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Alteration of Acetylcholinesterase Mediatory Cognitive Behavioral Pattern through Phytomedicine (MEC-01) in Wistar Rats

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ABSTRACT

MEC-01 is a phytomedicine composed with C. pluricaulis, B. monnieri, C. asiatica, A. racemosus, N. jatamansi and Withania somnifera. The aim of the present study was to find out underlying mechanism of MEC-01 on cognitive behavioral pattern in experimental animals. The total phenolics content, DPPH radical scavenging, brain lipid peroxidation and acetylcholine esterase in inhibitory actions of MEC-01 was studied in in vitro methods. Cognitive functions were assessed by passive avoidance and Morris water maze tests in normal and scopolamine induced memory impaired mice. The findings revealed that MEC-01 has enriched in phenolic compounds, inhibited free radicals scavenge, exerted protective actions against brain lipid peroxidation and inhibited central acetylcholine esterase enzyme activity. MEC has no lethality up to the oral single dose of 2000 mg/kg in mice. Moreover, MEC-01 exhibited improvement in retention of learning in normal and cognitive deficit animals. The results indicate MEC-01 can facilitate learning and memory and possesses significant antidementic properties that may be mediated via acetylcholine esterase attenuating neuronal functions.

Key-words: Antioxidant, Cognition, Dementia, Acetylcholine esterase, Phytomedicine.

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Introduction

Recent years have seen a sudden surge in an array of mental illnesses such as dementia and other memory related disorders. Dementia is a syndrome usually chronic, characterized by a progressive, global deterioration in intellect including memory, learning, orientation, language, comprehension and judgment due to disease of the brain (Ferri et al., 2005). Alzheimer's disease (AD), cerebrovascular ischemia, age, depression, stress, anxiety, emotions are the conditions that may lead to memory loss, amnesia or dementia (Kalaria et al., 2008). It is estimated that over 3.7 million people are affected by dementia in India and expected to double by 2030 (Shaji et al., 2010). Nootropic agents such as piracetam, nefiracetam, aniracetam and choline esterase inhibitors like donepezil are usually used to improve memory, mood and behavior (Singala and Balaram, 2002; Monczor, 2005;). However, the resulting adverse effects associated with these agents, like insomnia, risk of addiction, influenza, diarrhea, intense hyperactivity, headaches and heart palpation have limited their use (Lanni et al., 2008; Talih and Azaltouni, 2015).

Pharmacoepidemiological studies reveal that herbal learning and memory enhancing medicines are becoming very popular not only among Indian population but also Western community. Hyperforin, isolated from *Hypericum perforatum*, an herb used in Portuguese folk medicine, appears to enhance cognitive function (Homes et al., 2008). *Bacopa monniera* (Water hyssop) and *Centella asiatica* (Asiatic pennywort) has been used in Ayurvedic medicine to improve memory and intellectual functions (Manyam, 2009). *Withania somnifera* root is classed among the rejuvenative tonics in Ayurvedic medicine and is known to sharpen memory (Bhattarcharya and Muruganandam, 2003). Furthermore, mixed formulations are generally used in the Vedic medicines, based on the concept that such combinations not only provide synergistic therapeutic actions but also minimize the adverse effects. On the basis of this hypothesis, a new formulation (MEC-01) has been prepared for cognitive and neuroprotective therapeutic action with standardized powdered extract of *Convolvulus pluricaulis* (Nahata et al., 2008), *Bacopa monnieri, Centella asiatica, Nardostachys jatamansi* (Joshi and Parle, 2006), *Asparagus racemosus* (Ojha et al., 2010) and *Withania somnifera* (Table 1). The aim of the present study was, therefore, to find out the memory enhancing properties of MEC-01 in cognitive deficit animals and intended to establish its mode of actions in animal models. This is the first report of MEC-01 on cognition in animals.

Materials and methods

Chemicals and reagents

Acetylthiocholine chloride, butylated hydroxyl toluene, DPPH (1,1-diphenyl-2-picrylhydrazyl), DTNB or 5.5'-dithiobis(2-nitrobenzoic acid), gallic acid, α-tocopherol, thiobarbituric acid, scopolamine HBr were obtained from Sigma Chemicals Co.(St. Louis, MO, USA). Other analytical grade reagents were purchased from Merck, India.

Animals

Swiss male adult albino mice were used. In the present experiments, recommended guidelines for the care and use of the animals were strictly followed (CPCSEA, 2003). The permission from Institutional Animal Ethic Committee was also obtained (IAEC/AH-2/2011/UCM-72). The room temperature was maintained at 23°±2°C and humidity between 40 and 60%. The light cycle was also maintained (12:12h). The animals were fed supplementary feed for animal and water *ad libitum*. The food was also withdrawn as per experimental protocol.

Test drug preparation

The standardized powdered 50% hydro-ethanolic extracts were weighing proportionately as given in Table 1.

Table 1: Ingredients and composition of MEC-01

Botanical Name	Family	India Name	Parts Used	Quantity (%)
Convolvulus pluricaulis	Convulvulaceae	Shankpushpi	Whole plants	20
Bacopa monnieri	Scrophulariaceae	Brahmi	Leaves	20
Asparagus racemosus	Asparagaceae	Shatavari	Roots	20
Centella asiatica	Apiaceae	Mandukparni	Leaves	20
Nardostachys jatamansi	Valerianaceae	Jatamansi	Rhizomes	10
Withania somnifera	Solanaceae	Ashwagandha	Roots	10

***In vitro* experiments**

Determination of total phenolics content

To 5 ml of Folin-Ciocalteu reagent, 1.0 ml of MEC-01 (1mg/ml) was added, mixed and incubated in the dark for 3 minutes. Thereafter, 5 ml of sodium carbonate (75 g/litre) solution was added, mixed and further incubated in the dark for 1 h. The absorbance was read at 765 nm. The total phenolic was expressed as mg of Gallic acid equivalent per gram (mg GAE/g) of extract (Sur et al., 2015).

DPPH radical scavenging activity

To 2.5 ml of MEC-01 solution at different known concentrations was mixed with 1 ml of 0.3 mM DPPH solution and was allowed to stand in dark for 30 minutes. The absorbance values were measured at 518 nm. The antioxidant activity of the extract was expressed as IC_{50} (Sur et al., 2015).

Anti-lipid peroxidation

To 1 ml of MEC-01 solution at different concentrations, 1 ml mouse brain homogenate (250 µg protein/ml) and 1 ml 0.25 mM copper chloride in Tris-HCl buffer (pH 7.4) was added and mixed. The mixture was incubated at 37°C for 5 min. Thereafter, 3 ml of 0.37% thiobarbituric acid was added. Finally, the solutions were heated in boiling water-bath for 15 min, centrifuged and measured the absorbance at 525 nm. The IC_{50} was expressed µg/ml of extract (Chan et al., 1982).

Acetylcholine esterase activity

The reaction mixture contained 1.5 ml of phosphate buffer (pH 7.2), 1 ml of 0.5 mM DTNB, 0.2 ml test extract at different concentrations and 0.02 ml of suspension of rat brain homogenate (20% w/v in phosphate buffer; pH 7.2). The mixture was mixed and incubated for 15 min at 25ºC. The reaction was initiated by the addition of 0.2 ml of 10 mM acetylthicholine chloride. Absorbance was read at 412 nm after 30 sec, 60 sec, 2 min and 3 min. The rate of reaction was calculated according to extinction coefficient and IC_{50} was expressed µg/ml of extract (Ellman et al., 1961).

***In vivo* experiments**

Acute toxicity

Single dose toxicity study was done on Swiss albino mice according to Organisation for Economic Co-operation and Development (OECD) guidelines No. 423 (2001) adopted for acute toxicity in animals up to 2000 mg/kg.

Passive avoidance test

Healthy adult male Swiss mice were grouped by randomized design and assigned to four groups of 6 mice each and treated (p.o.) for 15 consecutive days as follows:

Group I: Normal control: given 0.2 ml deionized water
Group II: Reference drug: Piracetam 100 mg/kg
Group III: Test drug: MEC-01 100 mg/kg
Group IV: Test drug: MEC-01: 200 mg/kg

The animals were placed on the elevated platform of Passive Avoidance Apparatus. Training (up to day 7) was carried out in two similar sessions. In brief, the mouse was gently placed on the upper platform set in the centre of the grid floor. Immediately after stepping down the animal received electric shock of 15 sec duration through the grid floor, and was then return to its home cage. The step down latency (SDL) was recorded. On the following day (24 h retention interval, *i.e.,* on 8^{th} day) the mouse was once again placed on the platform and SDL was recorded. Latency to step down (SDL) was further assessed a week later on 15^{th} day (Bhattarcharya and Muruganandam, 2003; Sapkota et al., 2010). Thereafter, Scopolamine HBr (Sigma, St. Louis, MO, USA) was given to all animals at the dose of 3 mg/kg, i.p., and the retention memory (SDL) was further noted after 24h of injection. The improvement of memory was scored by following formula: Inflexion ration = $(L_F-L_0)/L_0$

Where, L_0 = initial latency and L_F = final latency

Finally, the animals were sacrificed under deep anesthesia and the whole brain was removed rapidly. Thereafter, it was homogenized (20%) in phosphate buffer (pH 7.2), centrifuged at 4ºC. AChE was estimated from supernatant of homogenized brain following the method of Ellman and his colleagues (1961).

Morris water maze test

The apparatus was a circular tub filled with water up to a height of 30 cm at around 25^0C and rendered opaque by addition of milk. A small platform 6 cm width was fixed in the middle of water tub. The animals were grouped and treated as described before. Each mouse was subjected to 4 consecutive trials for 4 days (from 3-6th day) during which they were allowed to escape on to the hidden platform and allowed to remain there for 20 sec. The animal was released into the water and allowed 3 min to find the platform. Time to find the hidden

platform was considered as escape latency (EL). The escape latency was examined on day 7 and day 15 (Morris, 1984).

Statistical analysis

The data were expressed as mean ± S.E.M. and analyzed by t-test using statistical software SPSS version 17 (IBM, Chicago, USA). For comparing means *p* value <0.05 was considered as statistically significant.

Results

In vitro experiments

The total amount of phenolic content in MEC-01 was 98.319 mg GAE/g of extract. The 50% inhibitory concentration of MEC-01 on DPPH free radical formation was only 64.895 µg/ml and lipid peroxidation in brain tissue was only101.427 µg/ml. Moreover, IC_{50} of MEC-01 on AChE activity was 37.692 µg/ml (Table 2).

Table 2: Antioxidant effects of MEC-01

	MEC-01
Total Phenolics (mg GAE/g)	98.319 ± 0.24
DPPH Inhibition IC_{50} (µg/ml)	64.895 ± 0.78
Lipid Peroxidation IC_{50} (µg/ml)	101.427 ± 1.22
AChE IC_{50} (µg/ml)	37.692 ± 0.16

N=6 in each test; mean ± S.E.M; IC_{50}= 50% inhibitory concentration;

In vivo experiments

The findings of acute toxicological studies suggested that MEC has no lethality up to the single oral dose of 2000 mg/kg. MEC-01 was found to improve learning acquisition and retention of the learn task significantly in normal mice, with no cognitive deficit, when administered 15 days. In day 8, MCE-01 at the dose of 100 mg/kg and 200 mg/kg, showed 24% and 31% improvement respectively than control, while, piracetam improved 39% (Table 3). Further, MEC-01 and piracetam treated groups significantly enhanced SDL as compared to control dementia induced by scopolamine. MEC-01 at the dose of 100 mg/kg showed 194% memory retention and 200 mg/kg exhibited 223%; while, Piracetam (100 mg/kg) showed 229% withholding of learning ability than respective control (Table 3). Moreover, MEC-01 at the dose of 100 mg/kg and 200 mg/kg after scopolamine treatment attenuated AChE specific activity in mouse brain 27% and 41% respectively than control. Piracetam showed similar reduction (48%) in AChE activity (Table 4). In Table 5, searching of hidden place or escape latency time in Morris test were noted to reduce after the treatment of MCE-01 30.7% at 100 mg/kg, 40.4% at 200 mg/kg; while, 51.7% in piracetam (100 mg/kg).

Table 3: MEC-01 on step down latency in the passive avoidance test in mice

	Control 0.2 ml DW	Piracetam 100 mg/kg	MEC-01 100 mg/kg	MEC-01 200 mg/kg
SDL				
Day 7	6.8±0.94	6.3±0.88	6.5±0.76	5.8±1.35
Day 8	54.8±5.40	76.3±3.69*	68±6.07	71.8±4.31**
Day 15	32.5±4.08	68.8±5.76***	65.5±8.49**	68.8±4.82***
Day 16	20.3±2.60	66.8±2.90***	59.8±8.88***	65.6±3.59***
IL				
Day 8	8.4±2.04	13.2±1.39*	9.9±1.42	16.6±2.27*
Day 15	4.2±1.10	12.0±3.23***	9.4±1.60**	15.4±3.38***
Day 16	3.3±1.01	11.2±2.63***	7.6±0.97***	19.9±4.93***

N=6; mean ± S.E.M; SDL=Step down latency; IL=Inflexion ratio; Treatment= at day 15 all mice were treated with scopolamine HBr 3mg/kg, i.p; compared to control at same day; statistical analysis by t-test; significant level *p<0.05, **p<0.01, ***p<0.001

Table 4: AChE activity on scopolamine induced amnesia in mouse brain

	SC	Piracetam + SC 100 mg/kg	MEC-01 + SC 100 mg/kg	MEC-01 + SC 200 mg/kg
AChE	9.54±0.31	4.93±0.15***	6.92±0.26***	5.61±0.18***

N=6; mean ± S.E.M; AChE=Acetylcholine esterase (μmol/min/g brain tissue); SC= scopolamine compared to control; statistical analysis by t-test; significant level *p<0.05, **p<0.01, ***p<0.001

Table 5: MEC-01 on Escape Latency on Morris Water Maze Test

	Control 0.2ml DW	Piracetam 100 mg/kg	MEC-01 100 mg/kg	MEC-01 200 mg/kg
Day 7	34.6±1.81	20.5±1.83**	27.6±1.60**	23.1±1.32**
Day 15	55.6±3.98	26.8±1.81***	38.5±1.66**	33.0±1.39***

N=6; mean ± S.E.M; compared to control at same day; statistical analysis by t-test; significant level *p<0.05, **p<0.01, *p<0.001

Discussion

Neurodegenerative diseases comprise a condition in which nerve cells from brain and spinal cord are lost leading to either functional loss (ataxia) or sensory dysfunction (dementia). Free radicals have been reported for their great contribution to neuronal loss in cerebral ischemia, schizophrenia, Parkinson's disease and Alzheimer's disease including dementia (Cadet, 1998; Bayani et al., 2009). Antioxidants are exogenous or endogenous molecules those act against any form of oxidative stress and its associated ill effects on cellular system. There are clinical evidences that neurodegenerations can be ameliorated upon dietary intake or supplementary intake of natural antioxidants (Bickford et al., 2000; Peter et al., 2004). Phenolics are very important plant constituents because of their scavenging ability due to their hydroxyl groups (Sur et al., 2016). It has been reported that polyphenols have inhibitory effects on neurodegenerations and neuronal apoptosis. The test compound, MEC-01 possesses high amount of phenolic compounds. Previous reports also support the strong antioxidant and neuroprotective abilities of *Convolvulus pluricaulis, Bacopa monnieri, Centella asiatica, Nardostachys jatamansi, Asparagus racemosus* and *Withania somnifera* (Manyam, 1999; Bhattarcharya and Muruganandam 2003; Nahata et al., 2008; Lyle et al., 2009). Present study demonstrated that MEC-01is rich in phenolics compounds and strong free radical scavenging abilities. Moreover, induction of lipid peroxidation in neuronal tissues by copper cause alterations of the membrane lipids, proteins and DNA damage (Chan et al., 1982). Present works describes MEC-01ameliorated oxidative stress in mouse brain homogenate.

Cholinergic neuronal loss in brain is the major feature of AD and enhancement of central cholinergic activity by use of anticholinesterase is presently the mainstay of the pharmacotherapy of senile dementia (Scliebs, 2006). Anticholinergics induced transit impairment in memory in a passive avoidance test is widely employed to evaluate learning and memory performance. Passive avoidance is usually employed to describe experiments in which the animal learns to avoid a noxious event by suppressing a particular behavior. A prolongation of the step-down latency is defined as learning (Brioni et al., 1999). In this work, MEC-01 was found to improve learning acquisition and retention of the learn task significantly in normal mice indicates MEC-01 meets a major criterion of nootropic activity. Scopolamine, a muscarinic receptor antagonist, is reported to impair long term potentiation, and frequently used as amnesic agent for evaluation of anti-amnesic effect of new drugs (Hiramatsu and Kaori, 2000). MEC-01 and piracetam treated groups showed a significant increase in SDL or memory retention as compared to control dementia induced by scopolamine. Attenuation of scopolamine induced deficits in passive avoidance clearly indicates the prospective of MEC-01 as cognitive enhancer.

A considerable improvement in spatial learning in Morris water task was observed after MEC-01 treatment. In Morris water maze test, memory is developed progressively with repetitive trials which resemble the human interactions (spatial learning). The importance of hippocampal function for place navigation acquisition and of discrete cortical areas for long term storage of spatial memory has been demonstrated (Stwart and Morris, 1993). Therefore, MEC-01 has potential action to improve memory and that may be mediated through the neuronal functions of cortex and hippocampus (Sur et al., 2012).

It is generally accepted that acetylcholine esterase (AChE) inhibitors may also be beneficial in the treatment of Alzheimer's dementia (Ferreira et al., 2006). Previous reports suggested that *Convolvulus pluricaulis, Bacopa monnieri, Centella asiatica, Nardostachys jatamansi* etc. has potential AChE inhibitory actions (Dhingra, 2003; Mukherjee et al., 2007). In *in vitro* studies MEC-01 attenuated AChE activities. Further, potential inhibition of AChE activity in the brain was observed by MEC-01 treatment as compared to scopolamine induced dementia.

The decrease in AChE activity indicates an increase in the basal level of acetylcholine, which might be helpful in maintaining the learning and memory functions in the dementia group (Peng et al., 1997). Therefore, it can suggest, MEC-01 has learning and memory enhancing ability that may be mediated through acetyl cholinesterase inhibition or regulatory pathways.

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References

1. Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M *et al.* Global prevalence of dementia: a Delphi consensus study. Lancet 2005; 366(9503): 2112-2117.
2. Kalaria RN, Maestre GE, Arizaga R, Friedland RP, Galasko D, Hall K, *et al.* Alzheimer's disease and vascular dementia in developing countries: prevalence, management, and risk Factors. Lancet Neurol. 2008; 7:812-826.
3. The Dementia India Report 2010: Prevalence, impact, cost and services for dementia. (Eds) Shaji KS, Jotheeswaran AT, Girish N, Bharat S, Dias A, Pattabiraman M, Varghese M. New Delhi: ARDSI, 2010.
4. Singala J, Balaram R. Nootropics. Indian J Pharmacol. 2000; 34:437-440.
5. Monczor M. Diagnosis and treatment of Alzheimer's disease. Curr Med Chem – Central Nervous System Agents. 2005; 5:5-13.
6. Talih F, Azaltouni J. Probable nootropic induced psychiatric adverse effects: a series of four cases. Innov Clin Neurosci. 2015; 12:21-25.
7. Lanni C, Lenzken SC, Pascale A. Cognition enhancers between treating and doping the mind. Pharmacol Res. 2008; 57:196-213.
8. Homes MJ, Perry NS, Houghton PJ. Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders. Phytother Res. 2003; 17: 1-18.
9. Manyam B. Dementia in Ayurveda. J Altern Complement Med. 1999; 5: 81-88.
10. Bhattarcharya SK, Muruganandam AV. Adaptogenic activity of *Withania somnifera*: an experimental study using a rat model of chronic stress. Pharmacol Biochem Behav. 2003; 75:547-555.
11. Nahata A, Patil IK, Dixit VK. Effect of *Convulvulus pluricaulis* Choisy on learning behaviour and memory enhancement activity in rodents. Natural Product Res. 2008; 22:1472-1482.
12. Joshi H, Parle M. *Nardostachys jatamansi* improves learning and memory in mice. J Med Food. 2006; 9:113-118.
13. Ojha R, Sahu AN, Muruganandam AV, Singh GK, Krishnamurthy S. *Asparagus recemosus* enhances memory and protects against amnesia in rodent models. Brain Cognition 2010; 74:1-9.
14. Committee for the Purpose of Control and Supervision on Experiments on Animals. CPCSEA guidelines for laboratory animal facility. Indian J Pharmacol. 2003; 35:257-274.
15. Sur TK, Hazra AK, Bhattacharyya D, Hazra A. Antiradical and anti-diabetic properties of standardized extract of Sunderban mangrove *Rhizophora mucronata*. Pharmacognosy Res. 2015; 11: 389-394.
16. Chan PC, Peller OG, Kesner L. Copper (II)-catalyzed lipid peroxidation in liposome sans erythrocyte membranes. Lipids 1982; 17:331-337.
17. Ellman GL, Lourtney DK, Andres V, Gmelin G. A new and rapid colorometric determination of acetylcholinergic activity. Biochem Pharmacol. 1961; 7:88-95.
18. Organization for Economic Co-operation and Development (OECD) Guideline No. 423. Acute oral toxicity in animals. OECD/OCDE No. 423, adopted 17th December, 2001.
19. Sapkota SP, Sur TK, Debnath PK, Bhattacharyya D. Effect of *Peureria tuberosa* on chronic foot shock stress in Wistar rats. Nepal Med Coll J. 2010; 12:234-238.
20. Morris R. Development of a water maze procedure for studying spatial learning in the rat. J Neuroscience Methods. 1984; 11:47-60.
21. Cadet JL. Free radical mechanisms in the central nervous system: An overview. Int J Neurosci. 1998; 40:13-18.
22. Bayani U, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol. 2009; 7:65-74.
23. Bickford PC, Gould T, Briederick L, Chadman K, Polloch A, Young D. Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats. Brain Res. 2000; 886: 211-217.

24. Peter PZ, Anthon JC, Khachaturian AS, Stone SV, Deborah G, JoAnn, T *et al*. Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements -the Cache county study. Arch Neurol. 2004; 61:82-88.
23. Sur TK, Hazra A, Hazra AK, Bhattacharyya D. Antioxidant and hepatoprotective properties of Indian Sunderban mangrove *Bruguiera gymnorrhiza* L. leave. J Basic Clinical Pharmacy. 2016; 7:75-79.
24. Lyle N, Gomes A, Sur TK, Munsi S, Paul S, Chatterjee S, Bhattacharyya D. Possible role of antioxidant property of *Nardostachys jatamansi* in alleviation of chronic fatigue syndrome. Behav Brain Res. 2009; 202:285-290.
25. Scliebs RA. The significant of the cholinergic system in the brain during aging and in Alzheimer's disease. J Neural Transm. 2006; 113:1625-1644.
26. Brioni JD, Hock FJ, McGaugh JJ. Drug effects on learning and memory. In: Vogel GH, Vogel WH, editors. Drug Discovery and Evaluation: Pharmacological Assays. New York: Springer Verlag, 1997.
27. Hiramatsu M, Kaori I. Improvement by low doses of nociceptin on scopolamine induced impairment of learning and /or memory. Eur J Pharmacol. 2000; 395:149-156.
28. Stwart CA, Morris RGM. The water maze. In Behavioural Neuroscience: A practical approach. Vol I, New York: Oxford University Press, 1993.
29. Sur TK, Maroo N, Pant J, Mukherjee B, Hazra A. Effect of MEC-01 (herbal formulation) on learning behavior and memory enhancement activity in rodents. Traditional medicine and globalization. 12th Int Congress of Ethnopharmacol. Kolkata, Feb 17-19, 2012.
30. Ferreira A, Proenca C, Serralheiro ML, Araijo ME. The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J Ethanopharmacol. 2006; 108:31-37.
31. Dhingra D, Parle M, Kulkarni SK. Medicinal plants and memory. Indian Drugs 2003; 40: 313-319.
32. Mukherjee PK, Kumar V, Mal M, Houghton PJ. Acetylcholinesterase inhibitors from plants. Phytomed. 2007; 14: 289-300.
33. Peng WH, Hsich MT, Wu CR. Effect of long term administration of berberine of scopolamine induced amnesia in rats. Jpn J Pharmacol. 1997; 74: 261-265.