

Neuroprotective Effect of *Aegle marmelos* Leaf Extract in Scopolamine Induced Cognitive Impairment and Oxidative Stress in Mice

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Abstract: Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by a gradual decline in memory associated with shrinkage of brain tissue, with localized loss of neurons mainly in the hippocampus and basal forebrain and diminished level of central cholinergic neurotransmitter. In the present study we investigated the Neuroprotective effects of Methanolic leaf extract of *Aegle Marmelos* (MEAM) against scopolamine-induced memory impairment and oxidative damage in Swiss albino mice. Pole climbing apparatus and Morris water maze performance tests were used to assess memory performance tasks and Actophotometer was used to assess gross locomotor activity. Scopolamine (0.4mg/kg, i.p.) has been used for disease induction and Donepezil (5mg/kg, oral) has been used as standard drug. Various biochemical parameters such as lipid peroxidation, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) activity, Catalase activity and Acetylcholinesterase were also assessed. Different concentrations of MEAM treated groups has shown significant ($p < 0.01$) decrease in latency time of Morris water maze and also significant ($p < 0.05$) decrease in latency time of pole climbing apparatus on the 9th day has been observed as compared to 1st day session and also did not cause significant alteration in the locomotor activity as compared to disease control group. Increased oxidative stress in brain after scopolamine treatment, as observed by increase in MDA and decrease in % inhibition of DPPH and % H₂O₂ scavenging activity, was reversed in the groups treated with different concentrations of methanolic leaf extract of *Aegle marmelos*.

Key words: *Aegle marmelos* (MEAM) • Alzheimer's Disease • Scopolamine

INTRODUCTION

Dementia is generally defined as the "loss of intellectual abilities (medically called cognitive function) of sufficient severity to interfere with social or occupational functioning". Dr Philippe Pinel the founder of modern psychiatry, first used the word 'dementia' in 1797. Dementia leads to deterioration in all the components of intellectual function. These contributory diseases may be located primarily outside the brain, as in certain thyroid diseases or may be due to abnormalities of the brain such as multiple strokes, increased pressure in the brain and degenerative disorders, wherein brain cells are damaged and die. When dementia is the result of certain known causes, it is called secondary dementia [1].

Alzheimer's disease is the most common form of dementia in the elder people. This condition is characterized by a progressive loss of memory, deterioration of virtually all intellectual functions, increased apathy, decreased speech function, disorientation and gait irregularities. Alzheimer's disease is caused by changes in the cerebral cortex, basal forebrain and other areas of the brain. The brain tissue of Alzheimer's patients is marked by moderate-to-severe changes that involve characteristic plaques and tangles. Females are slightly more likely than males to develop Alzheimer's disease. Individuals with Down's syndrome are more likely to develop Alzheimer's disease than the general population. Alzheimer's disease attacks every socioeconomic and ethnic group [1].

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Scopolamine is a muscarinic antagonist which induces central cholinergic blockade and impairs learning and memory. Currently, the treatment for Alzheimer's disease was the administration of acetylcholinesterase inhibitors that increase the availability of acetylcholine at cholinergic synapses. Such acetylcholinesterase inhibitors as physostigmine, tacrine and donepezil antagonize the negative effects of scopolamine on spatial memory in various behavioral tests [2].

Common types of dementia include Alzheimer's disease, Vascular dementia, Dementia with Lewy bodies (DLB), Mixed dementia, Parkinson's disease, Frontotemporal lobar degeneration (FTLD), Creutzfeldt-Jakob disease, Normal pressure Hydrocephalus and Mild cognitive impairment (MCI) [3-8].

Possible causes of memory problems include: Depression, Medication side effects, Excess alcohol use, Thyroid problems, Poor diet, Vitamin deficiencies, certain infections, Alzheimer's disease and related dementias.

Aegle marmelos commonly known as Bael belongs to Family Rutaceae is a plant of Indian origin having tremendous therapeutic potential. It is known by the several other names in the different parts of the country and also outside of the country. In India it found in Sub-Himalayan tracts from Jhelum eastwards to West Bengal, in central and south India [9]. Leaves, fruits, stem and roots of *Aegle marmelos* have been used in ethno medicine to exploit its medicinal properties including astringent, antidiarrheal, antidysenteric, demulcent, antipyretic and anti-inflammatory activities etc [10].

Scopolamine hydrobromide is one of the major antimuscarinic agents that inhibit the action of acetylcholine (ACh) on autonomic effectors innervated by postganglionic cholinergic nerves as well as on smooth muscles which lack cholinergic innervations [11].

The main purpose of present study was to determine effect of *Aegle marmelos* leaf extract in scopolamine induced cognitive impairment and oxidative stress in mice.

MATERIALS AND METHODS

Drugs and Treatment: Scopolamine (Cadila Health Care Ltd), Donepezil (Alkem Laboratories Ltd). Other chemical and reagents were of analytical grade.

Preparation of Plant Extract: The leaves were dried under shade and coarsely ground to powder and were stored in an air tight container. Suitable amount of leaf powder was taken and then phytochemicals were extracted with methanol using Soxhlet apparatus. The solvent was

completely air dried to obtain dark green colored viscous substance, which was stored in air tight container for further use.

Animals: Swiss albino mice (16-25gms) were procured from Mahaveer Enterprises, Hyderabad. They (36) were housed into group of six mice per cage and maintained at $24^{\circ}\text{C}\pm 1^{\circ}\text{C}$ with relative humidity 45-55% and 12:12 hours' dark/light cycle. The animals had free access to food (standard chew pellets) and water ad libitum. Food but not water was withdrawn 3 hours before and during the experiment. The Institutional Ethics Committee of Synapse Life Sciences approved all the experimental procedures.

Experimental Design: Memory impairment was induced by scopolamine (0.4mg/kg i.p.) and given 30 min after administration of each drug. Scopolamine was dissolved in sterile water for injection. Donepezil was dissolved in 0.1% CMC solution. All drugs were prepared fresh daily. Doses were given according to the respective mice weights shown in Table 1. Animals (36) were weighed and kept in cages accordingly and randomly divided into 6 groups (n=6). On day 1, the training sessions for all the animals were given. This was followed on 3rd, 5th, 7th, 9th days.

In vivo Methods (Behavioral Assessment)

Assessment of Cognitive Performance

Morris Water Maze Test: Method was carried out in a circular pool (90 cm in diameter and 50 cm in height) of water with a featureless inner surface. The 1st day of the experiment was dedicated to swimming training for 60 sec in the absence of the platform. During the 4 consecutive days the mice were given the trial session with the platform in place. Once the mouse located the platform, it was permitted to remain on it for 10 sec. If the mouse did not locate the platform within 120 sec, it was placed on the platform for 10 sec and then removed from the pool. One day after the final training trial sessions (on day 5), mice were individually subjected to a probe trial session in which the platform was removed from the pool and mice were allowed to swim for 120 sec to search for it and the latency time was determined [12, 13, 14].

Pole Climbing Test: Training and testing was conducted in a 25x25x40 cm chamber that was enclosed in a dimly lit, attenuated box. Scrambled shock was delivered to grid floor of the chamber. A 2.8 KHZ speaker and a 28 v light were situated on the top of the chamber. A wooden pole 2.5 cm in diameter was suspended by a counter balance

Table I: Experimental Design

Group-I	Normal Control	Vehicle (0.1% CMC).
Group-II	Disease control	Scopolamine (1.4 mg/kg) i.p.
Group-III	Standard	Donepezil (5 mg/kg) oral + Scopolamine (1.4 mg/kg) i.p.
Group-IV	Test-I	MEAM(100mg/kg)oral + Scopolamine (1.4mg/kg)i.p.
Group-V	Test-II	MEAM(200mg/kg)oral + Scopolamine (1.4 mg/kg) i.p.
Group-VI	Test-III	MEAM(400mg/kg)oral + Scopolamine (1.4 mg/kg) i.p.

weight through a hole in the upper layer centre of the chamber. A micro switch was activated when the pole was pulled down by 3mm, the response was recorded when a rat jumps on the pole and activates micro switch the activation of light and speaker together were used as conditioned stimulus[15].

Measurement of Locomotor Activity: Most of the CNS drugs influence the locomotor activities in man and animals. The locomotor activity of drug can be studied using actophotometer which operates on photoelectric cells which were connected in circuit with a counter when the beam of light falling on photocell was cut off by the animal a count was recorded. Animals were placed individually in the activity cage for 10 min and the activity was monitored. The test was done after 30 min of drug administration. The photo cell count was noted and decrease or increase in locomotor activity was calculated [16, 17].

Histopathological Studies: After 8 days treatment, the brains of different groups were perfusion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and postfixed in the same fixative overnight at 48°C. The brains were then routinely embedded in paraffin and stained with Hematoxylin-Eosin. The hippocampal lesions were assessed microscopically at 40 magnification [18].

In vitro Methods: On 9th day, after behavioral assessments, animals were scarified by cervical dislocation. The brains were removed. Each brain was separately put on ice and rinsed with ice-cold isotonic saline. A (10% w/v) homogenate was prepared in 0.1M phosphate buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm for 15 minutes and aliquots of supernatant was separated and used for biochemical estimation [18].

Preparation of Brain Homogenate: Brain tissue samples were thawed and homogenized 10 times (w/v) with ice cold 0.1M phosphate buffer (P^H 7.4). Aliquots of homogenates from mice brains were separated and used

to measure protein lipid peroxidation and glutathione. The remaining homogenates were centrifuged at 3, 000 rpm for 30 min and the supernatant was then used for enzyme assay [19].

Estimation of Cholinergic Status in the Mice Brain: Brain tissue homogenate was incubated for 5 min with 2.7ml of phosphate buffer and 0.1ml DTNB (5, 5-dithiobis (2-nitrobenzoic acid)) reagent. Then the supernatant obtained was added with 0.1ml acetylthiocholine iodide substrate. The resulting yellow color was due to reduction of DTNB by certain substances in brain homogenate and due to non-enzymatic hydrolysis of substrate. DTNB reagent containing solution was used for zeroing the colorimeter. The absorbance was read at 420nm. Cholinergic concentration was spectrophotometrically determined [20].

Biochemical Estimation of Markers of Oxidative Stress: DPPH Radical Scavenging Assay: The free radical scavenging activity of the test drug was measured *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. About 0.3mM solution of DPPH was dissolved in 100% ethanol and 1ml of this solution was added to 3ml of the fraction dissolved in ethanol. The mixture was shaken and allowed to keep at room temperature for 30 min and the absorbance was measured at 517nm using a spectrophotometer. The percentage of scavenging activity was determined [13, 21, 22].

Hydrogen Peroxide Scavenging Assay (Catalase Activity): Hydrogen peroxide solution (2mM/L) was prepared with standard phosphate buffer (P^H 7.4). The test drugs dissolved in distilled water were added to 0.6ml of hydrogen peroxide solution. Absorbance was determined at 230nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity was determined [19, 23].

TBARS (Thiobarbituric Acid Reactive Substance) Assay: The reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a secondary product of lipid peroxidation has been widely adopted as a sensitive

assay method for measurement of lipid peroxidation in biological fluids. The tissue homogenate (0.5ml) was supplemented with 0.5ml of phosphate buffer and then with 1ml of 10% trichloroacetic acid. The mixture was centrifuged at 3000rpm at 4°C for 10 min. The supernatants of the tissue homogenates were incubated with 1 ml of 0.8% w/v of the thiobarbituric acid at 100°C for 15 min. After a cooling period, TBARS concentration was spectrophotometrically determined at 532nm. The levels of lipid peroxides were expressed as nano moles of TBARS. Standard Figure was plotted using TEP (1, 1, 3, 3-tetraethoxy propane) [19, 24,].

Statistical Analysis: All data were expressed as mean ± SD. The data were analyzed using ANOVA, followed by Dunnett-test where readings of the control, standard and test drug induced animals were compared against scopolamine induced group and the level of significance was set at P<0.05.

RESULTS

Morris Water Maze Method: Over the 4 days of water maze testing, on day 5, mice in disease control group exhibited longer swim latency time in comparison with other groups receiving test and standard drugs. Swim latencies exhibited by all groups of mice except disease control group were gradually decreased from day 1 to day 5. Significant (p< 0.01) reduction was observed with different concentrations of MEAM. Standard drug group has shown significant (p< 0.001) reduction in latency time as shown in Fig. 1.

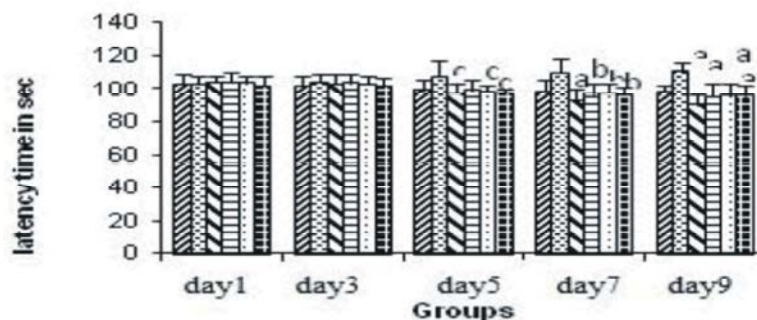


Fig. 1: Morris Water Maze Test:

Fig. 1: Effect of *Aegle marmelos* on latency time in Morris water maze test compared to the drug induced memory impairment group (Mean ± SD, n = 6). Values were expressed as Mean ± SD of Time spent in seconds. C (*p<0.05), b (**p<0.01), a (**p<0.001) compared with scopolamine.

Pole Climbing Test: Methanolic leaf extract of *Aegle marmelos* showed significant (p<0.05) decrease in latency time on day 7 and 9, as shown in Fig. 2. Moreover the higher latency time induced by scopolamine was significantly reversed by treatment group compared with scopolamine group and combination effect showed lesser efficacy than standard donepezil which was used as the positive control.

Locomotor Activity: In the present series of experiments, the mean scores of locomotor activity for each mouse were relatively stable and showed no significant variation among different groups. The mean scores in control, scopolamine treated groups remain unchanged. The MEAM did not cause significant alteration in the locomotor activity as compared to scopolamine treated mice as shown in Fig. 3.

AChE Enzyme Estimation (Ellman's Method): Anticholinesterase activity has been expressed in % inhibition of enzyme. Scopolamine treatment decreased brain AChE significantly. Improvement of AChE was observed in standard and MEAM treated groups compared to scopolamine treated group as shown in Fig. 4.

DPPH Method: Antioxidant activity has been expressed in % inhibition of DPPH activity. Scopolamine treatment decreased brain antioxidant activity significantly. Improvement of antioxidant activity was observed in standard and MEAM groups compared to disease control as shown in Fig. 5.

Fig. 2: Pole Climbing Test:

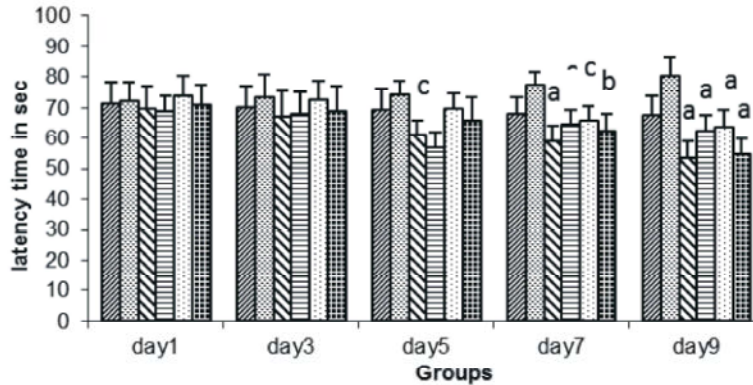


Fig. 2: Effect of *Aegle marmelos* on Conditioned Avoidance Response in Pole climbing test compared to the drug induced memory impairment group (Mean \pm SD, n = 6). Values were expressed as Mean \pm SD of Time spent in seconds. C (*p<0.05), b(**p<0.01), a(****p<0.001) compared with scopolamine

Fig. 3: Locomotor Activity:

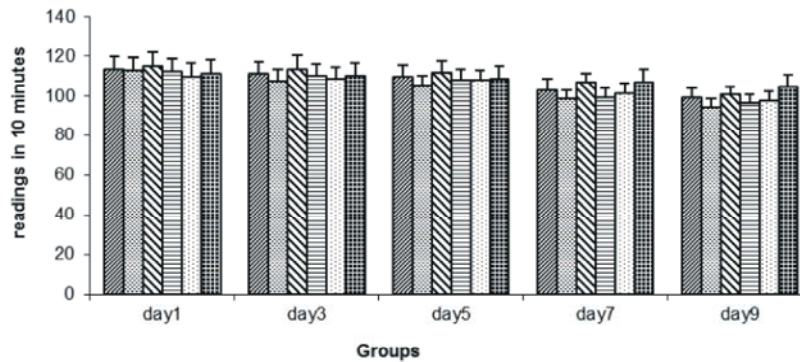


Fig. 3: Effect of *Aegle marmelos* on Locomotor activity compared to the drug induced memory impairment group (Mean \pm SD, n = 6). Values were expressed as Mean \pm SD of reading in 10 minutes on Actophotometer

Fig. 4: AchE Enzyme Estimation (Ellman's Method):

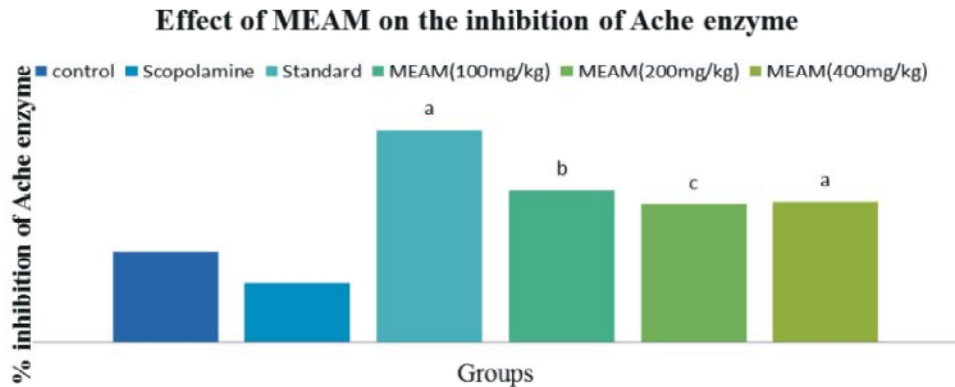


Fig. 4: Effect of *Aegle marmelos* on % inhibition of AChE enzyme compared to the drug induced memory impairment group (Mean \pm SD, n = 6). Values were expressed as Mean \pm SD of % inhibition of AChE enzyme. c(*p<0.05), b(**p<0.01), a (****p<0.001) compared with scopolamine

Fig. 5: DPPHMethod:

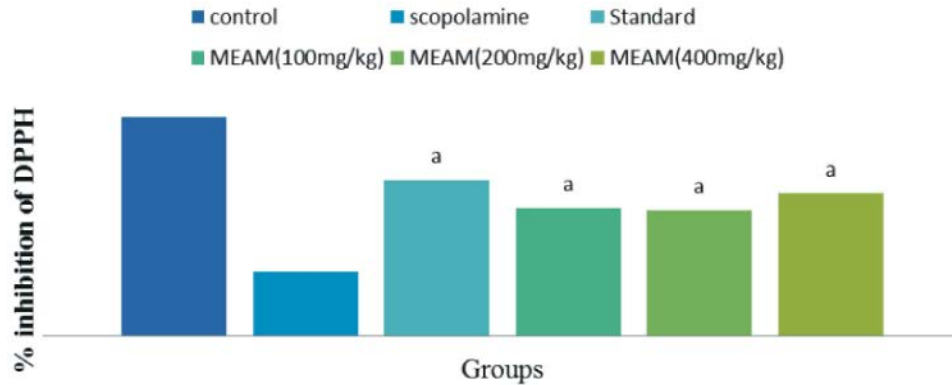


Fig. 5: Effect of *Aegle marmelos* on % inhibition of DPPH compared to the drug induced memory impairment group (Mean \pm SD, n = 6). Values were expressed as Mean \pm SD of % inhibition of DPPH. a(***p<0.001) compared with scopolamine.

Fig. 6: Catalase Activity:

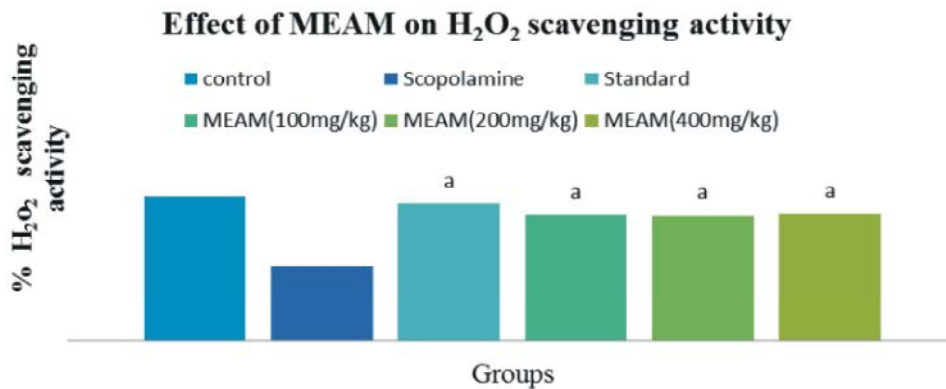


Fig. 6: Effect of *Aegle marmelos* on % of H₂O₂ scavenging activity compared to the drug induced memory impairment group (Mean \pm SD, n = 6) Values were expressed as Mean \pm SD of %H₂O₂scavenging activity. a (***p<0.001) compared with scopolamine.

Fig. 7: TBARS Assay:

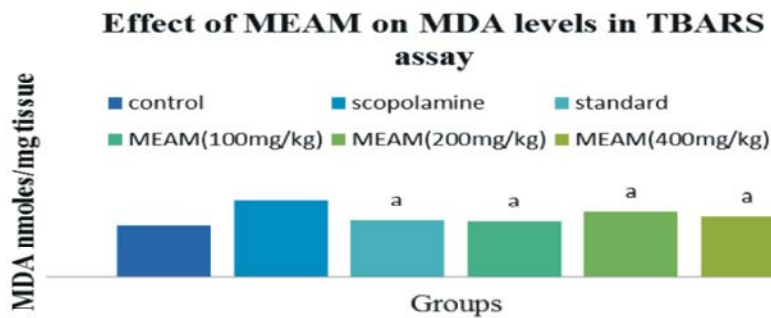


Fig. 7: Effect of *Aegle marmelos* on MDA levels in TBARS assay compared to the drug induced memory impairment group (Mean \pm SD, n = 6) .Values were expressed as Mean \pm SD of MDA levels. A (***p<0.001) compared with scopolamine

Fig. 8: Histopathological Studies:

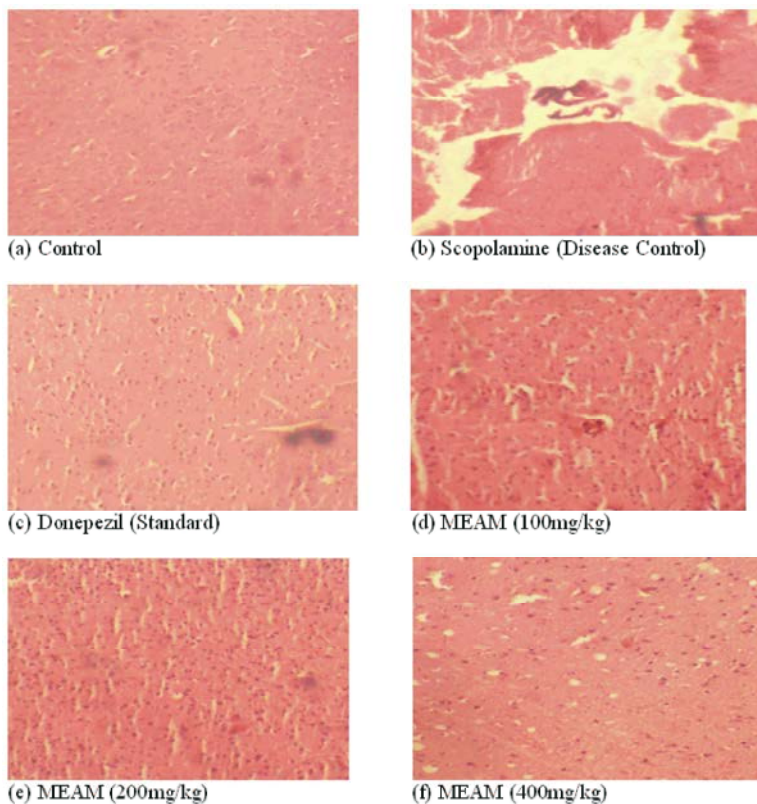


Fig. 8: The above slides a, b, c, d, e and f are Normal Control, Scopolamine (Disease Control), Donepezil (Standard), MEAM(100mg/kg), MEAM(200mg/kg) and MEAM(400mg/kg) respectively represent the histopathological sections of the brain tissue showing neurological lesions

Catalase Activity: Catalase activity has been expressed in %H₂O₂ scavenging activity. Scopolamine treatment decreased brain catalase activity significantly. Improvement of catalase activity was observed in standard and MEAM treated groups compared to scopolamine treated group as shown in Fig. 6.

TBARS Assay: Scopolamine treatment significantly increased the brain MDA levels compared to control group. Standard drug donepezil and MEAM treatment significantly decreased brain MDA levels compared to scopolamine treated group as shown in Fig. 7.

DISCUSSION

Acetylcholine (ACh) is a neurotransmitter that has long received much attention in memory research. ACh acts on cholinergic receptors that are widely distributed throughout in the brain. Cholinergic antagonism was reported to produce cognition deficit which imitates

Alzheimer's disease similar to hippocampal lesion-induced cognitive deficits. Scopolamine, acetylcholine receptor antagonist, was reported to impair cognitive performances especially spatial learning and memory. It exerts amnesic effect equally in various behavioral models of memory including Morris water maze and pole climbing apparatus [16]. Therefore, scopolamine was considered as reliable tool to study neuroprotective effects of candidate molecules.

The present study investigated the neuroprotective effect of methanolic leaf extract of *Aegle marmelos* (MEAM) in the prevention of dementia of Alzheimer's type using scopolamine induced mice. Salient findings of this study were that pre and Post scopolamine treatment with MEAM improved cognition, decreased malondialdehyde and increased activities of catalase and antioxidant activity. This illustrates that intraperitoneal administration of scopolamine was characterized by progressive deterioration of learning and memory, oxidative stress and decrease in acetylcholine turnover.

The etiology of AD has not been revealed clearly. Nevertheless, lots of hypotheses and biochemical evidences have been provided to support the theory of a cascade of events in AD that includes the onset of oxidative stress and excitotoxicity.

From Morris Water Maze Test, Swim latencies exhibited by all groups of mice except disease control group were gradually decreased from day 1 to day 5. Significant ($p < 0.01$) reduction was observed with MEAM treated groups. Standard drug group has shown significant ($p < 0.001$) reduction in latency time.

From Pole Climbing Test, test groups have showed significant ($p < 0.05$) decrease in latency time on day 7 and on day 9, standard drug group has shown significant ($p < 0.001$) decrease in latency time with mice compared to disease control group.

From Locomotor Activity Test, the MEAM did not cause significant alteration in the locomotor activity as compared to scopolamine treated mice. In comparison with Donepezil, administration of test drugs treated group had almost equal behavioral test responses which indicates therapeutic efficacy of ameliorative property of MEAM against memory loss.

The present study therefore demonstrates the probability by which MEAM showed neuroprotective activity by increasing the performance of learning and memory which was clear from behavioral test results. Oxidative stress in brain generates oxygen radicals like superoxide anion, hydroxyl radical and hydrogen peroxide, which act on polyunsaturated fatty acids in brain, thereby propagating the lipid peroxidation. The major antioxidant and oxidative free radical scavenging enzymes like glutathione, SOD and catalase play an important role to reduce oxidation stress in brain.

Our results also suggest that the MEAM reduced oxidative stress by reducing lipid peroxidation and increasing the endogenous antioxidant enzymes in brain. Other important activity has been shown by the test drugs was that it has acetylcholinesterase (AChE) inhibiting activity. This activity tends to allow the more retention of acetylcholine in the brain, which was important for the cognitive functions, learning and memory.

CONCLUSIONS

The present study demonstrated that the activity of methanolic extract of *Aegle marmelos* had potential

therapeutic effects on improving the anti-amnesic activity in mice through inhibiting lipid peroxidation, augmenting endogenous antioxidant enzymes and decreasing acetylcholinesterase (AChE) activity in brain. Further study was warranted to find its potential use in humans.

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