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RESEARCH ARTICLE

Effect of standardized extract of *Marsilea minuta* on learning and memory performance in rat amnesic models

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Abstract

Context: *Marsilea minuta* Linn (Marsileaceae) is a common Indian hydrophytic plant. Traditionally, the plant has been used as a sedative for the treatment of insomnia and other mental disorders. Background information of this plant has encouraged us to investigate its antiamnesic activity in rat.

Objective: Standardized ethanol extract of *M. minuta* was investigated for their putative role in learning and memory performance in normal and amnesic rats.

Materials and methods: Ethanol extract of *M. minuta* (EMM) was standardized for marsiline using HPLC. The effect of standardized extract of *M. minuta* (1.15% w/w marsiline) was tested in amnesic rat using elevated plus maze (EPM) and passive avoidance (PA) test. Amnesia was induced after scopolamine (1 mg/kg, s.c.) and electroconvulsive shock (150 mA, 0.2 s) treatment. Behavioral studies were further substantiated with acetylcholinesterase (AChE) activity and radioligand muscarinic receptor binding studies in rat brain regions.

Results: Oral administration of EMM at 200 and 400 mg/kg/day for 3 days significantly reversed the amnesia whereas, no per se effect was observed. In comparison to control, AChE activity in frontal cortex and hippocampus was found to be significantly ($P < 0.05$) inhibited by EMM. EMM at doses 200 and 400 mg/kg has significantly ($P < 0.05$) increased (+34 % and +40 % change in affinity, respectively) the binding of 3H-QNB in frontal cortex indicating the up regulation of the muscarinic receptors.

Discussion and conclusion: These findings suggest that standardized extract of *M. minuta* have excellent antiamnesic activity, probably mediating through central cholinergic system.

Keywords: Acetylcholinesterase, antiamnesic, electroconvulsive shock, muscarinic receptor, scopolamine

Introduction

Cognitive impairment is a major neurodegenerative disorder affecting large number of world population and imposes enormous health and socio-economic burden on society. One of the major challenges of the third millennium will be to restore normal brain functions in individuals that suffer from neurodegenerative diseases and to reverse the physiological decline of brain functions related to ageing (Le Merrer & Nogues, 2000). However, cognitive neuropharmacology, which should provide the theoretical support and set of reliable assays for the discovery of cognition enhancing drugs, is still in its infancy (Le Merrer & Nogues, 2000). With this limitation, Certain new classes of drug that facilitate the integrative functions of the central nervous system (CNS), particularly the intellectual performance, learning capacity and memory has discovered and called as nootropic drug (smart drug) (Giurgea, 1973). Nootropic agents are successfully used for the treatment of cognition impairment patients. However, There has been a phenomenal rise in the interest of scientific community to explore the pharmacological actions or to confirm the veracity of claims made about herbs in the official book of Ayurveda (Indian traditional medicine system) for the treatment of mental

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and neurological disorders (Nadkarni, 1976). Hence, it is worthwhile to explore the utility of traditional medicines for improvement of cognitive functions.

Marsilea minuta Linn. (Marsileaceae) is one of the common Indian hydrophytic species widely found in wet and flooded low lands. The plant as a whole is used as sedative for the treatment of insomnia and other mental disorders (Sivarajan & Balachandran, 1994). The decoction of this plant was prescribed to the patients suffering from mental disorders along with their meal as a routine procedure in some mental clinics (Chatterjee et al., 1963). Marsiline (an ester of 1-triacontanol and hexacosanoic acid), a major centrally active principle was first reported by Chatterjee et al. (1963) and found to be responsible for sedative and anticonvulsant properties. In our previous study we have demonstrated the modulation of monoaminergic neurotransmitter system for exhibiting antidepressant (Bhattamisra et al., 2008), anxiolytic activity (Bhattamisra et al., 2007) and anti-aggressive (Tiwari et al., 2010) activity after *M. minuta* extract treatment. In the light of the above, the present study was undertaken to investigate the effect of standardized extract of *M. minuta* on learning and memory of rats using behavioral and neurochemical studies.

Materials and methods

Materials

Whole plants of *M. minuta* were collected during the month of July 2004 from Berhampur, Orissa, India. A specimen copy (Sept-2004-1) was deposited in the herbarium, Department of Botany, Banaras Hindu University. Piracetam was procured from UCB Pharma, India (Nootropil, Batch no: BN 9068728). Acetylthiocholine, dithiobisnitrobenzoic acid (DTNB) reagent, atropine sulphate and scopolamine hydro bromide were purchased from Sigma, St. Louis, MO, USA. L-[Benzilic-4,4′-3H]-quinuclidinyl benzilate (QNB) was obtained from NEN (Boston, MA, USA). All other reagents and chemicals were of analytical grade.

Animals

Male Wistar rats (200–250 g) were obtained from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (Regd. no. 542/02/ab/CPCSEA). Animals were housed six per cage in a room with controlled ambient temperature (25 ± 1°C), relative humidity (45–55%) and a 12 h light/dark cycle. They were fed with food pellet (Hindustan Lever, Mumbai, India) and water *ad libitum*. The experiments were conducted in between 8 a.m. and 2 p.m. All experiments were performed in accordance with the norms of committee for the purpose of control and supervision of experiments in animal (CPCSEA), India, and approved by Institutional animal ethics committee.

Preparation of extract

About 500 g of whole plant powder was thoroughly extracted with 2.5 l of 90% ethanol using Soxhlet apparatus for 48 h. The extract was concentrated *in vacuo* at 50°C and then lyophilized (yield 16.3% w/w), which was further stored at –20°C until required. Ethanol extract of *M. minuta* (EMM) was tested for presence of steroids, flavonoids, alkaloids, and saponins (Wagner et al., 1984). Marsiline was isolated as previously described (Chatterjee et al., 1963). Marsiline was confirmed by comparing the spectroscopic data (IR, ^{1}H-NMR, EIMS) obtained, with that of earlier reported spectroscopic data. EMM was standardized for marsiline in a Perkin Elmer's HPLC with diode array detector. The standardized EMM (1.15% w/w of marsiline) was used for the pharmacological evaluation.

Drug treatment

EMM was reported to be safe up to a maximum dose of 1 g/kg by both oral and intraperitoneal route in rodent (Chatterjee et al., 1963; Gupta et al., 2002). Two dose levels of 200 and 400 mg/kg of EMM was administered orally, as polyethylene glycol (PEG) suspension, once daily for three consecutive days. Control rats were received vehicle (10% v/v PEG, 10 ml/kg/day, p.o.) for three consecutive days. Experiments were conducted on day 3, 1 hour after the last dose administration. The effects of positive nootropic agent, piracetam (500 mg/kg, i.p.) were tested 30 min after treatment.

Transfer latency on elevated plus maze

The elevated plus maze (EPM) as described in Vogel and Vogel (2002), was kept in a dimly lit (20 lx) room for experiment. On day 3, rat was individually placed on the far end of one of the open arms, facing away from the center and the time taken by the rat to enter one of the closed arms (transfer latency) was recorded with the help of a stop watch. The rat was left in the enclosed arm for 10–15 s and returned to its home cage afterward. On day 4 and day 11, the procedure was repeated and transfer latency was recorded. This test was first developed to assess the learning acquisition and memory retention (Itoh et al., 1990).

Passive avoidance test

This test was developed by King and Glasser (1970) to assess the long term memory retention. The step through passive avoidance (PA) behavior was evaluated by using light- dark apparatus following the procedure described in Vogel and Vogel (2002). On day 3, a rat was placed in the white box and the time taken to enter into the dark box was noted. As soon as the rat entered the dark box, the guillotine door was closed and a foot electric shock (0.5 mA, 3 s) was delivered. The rat was then returned to home cage. On day 4 and day 11, individual rat was again placed in the white box and was given a 5 min inhibition period. Latency to step through to the dark chamber was recorded. Electric shock was not delivered on day 4 and day 11. If the animals remained in the white box for a 5 min test period, the maximum score of 300 s was assigned.

Effect on amnesic models

Amnesia was developed in rat on administering scopolamine hydro bromide (1 mg/kg, s.c.) and electroconvulsive shock (ECS) (150 mA, 0.2 s) immediately after the learning trial on day 3 (Itoh et al., 1990). The protective effect of EMM on the amnesic models was evaluated on day 4 and day 11, in both EPM and PA test.

Assay of acetylcholinesterase (AChE) activity in rat brain

Rats were decapitated and their brains were rapidly removed and frozen on dry ice. Various brain areas, frontal cortex, hypothalamus and hippocampus were dissected according to Glowinski and Iversen (1966). The tissue samples were weighed and stored at –20°C until homogenization. Each frozen tissue sample was homogenized using 0.1 M phosphate buffer (pH = 8) at 0°C. The homogenate was centrifuged at 10,000 × *g* (4°C) for 5 min. The total AChE activity in the aliquot of the homogenate was estimated in duplicate according to the method described by Ellman et al. (1961).

Radioligand receptor binding assay

Rats were sacrificed by means of quick decapitation. Whole of frontal cortex and hippocampus were isolated as described by Glowinski and Iversen (1966). The crude synaptic membranes were prepared following the procedure described previously (Seth et al., 1981). Briefly, brain regions were homogenized in 19 volumes of sucrose (0.32 M) followed by centrifugation at 50,000 × *g* (4°C) for 10 min. Subsequently the pellet was washed with deionized water and centrifuged at the same speed for 10 min to remove endogenous amines. Finally, the pellet was suspended in Tris-HCl buffer (40 mM, pH 7.4).

Binding incubations were carried out in triplicate in a final volume of 1 ml containing 40 mM Tris-HCl buffer (pH 7.4), the appropriate labeled and unlabeled pharmacological agents. The amount of tissue used per tube corresponded to 5–15 mg of the original wet weight and contained 300–400 μg of membrane protein as determined by the method of Lowry et al. (1951). At the end of 15 min incubation at 37°C, samples were filtered in glass fiber discs (25 mm diameter. 0.3 μm pore sizes, Gelman Inc., Ann Arbor, MI) and rapidly washed twice with 5 ml of Tris buffer. The filter discs were then dried and counted in 5 ml of scintillation mixtures using a Tricarb 2660 scintillation counter (Packard Instruments Co., Downers Grove, IL) at an efficiency of 38–43% tritium, to determine membrane bound radioactivity. Control incubations containing unlabeled competing ligand, were carried out simultaneously with the experimental series to determine the extent of nonspecific binding. The assay for muscarinic receptor was performed by using 1×10^{-9} M DL-[benzillic-4,4′-^{3}H] quinuclidinyl benzilate (QNB) (39 Ci/mmol) as the binding ligand and atropine sulphate as the competing compound in the control tubes. The method satisfied the requirements for saturability, specificity, reversibility and regional distribution (Seth et al., 1982). The values presented are representative of three separate runs, each in triplicate. Specific binding was calculated by subtracting non-specific binding from total binding. The values are expressed as pmoles bound/g protein.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). All data were analyzed statistically using one-way analysis of variance, followed by post-hoc Tukey's multiple comparison tests. Values of $P < 0.05$ were considered statistically significant.

Results

Transfer latency on elevated plus maze

As shown in Table 1, EMM (400 mg/kg) treatment showed a tendency of reduced transfer latency (TL) in

Table 1. Effect of EMM on transfer latency in elevated plus maze test in rats.

Treatment	Dose (mg/kg)	Transfer Latency (s)		
		Day 3	Day 4	Day 11
Vehicle (12)	–	41.69 ± 2.52	43.22 ± 4.37	40.12 ± 7.61
EMM (6)	200	40.31 ± 2.53	39.37 ± 2.99	43.43 ± 4.55
EMM (6)	400	40.06 ± 4.52	31.98 ± 2.13[b]	44.21 ± 2.65
Piracetam (6)	500	44.55 ± 3.38	13.08 ± 4.98[a,b]	12.39 ± 2.64[a,b]
Vehicle + Scp (12)	± 1	44.21 ± 3.78	58.35 ± 3.08[a,b]	50.19 ± 4.20[a,b]
EMM + Scp (6)	200 + 1	43.44 ± 3.16	34.99 ± 3.03[a,bb]	24.53 ± 2.92[a,bb]
EMM + Scp (6)	400 + 1	42.92 ± 2.79	34.02 ± 2.50[a,bb]	23.88 ± 3.15[a,bb]
Piracetam+ Scp (6)	500 + 1	40.57 ± 4.01	24.07 ± 2.90[a,bb]	23.66 ± 1.81[a,bb]
Vehicle +ECS (12)	–	46.92 ± 9.12	71.8 ± 8.87[a,b]	78.59 ± 10.70[a,b]
EMM + ECS (6)	200	45.12 ± 4.12	32.10 ± 3.53[a,bb]	29.60 ± 1.17[a,bb]
EMM + ECS (6)	400	47.55 ± 3.48	30.44 ± 2.04[a,bb]	28.33 ± 4.11[a,bb]
Piracetam+ ECS (6)	500	38.19 ± 5.81	23.74 ± 1.88[a,bb]	21.99 ± 1.25[a,bb]

Transfer latency to enter into one of the enclosed arm was measured in elevated plus maze at day 3, 4 and 11. Values in parenthesis indicate number of animal used.

ECS, electro convulsive shock; EMM, ethanol extract of *Marsilea minuta*; Scp, scopolamine.

[a]$p < 0.05$ compared with day 1. [b]$p < 0.05$ and [bb]$p < 0.05$ compared with vehicle and vehicle+ amnesic agent respectively.

EPM on day 4. However, EMM at the dose of 200 mg/kg did not alter the TL in normal rat. Amnesic agents (scopolamine and ECS) treatment after the acquisition trial, had significantly ($P < 0.05$ vs. normal control) increased the TL in post acquisition phase (day 4 and 11) of the test. Pretreatment with EMM at the doses of 200 and 400 mg/kg have significantly ($P < 0.05$ vs. amnesia control) and dose dependently protected the amnesia induced by ECS and scopolamine on day 4 and 11 of the test. Piracetam (500 mg/kg, i.p.) improved memory retention ($P < 0.05$ vs. normal control) and reversed the amnesic effect induced by scopolamine and ECS in rats.

Passive avoidance behavior (step-through latency)

Step-through latency (STL) was measured in terms of time taken by the animal to enter the dark and electrified chamber of the apparatus. EMM (400 mg/kg, p.o.) and piracetam (500 mg/kg, i.p.) showed increased STL values ($P < 0.05$ vs. normal control) on day 11. Scopolamine and ECS, markedly decreased the STL on day 4 and 11 of the test indicating the impairment of cognition. Various doses of EMM (200 and 400 mg/kg, p.o.) administered for three consecutive days, showed dose dependent increase in STL values as compared to respective control groups. Piracetam not only facilitated the passive avoidance retention but also reversed the amnesia induced by the amnesic agents on day 4 and 11 of the test. The results are summarized in Table 2.

Effect on AChE activity

The inhibition of AChE activities by EMM in rat brain was shown in Table 3. EMM at dose 200 mg/kg, AChE activity in rat frontal cortex, hippocampus and hypothalamus was attenuated by 22, 26, and 12%, respectively ($P < 0.05$ vs. control group). AChE activity in frontal cortex, hippocampus and hypothalamus was inhibited by 29, 38, and 28%, respectively ($P < 0.05$ vs. control group), in EMM (400 mg/kg) treated rats. In all the three brain regions, no significant difference in AChE activity was observed in between EMM 200 and 400 mg/kg.

Radioligand receptor binding study

EMM at doses 200 and 400 mg/kg, significantly ($P < 0.05$ vs. control group) increased the binding level of [^{3}H]-QNB in frontal cortical membranes with a change of +34 and +40%, respectively. Although the observed percentage change by EMM 200 and 400 mg/kg in hippocampus membrane was +26 and +28%, respectively, it did not show statistically significant against control group. Whereas, no dose dependent effect was observed in between EMM 200 and 400 mg/kg in radioligand muscarinic receptor binding study. The results are summarized in Table 4.

Table 2. Effect of EMM on transfer latency in passive avoidance test (step through latency) in rats.

		Transfer latency (s)		
Treatment	Dose (mg/kg)	Day 3	Day 4	Day 11
Vehicle (12)	–	23.89 ± 2.31	29.88 ± 1.97	23.31 ± 1.65
EMM (6)	200	20.94 ± 1.94	22.43 ± 1.91	23.13 ± 3.6
EMM (6)	400	22.19 ± 2.01	28.32 ± 1.86[a]	26.65 ± 1.18[a,b]
Piracetam (6)	500	22.79 ± 1.57	41.79 ± 1.47[a,b]	32.47 ± 2.93[a,b]
Vehicle + Scp (12)	± 1	22.69 ± 1.85	21.68 ± 1.25[b]	18.64 ± 0.98 [b]
EMM + Scp (6)	200 + 1	19.87 ± 1.75	23.64 ± 1.58[a, bb]	22.65 ± 1.33[a,bb]
EMM + Scp (6)	400 + 1	18.65 ± 0.94	25.69 ± 1.97[a,bb]	23.24 ± 1.79[a,bb]
Piracetam+ Scp (6)	500 + 1	21.70 ± 2.25	31.02 ± 1.31[a,bb]	29.75 ± 1.81[a,bb]
Vehicle +ECS (12)	–	20.89 ± 1.19	19.98 ± 1.62[b]	20.98 ± 0.74[b]
EMM+ ECS (6)	200	21.62 ± 1.94	27.21 ± 1.89[a,bb]	24.56 ± 1.64[bb]
EMM+ ECS (6)	400	20.74 ± 1.32	29.42 ± 1.25[a,bb]	27.58 ± 1.49[a,bb]
Piracetam+ ECS (6)	500	22.92 ± 1.73	32.23 ± 2.96[a,bb]	31.69 ± 2.30[a,bb]

Latency to enter into dark chamber was measured at day 3, 4 and 11 in a light and dark apparatus. Values in parenthesis indicate number of animal used.
ECS, electro convulsive shock; EMM, ethanol extract of *Marsilea minuta*; Scp, scopolamine.
[a]$p < 0.05$ compared with day 1. [b]$p < 0.05$ and [bb]$p < 0.05$ compared with vehicle and vehicle+ amnesic agent respectively.

Table 3. Effect of EMM on AChE activity in rat brain regions.

		AChE activity (μmoles of substrate hydrolysed / min / g tissue)		
Treatment	Dose (mg/kg)	Frontal cortex	Hippocampus	Hypothalamus
Vehicle	–	9.25 ± 1.05	10.69 ± 1.65	15.72 ± 2.43
EMM	200	7.19 ± 0.87[a]	7.89 ± 0.75[a]	13.86 ± 2.37
EMM	400	6.57 ± 0.52[a]	6.68 ± 0.46[a]	11.27 ± 2.11[a]

Acetylcholinesterase activity was measured in frontal cortex, hippocampus, hypothalamus and whole brain content. n = 5 for each treatment group.
AChE, Acetylcholinesterase; EMM, ethanol extract of *Marsilea minuta*.
[a]$p < 0.05$ compared with vehicle-treated group.

Table 4. Effect of EMM on cholinergic-muscarinic receptors in rat brain regions.

Treatment	Dose (mg/kg)	Specific binding (pmoles bound/ g protein)	
		Frontal cortex	Hippocampus
Vehicle	–	725 ± 74.67	128 ± 13.71
EMM	200	975 ± 21.96[a] (+34)	162 ± 24.63 (+26)
EMM	400	1015 ± 35.31[a] (+40)	164 ± 32.09 (+28)

Radioligand receptor binding study for cholinergic-muscarinic receptor was studied in rat frontal cortex and hippocampus. n = 5 for each group. Values in parenthesis indicate percentage change of receptor binding with respect to vehicle-treated group. EMM, ethanol extract of *Marsilea minuta*. [a]$p < 0.05$ compared with vehicle-treated group.

Discussion

In the present study, the antiamnesic like effect of *M. minuta* was evaluated in elevated plus maze and passive avoidance tasks. In both the tests, it has facilitated the acquired learning and retention in cognitive deficit rats. Further the neurochemical mechanism was studied for muscarinic receptor, where EMM showed significant up regulation of muscarinic receptor in frontal cortex. EMM has also showed the cholinomimetic effect by inhibiting the AChE activity in frontal cortex and hippocampus.

The EPM served as the exteroceptive behavioral model (wherein the stimulus existed outside the body) for screening long-term memory. In EPM, rats show natural aversion to open and high spaces and therefore, spend more time in enclosed arms. Itoh et al. (1990) suggested that transfer latency (TL) might be shortened if the animal had previous experience of entering the enclosed arm and the shortened TL could be related to memory. TLs on day 3 and 4 are taken as acquisition and retrieval, respectively. EMM did not improve the memory in absence of cognitive deficit except a minimal improvement observed in EMM (400 mg/kg) treated rats on day 4. However, various doses of EMM significantly and dose dependently shortened the TL on post-acquisition phase (day 4 and 11) and reversed the cognitive deficit induced by scopolamine and ECS. Piracetam exhibited significant improvement in amnesic models and also showed marked *per se* effect in the EPM model.

Passive-avoidance response is extensively used for the screening of drugs affecting learning and memory (Dilts & Berry, 1967). The test is suitable for assessing the long-term memory. It involves training rodents to avoid punishment (normally an electric shock) by curbing a normal behavior (such as the exploratory behavior). In passive avoidance test, animals on exposure to first trial acquire the information that entry into dark chamber results into noxious experience of electric shock and cognitive ability of the animal is reflected in avoiding the entry into dark chamber (a judgment based on successful retention and recalling of the acquired information) (Das et al., 2003). Amnesic agents like scopolamine and ECS administration in first trial (acquisition) resulted into impaired learning (amnesia) i.e., no significant increase in the transfer latency time on second trial (retention). EMM had no significant per se effect though, EMM (400 mg/kg) showed significant increase in step-through latency on day 11 of the test. Both the doses of EMM significantly and dose dependently reversed the amnesic effect of scopolamine and ECS.

Piracetam meet the major criteria for nootropic activity, namely improvement of memory in absence of memory deficit subjects. In our both tests viz. EPM and PA, piracetam showed improvement in learning and memory in normal and cognition impaired rats. Mechanistically, piracetam improves the cognition performance in aged animals by positive effect on NMDA receptor density in the hippocampus, and on muscarinic cholinergic receptor densities in the frontal cortex, straitum, and a lesser extent in hippocampus. Cognitive performance by piracetam not only depends on cholinergic function but it also depends on the effect on membrane fluidity in the frontal cortex, the hippocampus, and the striatum and its effect on NMDA densities in the hippocampus (Scheuer et al., 1999). EMM does not show consistence improvement in cognitive performance in normal rats in both the behavioral tests, probably it did not have the associated mechanisms as in case of piracetam. Alteration in cholinergic transmission may not only responsible for improvement in cognitive behavior in a normal rat whereas, improvement in cholinergic transmission in cognitive deficit rat is essential for improvement in learning and memory. EMM showed improvement in cognitive behavior in amnesic rats indicating its role in cholinergic transmission which is substantiated with the receptor binding study. This suggests that EMM is not a nootropic agent rather it is an antiamnesic agent.

Learning is defined as the acquisition of information and skills. Subsequent recall of this information is called memory. Impairment of learning and memory, as the most characteristic manifestation of dementia, could be induced chemically in experimental animals by administration of several amnesic agents. This experimental animal model of scopolamine-induced amnesia has been extensively used in research to screen for drugs with potential therapeutic value in dementia (de Angelis & Furlan, 1995; Rubaj et al., 2003).

The amnesic effect of ECS is known to produce marked depletion of rat brain acetylcholine induced by electroshock. EMM extract showed significant and dose dependent reversal of amnesia induced by scopolamine and ECS. Hence, this evidence supports the cholinomimetic effect of EMM either by augmenting the liberation of acetylcholine (ACh), muscarinic activity and/or by inhibiting the ACh metabolism (anticholinesterase activity).

The experimental and clinical evidence suggests that the cholinergic system in the brain plays a significant role in cognitive function, particularly in memory formation (Baskin et al., 1999; Mesulam, 2004). The clinical discovery that memory deficits in Alzheimer's disease (AD) are concomitant with a loss of cholinergic markers in CNS (Perry et al., 1981) has sparked growing interest in the role that ACh plays a major role in learning and memory. Psychopharmacological studies in human and animal subjects have shown concordantly that systemic cholinergic blockade results in deficits of attention, learning and memory (Blockland, 1996). Conversely, augmentation of cholinergic neuronal systems within the CNS has been shown to improve cognitive performance in rats (Smith et al., 1996), and monkeys (Rupniak et al., 1997). AChE is an important regulatory enzyme that rapidly hydrolyses ACh at brain cholinergic synapses as well as at the neuromuscular junction (Grisaru et al., 1999). Blockade of AChE results an increased level of ACh at synapse and augmentation of cholinergic neurotransmission. Among the possible strategies for enhancing brain cholinergic activity, AChE inhibitors such as physostigmine, tacrine, donepezil, and rivastigmine are the mainstay of the pharmacotherapy of senile dementia of Alzheimer type (Enz et al., 1993; Siddiqui & Levey, 1999).

In connection to cognitive performance, cholinergic innervations in both hippocampus and frontal cortex of brain play a distinct role. Hippocampus can often support recall (consolidation) of the specific details of a given episode, while neocortical representations can usually only support a general feeling of familiarity, without the ability to recall specific details (Norman & O'Reilly, 2003). Central cholinergic system particularly in hippocampus plays an undisputed key role in regulation of learning and memory, which are the key constituents of cognitive behavior (Blockland, 1996). It has been found that cholinergic neuronal loss in the hippocampal area is the major feature of Alzheimer's disease (Enz et al., 1993; Siddiqui & Levey, 1999), while profound depletions in all cholinergic markers measured (ACh, choline, ChAT, AChE) were found both in frontal and temporal cortex (Giacobini, 2003) of AD patients.

On prior treatment with EMM extract significantly attenuated the AChE activity in frontal cortex and hippocampus. Therefore, the findings of the present investigation indicate that anticholinesterase activity of EMM may be responsible for the antiamnesic effect.

For further confirmation, we have investigated the cholinergic muscarinic receptor binding study in frontal cortex and hippocampus region of rat brain. EMM treatment significantly increased the binding of [^{3}H]-QNB to the frontal cortical region indicating the up-regulation of muscarinic receptor. The significant up-regulation of muscarinic receptor in frontal cortex with minimal change in hippocampus was observed in both the doses of EMM. Decreased total muscarinic receptors have been reported in samples of cerebral cortical tissue from patients with Alzheimer's disease, but not in individuals without the disorder (Russell, 1996). On the contrary, the memory enhancers like piracetam, shilajit and *Withania somnifera* extract up-regulate the muscarinic receptor density in frontal cortex (Pilch & Müller, 1988; Schliebs et al., 1997). In the present investigation, we have observed the anticholinesterase activity of EMM in both frontal cortex and hippocampus. Hence, the finding support the cognition enhancing activity seen with EMM extract and this could also account for the protection of scopolamine induced amnesia.

The neurological basis of learning and memory remains controversial, despite extensive experimental and clinical studies. The role of the central cholinergic system is fairly well established, the role of other neurotransmitter system cannot be ignored. It has been reported earlier that increase in the serotonergic transmission can interfere with learning acquisition and memory consolidation (Ogren, 1982). Nootropics have also been demonstrated to interfere with serotonergic transmission and have an inhibitory effect on noradrenaline function (Ogren, 1982).

In our previous studies, we have reported the significant inhibition of brain serotonin and nor-adrenaline level by EMM which could be the reason for its anxiolytic activity (Bhattamisra et al., 2007). This neurochemical basis concerning the inhibitory effect on brain serotonin and noradrenaline level also supports the cognition enhancement effect of EMM. In addition to this, the significant reversal of scopolamine and ECS induced amnesia by EMM also suggests involvement of central cholinergic system. Hence, in the present investigation the behavioral studies were supplemented with the neurochemical studies pertaining to the cholinergic system.

Conclusions

The present investigation, thus, establishes the anti-amnesic activity of *M. minuta* and also suggests that EMM augment the acetylcholine function by anticholinesterase and muscarinic agonistic activity. In addition inhibitory effect on serotonergic and noradrenergic function in brain may also be responsible for the observed antiamnesic effect. However, further investigations using more experimental paradigms and receptor binding study are required for complete understanding the anti-amnesic effect of EMM.

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Declaration of interest

The authors report no declarations of interest.

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