APPRAISAL OF SCOPOLAMINE-INDUCED ANTIAMNESIC EFFECT OF SPHAERANTHUS INDICUS IN MICE

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Summary

The effect of Ether extract of *Sphaeranthus indicus* (SI) was investigated in mice for memory enhancing activity using various experimental paradigms of learning and memory *viz*. Transfer latency (TL) on elevated plus maze and passive avoidance. Ether extract of *Sphaeranthus indicus* (EESI) at a doses of 150 and 300 mg/kg significantly ($P<0.01$) enhanced the learning and memory activities against the scopolamine induced dementia after 9 days of treatment. Further, extract had produced a significant ($P<0.05$) decrease in *Acetylcholinesterase* level in cortex, midbrain, medulla and cerebellum of brain in animals which led us to conclude that the memory enhanced activity as evidenced by learning and retrieval was due to cholinergic facilitatory effect in animals. These results indicate a possible memory enhancing action of *Sphaeranthus indicus* which qualitatively comparable with that of piracetam.

Key words: Ether extract of *Sphaeranthus indicus* (EESI), Scopolamine, and Acetylcholinestrase.

Introduction

Dementia is characterized by progressive cognitive function deficits with multiple cortical dysfunctions including difficulties in memory, judgment, orientation, comprehension, learning capacity and language.[1] The most well know type of dementia is Alzheimer's disease (AD), which proceeds at moderate to severe form and destroy brain. Brain aging is known to be related to excessive neuronal loss, decrease in acetylcholine level, increased inflammation and oxidative stress,[2] senile plaques and neurofibrilary tangles, [3] myeloid-β peptide induced neurotoxicity, [4] increased level of lipid peroxidation.[5] Furthermore it was reported that amyoloid- β deposition causes selective neurological loss and is related to dysfunction and degeneration of basal forebrain cholinergic neurons. The activity of acetylcholinestrase is responsible for Ach hydrolysis has been shown to be increased within and round the amyoloid plaque.[6] It has been suggested that AchE play a pathogenic role in AD. [7] An estimated 4.5 million Americans have AD. The National Institute of Health predicts if the current trend continues, there will more than 8.5 million AD patients by year 2030 in USA alone, by 2050 the number of individual with AD could range from 11.3million to 16 million. [8] Nowadays most accepted treatment strategy include: Rivastigmine, Donepezil, Velnocrin, Tacrine, galanthamine, and memantine used for improving, mood, and behavior, however use of these resulting many adverse effects that made their use limited to symptomatic treatment only. It is worthwhile to explore the use of traditional medicine in treatment of various cognitive disorders. *Sphaeranthus indicus* is known as 'Mundee' in Ayurveda is in many mental disorders, epilepsy, anthelmentic, alexipharmic, antidote for poison, insanity, tubercular glands, looseness of breast, immunomodulator, inflammation, wound healing, antihyperglycemic, anti-oxidant, hypersensitivity, etc. [9] A large number of constituents have been isolated from the extracts of the whole herb, flowers and leaves. Essential oil, obtained by steam distillation of the whole herb, contains ocimene, α-terpinene, methyl-chavicol, α- citral, geraniol, α-ionone, β-ionone, d-cadinene, p-methoxycinnamaldehyde and an alkaloid sphaeranthine phenylurethan, n-pentacosane. [10]

The alcoholic extract of powdered caputula contains stigmasterol, β-sitosterol, hentriacontane, sesquiterpene lactone, sesquiterpine glycoside, sphaeranthanolide, flavone and isoflavone glycosides. [9] A novel flavonoid *C*-glycoside, 5-hydroxy- 7-methoxy-6-*C*-glycosylflavone and isoflavone glycoside,[11] 5,49-dimethoxy-39-prenylbiochanin 7-*O*-b-D-galactoside, Eudesmanoids11a,13-dihydro-3a,7adihydroxy-4,5-epoxy-6b, 7-eudesmanolide,11a,13-dihydro-7a-acetoxy-3b-hydroxy- 6b, 7-eudesm-4-enolide 2a and 3-keto-b-eudesmol.[12] Hence, in the present study, we undertaken to evaluate the effect of petroleum ether extract of *Sphaeranthus indicus* on memory in mice by employing EPM and passive avoidance paradigm and also to evaluate the on the changes in central cholinergic system content of in various parts of their brains.

Materials and methods

Plant collection and extraction

Sphaeranthus Indicus was collected from fields Mangalore, India, and it was identified by botanist Dr. Rajanna, GKVK, University of Agricultural Science, Bangalore India. A specimen voucher (PC2007558) is deposited in museum in the Department. The whole plant was shade dried, powdered and subjected to successive solvent extraction in soxhlet apparatus using Petroleum ether (60/80), the extract was concentrated using rotary evaporator to get semisolid residues which was used for animal studies and preliminary phytochemical tests.

Preliminary photochemical screening of extracts

An aliquot of sample was spotted onto the silica gel plate (Silica gel coated TLC plates; Merck) with a developing solvent system of ethylacetate: glacial acetic acid: formic acid: water (100:11:11:27). The spots were checked under a UV detector at 254 nm and 365 nm. Plates were visualized under UV light and after spraying with chromogenic agents *viz* Dragendorff's reagent (alkaloids), iron (III) chloride (phenolic compounds, including flavonoids), anisaldehydesulfuric acid (terpenes and steroids), etc. The presence of flavonoids was further confirmed by spraying the plates with 5% $AlCl_3$ in ethanol.

Animals

Swiss albino mice of either sex weighing 18-25g were procured from Raghavendra Enterprises, Bangalore, India. They had free access to food and water, and were maintained under standard laboratory conditions with alternating light and dark cycles of 12 h each. The animals were acclimatized for at least 7 days before behavioral experiments. The experimental protocol was approved by the Institutional Animals Ethics Committee (IAEC), PES College of Pharmacy, Bangalore, India. The care of laboratory animals was taken as per CPCSEA (committee for the purpose of control and supervision on experiments on animals) guidelines, Ministry of Environment & Forests (Animal Welfare Division) Government of India.

Acute toxicity studies

The acute oral toxicity of Ether Extract of *Sphaeranthus Indicus* (EESI) and was determined in female albino mice (18-22g). Each group consisting of three mice were administered with different doses of the extract up to 2000mg/kg by oral route. Then the mortality with each dose was observed for 48 hours. Outcome of this was used for the treatment protocol.

Drugs and chemicals

Scopolamine hydro bromide (Sigma-Aldrich, USA), Diazepam (Ranbaxy, India), Piracetam (Micro labs, India), 5, 5' Dithio-bis (2-nitrobenzoic acid) (DTNB) (Sigma, St. Louis, MO, USA), Acetylthiocholine Iodide (Sigma, St. Louis, MO, USA). All other chemicals and reagents are of laboratory grade.

Elevated Plus-Maze Test

The elevated plus maze served as simple behavioral model to evaluate learning and memory in mice. The procedure and techniques and end point for testing memory was followed in accordance with standard literatures.[13] The apparatus consisted of two open arms (16 cm X 5 cm) and two covered arms (16 X5X12). The arms extended from a central platform (5 cm X 5 cm), and the maze was elevated to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was taken as the time taken by the mouse to move into any one of the covered arms with all its four legs. TL was recorded on the first day. The cut off time is 90 s. Memory retention was examined 24 h after the first day trial on the second day. Significant reduction in TL value of retention indicated improvement in memory.

Passive Shock Avoidance Paradigm

Passive avoidance apparatus is based on negative reinforcement was used to examine long term memory. The apparatus consisted of a rectangular box (27 X 27 X 27 cm^3) having three walls of wood and one wall of Plexiglas featuring a grid floor (3 mm stainless steel rods set 8 mm apart), with a wooden platform (10 X 7 X1.7 cm^3) at the center of the grid floor an electric shock was delivered to the grid floor. During training sessions, each mouse was placed on the wooden platform, immediately after mouse stepped down on the grid, an electric shock of 1.8 mA, for 0.2sec was delivered and it was recorded as step-down latency (SDL). SDL was defined as the time taken by the mouse to step down from wooden platform to grid floor with its entire paw on the grid floor. All the animals were submitted to a single training session. Mice showing SDL in the range (2–15 s) during the first test were used for the second session and the retention test. The second session was carried out 90 min after the first test. In this test animals were trained to remain on the platform for a period of 60s and the SDL were noted. Retention was tested after 24 h (i.e. 9^{th} day after last dose) in a similar manner, except that the electric shocks were not applied to the grid floor. SDL was recorded with an upper cutoff time of 300 s. Significant increase in SDL indicate improvement in memory. During the study number of step down errors were counted. [14]

Estimation of Brain Cholinestrase

AChE inhibitory activity of the extracts was measured by the spectrophotometric method developed by Ellman et al. (1961). The cerebral cortex, midbrain, medulla oblongata and cerebellum were dissected on ice and weighed as described earlier.[15] The different parts of brain were homogenized in a tissue homogenizer using phosphate buffer at a pH of 8.0. The homogenate was centrifuged at 3000rpm for 10min. The homogenate mixed DTNB (Sigma, USA), and Acetylthiocholine iodide (substrate). Thiocholine released because of the cleavage of substrate by AChE was allowed to react with the –SH reagent DTNB, which is reduced to thionitrobenzoic acid, a yellow colored anion with an absorption maxima at 412nm was measured utilizing a UV 160A, UV–visible recording spectrophotometer, Shimadzu (Japan). The extinction coefficient of the thiobenzoic acid is 1.36×10^{-4}/molar/centimeter. The rate in moles of the substrate hydrolyzed per minute per gram of tissue was calculated.

Statistical analysis
Values are expressed as mean ± SEM from 6 animals. Statistical differences in mean were analyzed using one way ANOVA followed by Dunnett's test. $p<0.05$ was considered significant.

Results

Phytochemical investigation
The yield value for ether extract was 3%. Blue-colored spots was observed on plate indicated that all extracts contained phenolic compounds including flavonoids and exposure of the extracts to Dragendorff reagent for alkaloid identification gave a positive result. Other phytochemical investigation of extract revealed the presence of glycosides, phenolic tannins, carbohydrates, proteins, saponins and triterpenoids.

Acute toxicity studies
In acute oral toxicity there was no mortality recorded in all the groups with EESI was found to be safe till 2000 mg/kg in mice. Animals showed a slight depression and urination.

Effect on transfer latency in elevated plus-maze
The ether extract of SI was found effective in enhancing the learning and memory in scopolamine induced dementia. The treatment with 150 and 300 mg/kg significantly ($p<0.01$) decreased the TL during learning phase by 62% (23.56± 0.78), 50.1% (31.58 ±4.8) and piracetam by 33.5 % compared to scopolamine (63.2±4.8). In Retention phase, animals showed a significant decrease ($P<0.01$) in TL was with both doses by 42.7% (17.73±3.07) and 57.6% (16.5±2.05) when compared to control 22.2 % and piracetam 24.4 %. Results were depicted in Fig.no.1.

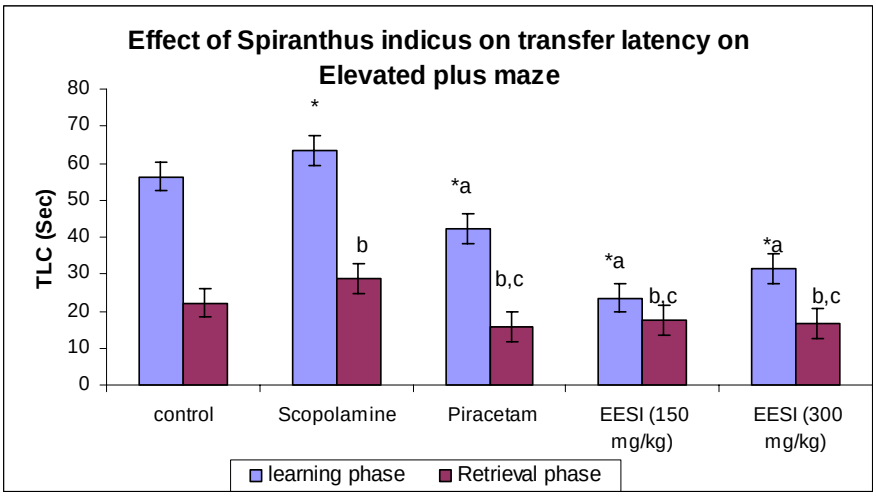

Fig.1. Effect of *Sphaeranthus indicus* on TL in elevated plus maze. Statistical analysis: one way ANOVA followed by Dunnet's test. *($P<0.01$) Compared to control, [a] ($P<0.01$) scopolamine Vs. all in acquisition phase, [b] & [c] ($P<0.01$) scopolamine Vs. all in retrieval phase

Effect on step-down latency using passive avoidance

Ether extract of *Sphaeranthus Indicus* administered with 150 and 300 mg/kg for 7 days significantly increased SDL (P<0.01) during the acquisition by 78.9% (142.33±21.8) and 96% (225±15.66) were compared to control (84%) scopolamine (87.7%). The EESI was significantly (P<0.01) reversed the scopolamine induced dementia as seen from the SDL during retrieval phase by 71.8 % and 83.4% compared to control. Step down errors were also significantly (P<0.01) decreased in acquisition phase and where as in retrieval phase both doses significantly (P<0.01) decrease by 79.6% and 80% compared to control in scopolamine induced dementia. Results were depicted in Table no.1.

Treatment	Acquisition in sec.		Retrieval in sec.	
	SDL	SDE	SDL	SDE
Control	153±16.93	3.000±0.2	206±13.8	1.6±0.3
Scopolamine (0.4mg/kg)	24.33±2.98*	8.333±0.61*	40.18±3.74 ^{b}	4.33±0.4 ^{b}
Piracetam (500mg/kg)	198.17±22.79$^{*\,a}$	2.833±0.30^{*a}	232.17±9.26 $^{b,\,c}$	1.2±0.2 $^{b,\,c}$
EESI (150mg/kg)	151.33±21.8$^{*\,a}$	2.833±0.30$^{*\,a}$	142.33±12.65 $^{b,\,c}$	1.3±0.2 $^{b,\,c}$
EESI (300mg/kg)	225±15.66$^{*\,a}$	2.167±0.16$^{*\,a}$	242.33±10.24 $^{b,\,c}$	1.2±0.2 $^{b,\,c}$

Table No.1 : Effect of *Spiranthus indicus* on step down latency and step down errors in passive avoidance paradigm. Values=Mean ± SEM. SDL=Step down latency, SDE = Step down error. Statistical analysis: one way ANOVA followed by Dunnet's test. *(P<0.01) Compared to control, ^{a} (P<0.01) scopolamine Vs. all in acquisition phase, ^{b} & ^{c} (P<0.01) scopolamine Vs. all in retrieval phase

Effect on cholinesterase in various part of brain

Effect on cerebral cortex

Administration with Ether extract of SI at 150 and 300 mg/kg (oral) and piracetam (500 mg/kg, ip) significantly (P<0.01) decreased AChE activity in the cortex as compared to scopolamine and control group animals. Results are shown in (fig 2A)

Effect on midbrain

Treatment with Scopolamine (0.4 mg/kg) significantly (p<0.01) increased AChE activity compared to control. Upon seven day treatment with EESI (150 mg/kg and 300 mg/kg, p.o.) and piracetam (500 mg/kg, i.p.) exhibited significant (p<0.01) decline in AChE activity in midbrain fraction of mice brain homogenate compared to control (Fig no.2B)

Effect on medulla

Cholinesterase activity in medulla was significantly (P<0.01) increased with scopolamine at dose of 0.4mg/kg as compared to control. Upon treatment with EESI (150 mg/kg and 300 mg/kg, p.o.) and piracetam (500 mg/kg, i.p.) exhibited significant (p<0.01) decrease in AChE activity in medulla of mice brain (Figure no.2C).

Effect on cerebellum

EESI (150 mg/kg and 300 mg/Kg) and piracetam (500 mg/kg, i.p.) significantly (p<0.01) decreased AChE activity in the cerebellum compared to control (Fig no. 2D).

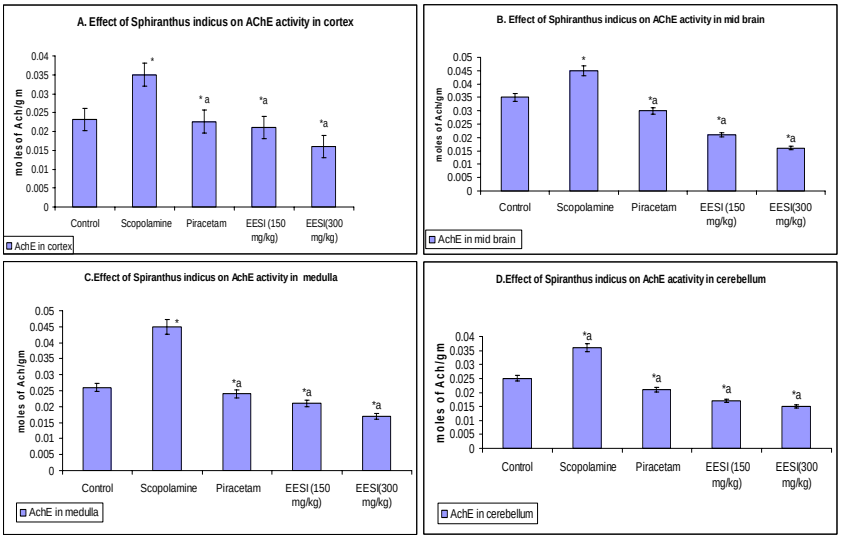

Fig. 2. Effect of *Sphiranthus indicus* cholinesterase in different parts of brain (A) Cortex, (B) midbrain (C)Medulla (D) cerebellum in mice. Values=Mean± mean. Statistical analysis: one way ANOVA followed by Dunnet's test. *(P<0.01) Compared to control, [a] (P<0.01) scopolamine Vs. all in acquisition phase, [b] & [c] (P<0.01) scopolamine Vs. all in retrieval phase

Discussion

Alzheimer disease is a genetically heterogeneous, neurodegenerative disease occurs progressively slow with the symptoms related to impaired neurotransmission and disintegration of neuronal circuits in the affected brain areas.[1] Patients with cognitive defects in AD are related with a progressive loss of cholinergic neurons and a subsequent decline in the levels of ACh in the brain particularly in the temporal and parietal neocortex and hippocampus [16] The Acetylcholine is said to have an effect on the memory, sleep, and concentration abilities, and also implicated in some severe diseases such as Alzheimer, Parkinson and epilepsy.[17] [18] Despite severity of this disease and high prevalence about 35 million people are suffering from AD, modern medicine is yet produce acceptable remedy for AD. There fore authors are motivated to explore for alternative approach for the management of AD in human.

In present study, treatment with *Sphaeranthus indicus* extract for 7 days improved the memory of mice as shown by enhanced SDL and TLC values as compared to control animals, also, the extract reduce the brain *cholinesterase* activity. Furthermore, pretreatment with SI for 7 days protected the animals from memory deficits produced by scopolamine. These findings suggest a possible neuroprotective role for *Sphaeranthus indicus.*

Piracetam, a Nootropics agent has facilitatory effect on integrative functions of the central nervous system, improves intellectual performance, learning capability and memory aged patients.[19] Passive avoidance paradigm is based on the negative reinforcement and is used to examine long term memory, where in which animal learn to avoid a noxious event by suppressing its normal exploratory behavior. Treatments with piracetam and Sphaeranthus Indicus have shown to improve learning and memory activity in mice, which meet the major criteria for nootropic activity. [20] Many epidemiological studies have confirmed that use of NSAID's have reduced the incidence of AD.[21] Several authors have reported that chronic treatment of anti-inflammatory drugs to elder patients with AD showed reduction on the symptoms.[22] *Sphaeranthus Indicus* has been reported to possess anti-inflammatory activity by suppressing ROS and Pro-inflammatory mediators.[23] This effect would taken care of the inflammatory section AD. *Sphaeranthus indicus* possess TNF-α inhibiting property which is one of the mediators of neuronal inflammation.

Developments of oxygen free-radicals are responsible for the process of age-related decline in cognitive performance in elderly persons Alzheimer's disease. *S I* has been reported to possess antioxidant property as well [24]. The neuroprotective effect of Sphaeranthus Indicus may be endorsed to its antioxidant property through of which vulnerable brain cells get exposed to less oxidative stress resulting in reduced brain damage and improved neuronal function.

Acetylcholine is considered as the most important neurotransmitter involved in the regulation of cognitive functions. There are extensive evidences linking the central cholinergic system to memory . The symptoms of dementia are presumed to be related to impaired neurotransmission and degeneration of neuronal circuits in the affected brain areas. [25] Cognitive deterioration occurring in patients with probable AD is associated with progressive loss of cholinergic neurons and consequent decline in levels of acetylcholine (ACh) in brain.[16] Selective loss of cholinergic neurons and decrease in cholinacetyltransferase activity was reported to be a characteristic feature of senile dementia of the Alzheimer's type. [26] *S I* at 150 and 300 mg/kg significantly inhibited the AChE activity in various parts of the mouse brain viz. midbrain, cerebellum, medulla oblongata and cortex, indicating its potential in attenuation of severity of Alzheimer's disease

The treatment with extracts EESI reduced significantly by inhibiting the AChE this could be owing to the presence of flavanoids, alkaloids or tannins, in these extracts. The *Sphaeranthus indicus* may prove to be effective in alleviating the symptoms of the AD by its multiple mechanisms. Further work is needed to isolate and determine exact mechanism of action associated with memory enhancing activity of *Sphaeranthus indicus.*

Conclusion

In the present study, we observed that *SI* extract (i) inhibited acetylcholinesterase enzyme activity in cortex medulla mid brain, cerebellum, and, (ii) ultimately improved memory of mice when tested on exteroceptive behavioral model, passive avoidance paradigm. Thus, a combination of anticholinesterase, anti-inflammatory, antioxidant and neuroprotective effects exhibited by *SI* may be responsible for the memory improving effect observed in the present study. However, investigations using more experimental paradigms may be required for further confirmation of nootropic potential of *SI* in the treatment of dementia and Alzheimer's disease.

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