antioxidant and acetylcholinesterase inhibitory properties of the leafy shoots of *C. decussata*, *C. diffusa*, *E. alsinoides*, *E. nummularius* and the roots of *C. ternatea.*

Materials and methods

Plant Materials:

The leafy shoots of *C. decussata* (CDS), *C. diffusa* (CDF), *E. alsinoides* (EAL), *E. nummularius* (ENM) and the roots of *C. ternatea* (CLT) were collected from Kolkata and surrounding areas. The extracts were made from the dried ground materials by refluxing with 100% methanol for 5 hours. The extracts were then evaporated to dryness. Different concentrations of the methanolic solutions of the

extracts were used for analyzing antioxidant activity in vitro and for studying acetylcholinesterase inhibitory activity. Each experiment was repeated three to five times.

Chemicals:

1,1 Diphenyl-2-picrylhydrazyl was procured from Sigma, USA. Nitroblue tetrazolium, 5,5′ dithiobis (2 nitrobenzoic acid), acetylthiocholine were obtained from Sisco Research Laboratories PVT. Ltd., India. All other reagents were of analytical grade.

Animals:

Brain from male mice, maintained in accordance with the guidelines of the committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Forests and Environment, Government of India, were obtained from the Central Drugs Laboratory, Kolkata, India

Acetylcholinesterase Activity:

Acetylcholinesterase activity was assayed modifying the method of Ellman et al. (1961), following Oh et al. (2004) and Siqueira et al. (2003). Brains from 3-4 weeks old mice were washed with cold sodium phosphate buffer (0.2M, pH 8), homogenized in buffer and centrifuged at 10,000 RPM at 4 ^{0}C. The supernatant was used as the enzyme source (AChE). The plant extracts were dissolved in methanol. AChE (1.4 ml) and plant extract solution (100 µl) were added to 1.44 ml buffer. The reaction was started by adding 30 µl 0.5 mM 5,5′ dithiobis (2 nitrobenzoic acid) (DTNB) and 30 µl 0.6mM acetylthiocholine iodide solution. The reaction mixture was incubated at 37 ^{0}C for 20 min. The optical density was measured at 412 nm immediately. The percentage inhibition of AChE activity by plant extract was calculated.

DPPH radical scavenging activity:

The antioxidant activity of the extracts on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined following the method described by Braca et al (2001).

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Superoxide radical ($O_2^{\cdot-}$) scavenging activity

Superoxide radical scavenging activity was determined following the method used by Dasgupta and De (2004) in the riboflavin-light-nitrobluetetrazolium (NBT) system (Beauchamp and Fridovich, 1971).

Metal chelating effect (Ferrous ion)

The method is based on the chelation of ferrous ions by the plant extract (Wang et al., 2003). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of plant extract (chelating agents) the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Measurement of colour reduction therefore allows estimating the metal chelating activity of the coexisting chelators. Fe^{2+} chelating ability is due to antioxidant activity of the plant extract.

Determination of total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH (Prieto et al., 1999). The antioxidant capacity is expressed as ascorbic acid equivalent (AAE). Extract (0.3ml) was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95^0 C for 90 min. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm against blank. Total antioxidant capacity of plant extract was measured from the regression equation ($y=0.0114x + 0.0664$) prepared from the concentration versus optical density of ascorbic acid.

Determination of total phenol content

Phenol content was determined by Folin-Ciocalteau reagent in alkaline medium and was expressed as gallic acid equivalents (GAE) (Sadasivam and Manikam, 1992). Total phenol content was calculated from the regression equation ($y = 0.0193x – 0.0006$) prepared from a range of concentrations of gallic acid and optical densities for the concentrations.

Determination of total flavonoid content

Total flavonoid content was determined following Kim et al. (2003) and was expressed as catechin equivalent (CE), calculated from the regression equation prepared from a range of concentrations of catechin and optical densities for the concentrations.

Statistical analysis

The results were statistically analyzed by Welch's t test to show significant differences in activity. Correlation coefficients to determine the relationship between two variables (concentrations and % inhibition; activity and total phenol/flavonoid content) were calculated using MS Excel software.

Results and discussion

Different concentrations (up to 500 μg/ml) of the extracts of the plants were tested for the acetylcholinesterase inhibitory activity. The plants except the root extract of *C. ternatea* showed good activity in the concentration range. But *C. ternatea* extract at 500 μg/ml concentration inhibited only 16.98 ± 1.04 percent enzyme activity. Although Taranalli and Cheeramkuczhi (2000) found that the ethanolic extract of the root parts of this plant significantly produced memory retention, increased rat brain acetylcholine content and acetylcholinesterase activity, the present result showed very weak activity. All other plant extracts inhibited AChE in a dose dependent manner. The IC_{50} values (the concentration of plant extract for inhibition of activity by 50%) are shown in Table I. CDS was found to have highest activity (statistically significant) in comparison to other plants (P value < 0.05). CDF recorded lowest activity. The difference in activity between EAL and ENM is statistically insignificant.

Antioxidant activity of the plant extracts were also tested in vitro using different systems of assay e.g. DPPH radical scavenging assay, superoxide radical scavenging assay, metal chelating (ferrous ion) assay and total antioxidant capacity. The IC_{50} values for scavenging DPPH radical, superoxide radical and ferrous ion chelation are shown in Table 2 - 4. Antioxidant activity of CLT was not studied as the plant extract did not show good acetylcholinesterase inhibitory activity. All other plant extracts showed good antioxidant activity highest being observed in CDS. All the extracts showed significant correlation between activity and concentration.

The plant extracts scavenged DPPH radical and the activity was proportional to the concentration (Table 2). The extracts on interaction with DPPH neutralized its free radical character. The colour changed from purple to yellow and its absorbance at wavelength 517 decreased. Highest activity was observed in CDS, followed by ENM, EAL and CDF. The differences in activities between CDS and other plants were statistically significant (P value < 0.05)

Highest superoxide radical scavenging activity was observed in CDS followed by EAL (Table 3). CDF and ENM were found to have lower activity. Statistically significant differences in activity between EAL and ENM (P value <

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0.01), CDS and CDF (P value < 0.05) and between CDS and EAL (P value < 0.01) were observed. Photochemical reduction of flavins generates $O_2^{-\cdot}$ which reduces NBT resulting in the formation of blue formazan (Beauchamp and Fridovich, 1971). The plant extracts inhibited the formation of the blue formazan in a dose dependent manner.

Table I: Acetylcholinesterase inhibitory activity

Plant material	Concentration (µg/ml)	%Inhibition ± sd	Regression equation (r*)	IC_{50} value (µg/ml)
CDS	133.33	40.16 ± 0.8	Y=0.111x + 29.566	
	200	54.08 ± 0.93		184.09
	266.66	61.61 ± 1.01	(0.9849)	
	400	75.97 ± 1.46		
	533.33	86.26 ± 0.93		
CDF	300	13.36 ± 1.92	Y=0.3726x – 88.672	
	350	50.43 ± 1.83		372.17
	400	66.88 ± 4.55	(0.9624)	
	500	92.23 ± 2.20		
EAL	100	8.06 ± 1.51	Y= 0.3463 – 30.456	
	200	32.20 ± 1.33		232.33
	250	57.97 ± 3.13	(0.9899)	
	300	70.06 ± 2.77		
	350	94.95 ± 5.24		
ENM	100	18.51 ± 2.60	Y=0.3056x – 16.809	
	200	41.55 ± 0.62		218.62
	250	56.32 ± 1.03	(0.9954)	
	300	75.89 ± 0.84		
	350	92.40 ±3.28		

* r = correlation coefficient of dose response

Table 2: DPPH radical scavenging activity of plant extracts.

Plant Material	Concentration (µg/ml)	%Inhibition ± sd	Regression equation (r*)	IC_{50} value (µg/ml)
CDF	16.13	21.62 ± 0.47		
	32.26	30.03 ± 0.38	Y=0.5837x+12.587	
	64.52	50.53 ± 0.40		
	96.77	73.99 ± 0.63	(0.9935)	64.1
	129.03	84.46 ±1.35		
CDS	16.13	27.21± 0.32		
	32.26	36.17 ± 0.90	Y=1.2737x+2.703	
	45.16	63.03 ± 0.48		37.13
	64.52	85.74 ± 0.35	(0.9804)	
EAL	16.13	18.24 ± 0.72		
	32.26	39.33 ± 1.01	Y=0.575x+18.689	
	48.39	52.86 ± 0.84		54.45
	80.65	70.77 ± 1.10	(0.9618)	
	96.77	77.64 ± 1.18		
	129.03	85.13 ± 1.39		
ENM	16.13	17.34 ± 0.51		
	32.26	46.10 ± 0.98	Y=0.6251x+19.135	49.38
	64.52	65.84 ± 1.58		
	96.77	88.05 ± 0.54	(0.9457)	
	129.03	90.07 ± 0.49		

* r = correlation coefficient of dose response

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Table3: Superoxide radical scavenging activity

Plant Material	Concentration (µg/ml)	% Inhibition ± sd	Regression equation (r*)	IC_{50} value (µg/ml)
CDS	150	36.46 ± 1.03		
	166.67	50.88 ± 0.71	Y=0.547x - 39.806	
	191.67	73.99 ± 0.71		164.18
	208.3	76.49 ± 0.61	(0.9622)	
	250	91.94 ± 1.47		
CDF	166.67	19.30 ± 1.48		
	266.67	47.00 ± 1.60	Y=0.1387x + 5.203	322.98
	333.33	60.13 ± 1.13		
	500	72.61 ± 0.65	(0.9698)	
	666.67	95.06 ± 1.74		
EAL	100	30.40 ± 0.92		
	166.67	55.17 ± 1.11	Y=0.16x + 22.402	172.49
	333.33	82.78 ± 0.13		
	500	97.31 ± 0.84	(0.966)	
ENM	250	38.65 ± 0.46		
	350	44.22 ± 1.61		
	500	61.65 ± 0.31	Y=0.0611x+25.686	397.94
	750	72.89 ± 0.75		
	1000	87.10 ± 0.48	(0.9915)	
	1250	100.0 ± 0.98		

* r = correlation coefficient of dose response

Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of plant extract (chelating agent) the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Measurement of colour reduction therefore allows estimating the metal chelating activity of the coexisting chelators. Fe^{2+} chelating ability is due to antioxidant activity of the plant extract. The plant extracts did not show good metal chelating property. Amongst the four plants, EAL was found to have highest activity. The activity of ENM was lower than EAL (Table 4). The activity between the two was statistically insignificant. The activities of the methanolic extracts of the two species of *Canscora* were poor. Different concentrations of extracts (up to 1000 µg/ml) of CDS and CDF were tested. It was found that 1000 µg/ml concentration of CDS and CDF disrupted complex formation between ferrozine and Fe^{2+} only by 10.55 ± 0.48 per cent and 32.42 10 ± 0.8 per cent respectively. So the IC_{50} values of these extracts were not calculated.

Total antioxidant capacity (AAE) of the plant extracts are shown in Table 5. Total antioxidant capacity (AAE) of all the plant extracts were good. Highest activity was found in CDS.

Table 4: Metal chelating property

Plant Material	Concentration (µg/ml)	% Inhibition ± sd	Regression equation (r*)	IC_{50} value (µg/ml)
EAL	200	8.97 ± 0.93	Y=0.2218x-54.952	
	300	16.29 ± 1.38		
	400	27.49 ± 0.94	(0.9323)	473.18
	450	38.6 ± 1.36		
	500	63.78 ± 2.0		
ENM	200	13.81 ± 0.93	Y=0.0624x + 0.047	
	400	24.13 ± 0.75		
	600	36.98 ± 0.13	(0.9977)	800.53
	800	48.56 ± 0.70		
	1000	64.01 ± 0.35		

* r = correlation coefficient of dose response

Total phenol and flavonoid content in the four plants were measured (Table 6). Significant correlation between total phenol content and acetylcholenesterase inhibitory activity (r = 0.838) and total phenol content and total antioxidant capacity (r = 0.9071) could be detected. Flavonoid contents were not related to any such activity in these four plants.

Table 5: Total antioxidant capacity (AAE)

Plant extract	**Total antioxidant capacity** (µg AAE / mg extract± sd)
CDS	42.61 ± 0.62
CDF	22.23 ± 0.72
EAL	31.63 ± 0.67
ENM	38.99 ± 0.24

Table 6: Total phenol and flavonoid content

Plant material	**Total phenol content (GAE)** (µg/µg plant extract ± sd)	**Total flavonoid content (CE)** (µg/µg plant extract± sd)
ENM	0.29 ± 0.007	0.107 ± 0.001
EAL	0.28 ± 0.01	0.129 ± 0.005
CDS	0.33 ± 0.01	0.0745 ± 0.001
CDF	0.26 ± 0.006	0.093 ± 0.004

Conclusion

Considering all the activities it can be concluded that *C. decussata* has the highest antioxidant (except metal chelating property) and acetylcholinesterase inhibitory activity than the other plants known as *Shankhapushpi* studied during the present investigation. The results also validate the use of some *Shankhapushpi* plants in Indian system of medicine for enhancing memory function. Further in vivo studies are required in this regard.

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