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Original Article

# *Entada Phaseoloides* Attenuates Scopolamine-induced Impairment of Spatial Memory in Mice.

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## Abstract

**Background:** *Entada phaseoloides* is a well-known medicinal plant, has been traditionally used in Ayurveda for centuries. The seed pulp of the plant has been reported for various pharmacological activities. Even though its effect on cognitive performance has not been reported so far. This study unveils the memory enhancing activity of *Entada phaseoloides*.

**Objectives:** Methanol extract from seeds of *Entada phaseoloides* was evaluated in Scopolamine induced learning and memory impairment using Morris water maze model in mice. Isolation of active compounds is also undertaken.

**Materials and Methods:** Scopolamine (0.4 mg/kg i.p.), a muscarinic cholinergic antagonist, the classic amnestic drug was used for impairing memory in mice. Mice (n=6/group) treated with methanolic extract of seed pulp of *Entada phaseoloides* (MEEP) (100 and 200 mg/kg) and the standard drug Tacrine (3 mg/kg, i.p.) were used. Morris water maze model was used for studying their anti amnesic property, following treatment. The level of the enzyme AChE and β-amyloid$_{1-42}$ in the hippocampus were determined biochemically by ELISA. Estimation of antioxidant enzymes viz. Superoxide dismutase (SOD), Glutathione (GSH), Catalase (CAT), oxidative stress markers like Lipid peroxidase (LPO), Nitric Oxide (NO), Catecholamine levels, Gene expression studies of Tropomyosin receptor kinase B (TrkB), Brain derived neurotrophic factor (BDNF), Nuclear Factor-κB p65 (NF-κB p65), Caspase-3 by Western Blotting and Acetylcholinesterase (AChE) and nuclear factor erythroid 2–related factor 2 (Nrf2) by Reverse transcriptase PCR were done following behavioural study.

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**Results:** MEEP and tacrine significantly improved memory dysfunction by decreasing escape latency during training and increase in time spent in target quadrant during retrieval in Morris water maze. Its treatment reversed the scopolamine induced hyper activation of acetyl cholinesterase activity, elevated SOD, GSH, Catalase levels and decreased LPO, NO and β-amyloid$_{1-42}$ in the hippocampus. Expression of proteins NF-κB p65, Caspase-3 were significantly down regulated whereas, BDNF and TrkB were up regulated in the MEEP and tacrine treated groups. MEEP suppressed mRNA expression of AChE and increased nuclear factor erythroid 2–related factor 2 (Nrf2) mRNA expression. Serotonin (5-HT) and Dopamine (DA) levels in the hippocampal tissue were also elevated. Different components of the seeds oleic acid, Entadamide A-beta-D-glucopyranoside, 1-O-protocatechuoyl-β-D-glucose and Entadamide A were isolated.

**Conclusion:** The results indicated that seeds of *Entada phaseoloides* could possibly be a promising therapeutic candidate for treatment of cognitive dysfunction in addition to its already established medicinal properties. Hence, *Entada phaseoloides* might be a good candidate for developing anti amnestic drug.

## Introduction

*Entada phaseoloides* (Linn.) Merr. (Family: Fabaceae) is a well-known medicinal plant distributed throughout the sub-Himalayan tract and in the monsoon forest of Western and Eastern Ghats. It is also abundant in Andaman & Nicobar Islands. The seeds are considered tonic, emetic, antiperiodic and anthelmintic (1). The paste of the seeds is applied to cure inflammatory glandular swellings. A glycoside of entagenic acid possesses anti-neoplastic activity (1).

In folk medicine, barks, stems, leaves, and seeds of *E. phaseoloides* have been used to treat haemorrhoids, stomach ache, toothache, spasm, gastritis, parotitis, and lymphadenitis (2). In India, the boiled seeds of ghila bean are consumed by Karbi tribes of Assam and Oceanic group of tribes such as Onges and Great Andamanese. The soaked seed kernels are roasted/boiled and eaten by northeast tribal sections such as Garo, Khasi, Naga and Kanikkars of Tamil Nadu and Kerala (3). The species is used medicinally in Malaysia, the Philippines and Java It possesses a wide range of ethnopharmacological properties contributed by phytochemical constituents of which most abundant are saponins, diterpenes, triterpenes and phenolics compounds (4). The extract was found to possess anti-inflammatory property as it acts on proliferative phase of inflammation (5).

In addition to this, it showed anti-arthritic (6), anti-diabetic and hypolipidemic activities (7), anti-ulcer (8), anti-toxicity (9), anti-complement and antimicrobial (10) properties and also possesses molluscidal activities (crude and processed product of *Entada phaseoloides*) (11). The seeds of *Entada phaseoloides* are very high in protein, carbohydrates and lipids contents (12). The nutritive value and antioxidant activity of *Entada phaseoloides* seeds were reported by us (13). Our study was directed towards the role of this plant in improving memory related disorders.

## Materials and Methods

### Chemicals, kits and reagents

Scopolamine hydrobromide, Tacrine hydrochloride (9-Amino-1, 2, 3, 4- tetrahydro-acridine hydrochloride hydrate), acetylthiocholine iodide, 5,5'-dithio-bis-nitro-benzoic acid (DTNB), Griess reagent, dopamine hydrochloride, serotonin hydrochloride and tetramethylbenzidine (TMB) were procured from Sigma-Aldrich, USA. Enzyme-linked Immunosorbent Assay (ELISA) Kit (RayBio®, USA), RIPA lysis buffer (Amresco, USA & Canada). All other chemicals and reagents used in the present work were of analytical grades.

### Animals

Adult male Swiss Albino mice (20–30 g) were selected

for the study and housed in polypropylene cages with clean bedding materials and safe drinking water with 12 h light-dark cycle, and was given standard laboratory feed and water *ad libitum*. All experiments were performed according to current guidelines for the care of laboratory animals by IAEC (Approval No. 770/ac/CPCSEA/FVSc, AAU/IAEC/11-12/118); effort was made to minimize suffering of the experimental animals throughout the study.

**Plant material**

The dried seeds of *Entada phaseoloides* were collected from local market and identified by taxonomist Dr. Iswar Chandra Barua, Principal Scientist, Department of Agronomy, Assam Agricultural University, Jorhat, Assam and a voucher specimen was deposited and kept at the herbarium of the Department of Agronomy, Assam Agricultural University, Jorhat-785013, Assam (AAU- EVM- NW-3).

**Extraction, isolation and characterization of the chemical constituents**

After removing the kernel from the seeds, they were shade dried, powdered mechanically, weighed and stored in airtight container. Then, 250 g of powdered material was soaked in 1000 mL methanol for 72 h in a beaker and mixture was stirred every 18 h using a sterile glass rod. Filtrate was obtained three times with the help of Whatman filter paper no. 1 and the solvent was removed by rotary evaporator (BUCHI, R-210, Labortechnik AG, Meierseggstrasse Switzerland) under reduced pressure leaving a dark brown residue (MEEP), and was stored in airtight container at 4°C for future use. The recovery percentage with respect to dry powder was 26.52% w/w.

The methanolic extract (10 g) was subjected to column chromatography (silica gel, 100-200 mesh, eluting with hexane/EtOAc mixture of increasing polarity) to give 40 column fractions. Column fractions were analyzed by TLC (silica gel 60 F254, hexane: EtOAc, 60:40), and fractions with similar TLC patterns were combined to give five major fractions ($F_1$, $F_2$, $F_3$, $F_4$, $F_5$). Fractions F4 was subjected to repeated column chromatography eluting with EtOAc: hexane (19:81) to yield compound 1. Fraction $F_3$ was subjected to Column chromatography(CC) on silica gel (100-200 mesh) using a hexane-EtOAc (10:0-6:4) to yield sub fractions $B_1$ and Compound 2. Sub fraction $B_1$ was then purified by preparative TLC with $CHCl_3$: MeOH (90:10) to get compound 3. Repeated purification of fraction $F_4$ on silica gel (230-400 mesh) using $CHCl_3$: MeOH (yu90:10) followed by Preparative HPLC, yielded compound 4.

**Oleic acid (1):**

Light yellow oil, $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 5.35 (2H, m), 2.33(4H, m), 2.01 (4H, m), 1.64 (4H, m),1.37-1.22(H, m), 0.88 (3H, t, J = 7.1 & 6.2 Hz). $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 180.55, 129.93, 129.63, 34.11, 31.91, 29.76, 29.66, 29.60, 29.53, 29.37, 29.32, 29.14, 29.05, 27.20, 27.13, 24.64, 22.67, 14.05. HR-ESI-MS m/z: 305.2456 (Calcd for $C_{18}H_{24}O_2Na$: 305.2451).

Entadamide A (2): White amorphous powder, $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$ : 7.62 (1H, d, J = 14.5 Hz), 6.44(1H, br s), 5.69(1H, d, J = 14.6 Hz), 3.71(2H,m), 3.46 (2H, m), 2.32 (3H,s). $^{13}$C NMR ($CDCl_3$, 75MHz) $\delta$: 165.80, 143.67, 115.31, 62.46,42.49, 14.60. HR-ESI-MS m/z: 162.0589 (Calcd for $C_6H_{12}NO_2S$: 162.0583).

Entadamide A-beta-D-glucopyranoside (3): White amorphous powder, $^1$H-NMR (500 MHz, $CD_3OD$) $\delta$ : 2.33 (3H, s), 3.27 (1H, m), 3.29 (1H, m,), 3.35 (1H, m), 3.37 (1H, m), 3.46 (1H, m), 3.72 (2H, m), 3.90(1H, m), 3.96 (1H, m), 3.99 (1H, m,), 4.29 (1H, d, J = 7.9 Hz), 5.85 (1H, d, J = 14.58Hz), 7.59 (1H, d, J = 14.58 Hz). $^{13}$C NMR (75 MHz, $CD_3OD$): $\delta$ : 165.58, 142.31, 114.83, 102.39, 75.8, 75.67, 72.97, 69.36, 68.23, 60.74, 38.92, 13.4. HR-ESI-MS m/z: 346.0946 (Calcd for $C_{12}H_{21}NO_7S$: 346.0931).

1-O-protocatechuoyl-$\beta$-D-glucose (4): Color less gummy, $^1$H-NMR (500 MHz, DMSO-$d_6$) $\delta$ : 6.91(1H, d, J = 8.68Hz), 6.59(1H, brs), 6.48 (1H, dd, J = 8.6 & 2.5), 4.47(1H, d, J = 6.8), 3.69(1H, m), 3.47(1H, m), 3.37(1H, m), 3.18(3H, m). $^{13}$C NMR (75 MHz, $CD_3OD$)

δ : 174.56, 152.15, 148.65, 129.14, 117.64, 117.12, 112.84, 103.74, 76.96, 76.45, 73.43, 69.74, 60.88.

**Acute toxicity studies**

The acute toxicity methanolic extract of *Entada phaseoloides* were performed according to Organization of Economic Corporation Development (OECD) Guidelines No. 423 in male albino mice (20-30 g). The extracts were administered orally at 2000 mg/kg to a group of mice (n=3) and the percentage mortality if any was recorded. The animals were kept under observation for next 14 days for mortality or gross abnormality with the given doses. Based on the acute toxicity study, 100 and 200 mg/kg oral dose were selected.

**Treatment Schedule**

The experimental mice were divided into 5 groups (n=6 in each group).

Group I: Normal control received only Saline,

Group II: Negative control, received scopolamine 0.4 mg/kg, i.p.

Group III: Standard control, received Tacrine 3 mg/kg, i.p.+ scopolamine, 0.4 mg/kg i.p.

Group IV: Methanolic extract of *Entada phaseoloides* 100 mg/kg, p.o.+scopolamine 0.4 mg/kg i.p. and

Group V: Methanolic extract of *Entada phaseoloides* 200 mg/kg p.o.+scopolamine 0.4 mg/kg i.p.

**Behavioural Studies**

**Morris Water Maze (14)**

The water maze contained a circular water pool with 150 cm diameter into Northeast (NE), Southeast (SE), Southwest (SW), and Northwest (NW) equally spaced quadrants along the circumference of the pool. In the North West quadrant, an escape platform (10 cm diameter) was kept 2 cm underneath the water surface. Throughout the acquisition trials the platform was maintained in a consistent area in North West quadrant. The mice were trained to locate this hidden platform within 60 s, it was gently guided to the platform and was allowed to stay there 15 s. Animals were given acquisition trials for four times per day for four consecutive days. To eliminate the quadrant effects, animals was positioned in each quadrant during each trial. Animals which failed to reach the platform in 20 s on the 4$^{th}$ trial day were discarded from the study. On the probe day (day 5), 24 h after the last acquisition trial, escape platform was removed and retention trial was conducted. The animals were allowed to swim for 60 s before the last behavioural test. Data obtained from behavioural tests were assessed through a video camera attached to a computerized tracking system (ANY-maze ™ software Stoelting Co. Video tracking). Time to reach hidden platform (escape latency), distance travelled to reach the platform were recorded during retention trials.

**Biochemical estimation**

Biochemical tests were conducted 24 h after the last behavioural test. The animals were sacrificed under anaesthesia, brains were removed; hippocampus was dissected and rinsed with ice-cold isotonic saline, followed by homogenization with ice-cold phosphate buffer (pH 8). The homogenates were centrifuged at 10,000 rpm for 15 min and the supernatant was used for the biochemical estimations or stored at -80°C. The total protein was estimated by the method of Bradford et al., 1976 (15).

**Estimation of Acetylcholinesterase activity**

The AChE activity was measured by the method of Ellman et al., 1961 (16). Change in absorbance per minute of the sample was read spectrophotometrically (MultiscanGo, Themo Fisher) at 420 nm.

**Estimation of β-Amyloid$_{1-42}$**

The level of β-amyloid$_{1-42}$ in the brain tissue was done by sandwich ELISA using mice β amyloid$_{1-42}$ (Bioassay Technology laboratory, Shanghai, China) kit.

**Assessment of oxidative stress and antioxidant status**

The pro-oxidant markers i.e. Lipid peroxidation (LPO) (17) and Nitric oxide (NO) (18), antioxidant proteins i.e. reduced glutathione (GSH) (19), Superoxide

dismutase (SOD) (20), Catalase (CAT) (21) were measured spectrophotometrically (MultiscanGo, Thermo Fisher) as per standard protocol.

**SDS-PAGE and Immunoblot analysis**

The expression of the TrkB, BDNF, NF-κB p65 and Caspase-3 proteins in the hippocampus was analyzed by Western Blotting. Thirty mg of hippocampus were homogenized in 5 mL of chilled lysis buffer (RIPA Buffer, Amresco, USA) and centrifuged at 23,000 × g for 20 min at 4°C. The protein concentration of the supernatants was quantified by Bradford reagent (Himedia) with Bovine serum albumin (BSA) as the standard. Samples with 50 µg of total protein were mixed with an equal volume of 2X Laemmli buffer, boiled for 5 min at 95°C, cooled, loaded and separated in 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS) by using the Hoefer Midi Gel apparatus (Harvard Apparatus, Holliston, MA) (Sambrook et al. 1989). The separated proteins were transferred to PVDF membranes for immunoblot analysis. After blocking with 3% Bovine serum albumin in 1X TBST for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies (TrkB, BDNF, NF-κB p65, Caspase-3 and β-actin) at a dilution of 1:500; Santa Cruz Biotechnology, Santa Cruz, CA. On the following day, the membranes with 1X TBST and then incubated with respective secondary antibodies conjugated to Horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Inc) for 1h at a dilution of 1:5000. The bands were visualized using TMB Blotting solution available commercially. The band intensities were quantified using Image J software (NIH, Bethesda, MD, USA).

**Reverse Transcriptase Polymerase Chain Reaction**

Expression of AChE and Nrf2 genes in the hippocampus of mice was studied by RT-PCR, following the method developed by Bodduluru et al., 2016 (22). Hippocampus Tissues (30 mg) was homogenized in TRIzol using micro pestle (Tarsons). The total RNA was isolated using TRIzol (Ambion). The RNA was then quantified using micro drop plate in spectrophotometer (Multiscan Go, ThermoFisher). The mRNA was reverse transcribed by Revert Aid First Strand cDNA synthesis kit (Thermo Scientific) with random hexamer primers. Initial step included 6.5 µl reaction mixture containing 3 µl RNA template, 0.5 µl Random hexamer and 3 µl nuclease free water with PCR condition of 65°C for 5 minutes. The following step includes, mixing of 2 µl 5X buffer, 0.5 µl dNTPs, 0.5 µl RNase Inhibitor (Ribolock) and 0.5 µl Reverse Transcriptase making the total volume of reaction to 10 µl. The PCR condition in final step comprised of three phases, initiation at 25°C for 5 min, elongation 42°C for 60 min and termination at 70°C for 15 min and 4°C hold for infinite time. Subsequently PCR was performed for above mentioned genes.

**Estimation of DA (Dopamine) and Serotonin (5-HT)**

DA and 5-HT concentrations were simultaneously estimated in the hippocampus with slight modification of the method by Sheikh et al. (2007) (23) using High Performance Liquid Chromatography. The animals were sacrificed and their brains rapidly were removed and dissected on an ice-chilled glass plate to remove the hippocampus. Each tissue sample was weighed and homogenized by sonication in 1000 µL 0.4 M perchloric acid. The homogenate was kept on ice for 1 h and then centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was preserved, and 20 L was injected to determine the concentrations of NE, DA and 5-HT using high performance liquid chromatography by electrochemical detector (HPLC-ECD) with minor modifications. The mobile phase consisted of 75 mM sodium phosphate monobasic monohydrate, 1.7 mM 1-Octanesulfonic Acid sodium salt, 25 µM EDTA, 100 µl Triethylamine, and 10% methanol, pH 3.0. An Acclaim $^{R}$ C30 5 µm (4.6×250 mm) (Dionex) was operated at 0.7 mL/min. The Coulochem III detector 5011A cell was set at: E1@-150 mV: E2@+225 mV, Dionex, Sunyvale, CA). External standard curves were used to quantify NE, DA and 5-HT contents in each sample and calculated using the area under the curve.

**Statistical analysis**

Results are expressed as Mean±SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple range tests, using Graph Pad Prism software version 5.0 (San Diego, CA, USA), $p<0.05$ were

considered to be statistically significant.

## Results

**Chemistry:**

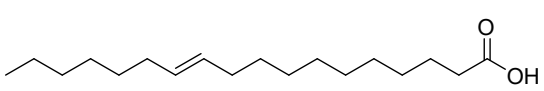
The fractionation and purification led to the isolation of 4 compounds in the crude methanolic extract from the seeds of *Entada phaseoloides*. The structures of isolates were established using IR, MS, 1D and 2D NMR spectroscopic techniques. After comparing their spectral data with those reported in the literature (24), they were identified as known compounds (Fig. 1) and confirmed as Oleic acid (1), Entadamide A (2), Entadamide A-beta-D-glucopyranoside (3) and 1-O-protocatechuoyl-β-D-glucose (4).

**Behavioural Study**

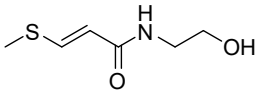
**Effect of MEEP on Morris water maze in mice**

**Escape Latency (Sec)**

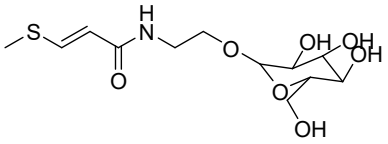
In Morris Water Maze, a significant decline in the escape latency could be observed ($p<0.001$) in MEEP (200 mg/kg) (6.373±0.029 sec), followed by tacrine and MEEP (100 mg/kg) (6.433±0.05 sec) when compared to scopolamine treated group (Fig. 2A).



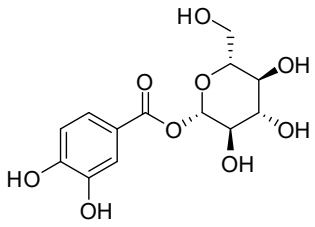

Oleic acid (**1**)




Entadamide A (**2**)




Entadamide A-beta-D-glucopyranoside (**3**)




1-O-protocatechuoyl-ß -D-glucose(**4**)

Fig. 1: The structures of the most active compounds identified by NMR as followed: Oleic acid (1), Entadamide A (2), Entadamide A-beta-D-glucopyranoside (3), 1-O-protocatechuoyl-β-D-glucose (4).

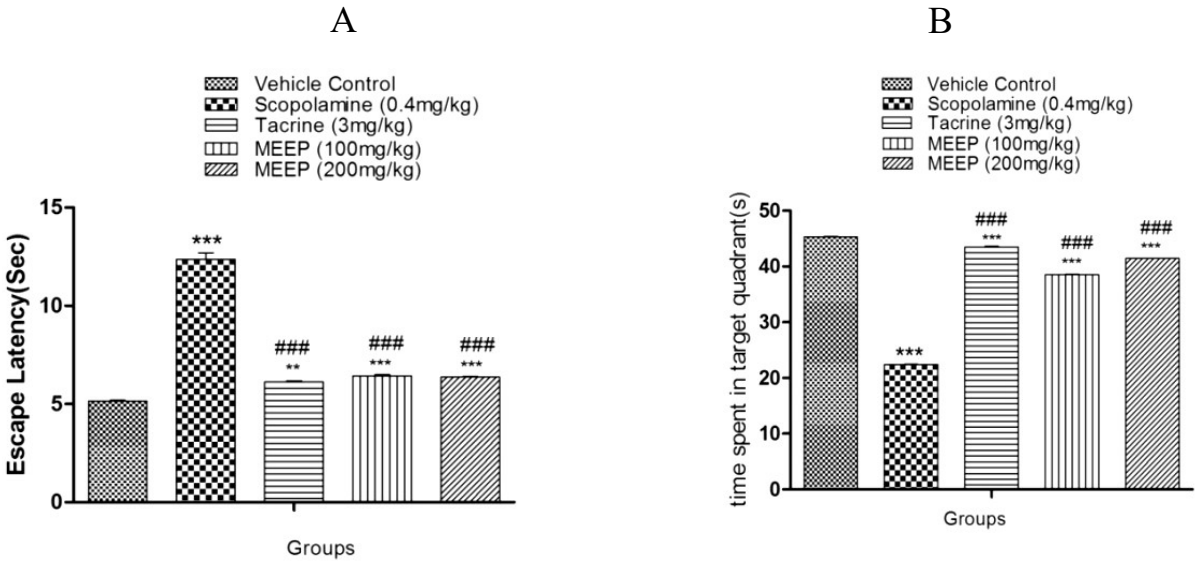



Fig. 2: Behavioural study by Morris Water Maze showing A) Escape Latency (Sec) B) Time spent in target quadrant (Sec). Values are expressed as percent fold change compared with control. Values represented as Mean±SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 compared with vehicle control group; #p<0.05, ##p<0.01, ###p<0.001 compared with scopolamine treated group.

**Time spent in target quadrant (Sec)**

During the probe trial, results clearly indicated that vehicle treated animals spent an average time of (45.33±0.06 sec) in the target quadrant. The Scopolamine-treated animals spent lesser time (22.41±0.03 sec) in the target quadrant in comparison to control group. MEEP (100mg/kg) (38.52±0.07 sec) and MEEP (200 mg/kg) (41.41±0.06 sec) shows significant ($p<0.001$) reversal of Scopolamine induced amnesia as the treated animals in the target quadrant (Fig. 2B).

**Effect of MEEP on brain Acetylcholinesterase activity and Beta Amyloid.**

Acetylcholinesterase levels in Scopolamine treated mice hippocampus were significantly elevated (208.3±3.38 nM/min/mg, $p<0.001$), as compared to control. On the other hand, a significant decline ($p<0.001$) in the level of the enzyme could be observed in Tacrine (124.0±1.15 nM/min/mg), MEEP 100 mg/kg (149.0±2.08 nM/min/mg) and MEEP 200 mg/kg (130.0±1.15 nM/min/mg) treated groups. The result is graphically presented in Fig. 3A. β-amyloid, a hallmark for Alzheimer's disease showed significant (1189±1.453 ng/L, $p<0.001$) elevation in the protein level in the hippocampus of scopolamine treated group when compared to control; while comparing the treated group with that of the scopolamine treated group, a decline in the protein levels was observed in MEEP 100 and 200 mg/kg (972.30±0.88 ng/L, 903.30±3.383 ng/L, $p<0.001$) and Tacrine (894±2.02 ng/L, $p<0.001$) (Fig. 3B).

**Effect of MEEP on oxidative stress markers and antioxidants in hippocampus of mice.**

Oxidative stress markers LPO and NO levels were elevated (230.0±5.29 nMol/mg, 3.240±0.04 µg/mg, $p<0.001$) in scopolamine treated group in comparison to control group. A reversal in LPO and NO levels could be observed in Tacrine (145.0±5.50 nMol/mg; 1.717±0.06 µg/mg), MEEP 100 mg/kg (155.0±4.04 nMol/mg; 2.147±0.09 µg/mg) and 200 mg/kg (152.0±6.08 nMol/mg; 1.823±0.06 µg/mg) treated groups at significantly decreasing ($p<0.001$) levels, when compared to scopolamine treated group. On

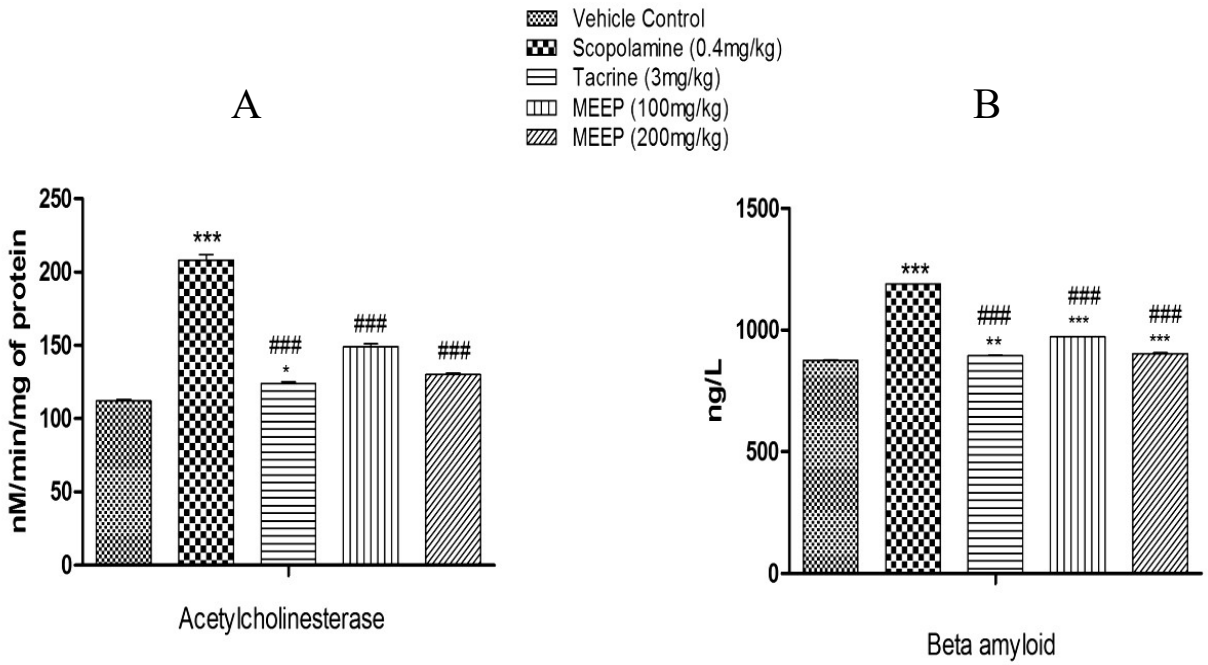



Fig. 3: Estimation of (A) Acetylcholinesterase and (B) Beta- Amyloid level in mice hippocampus with Scopolamine induced memory loss in Morris water maze model. Values are expressed as percent fold change compared with control and represented as Mean±SEM (n=6). *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with vehicle control group; #$p<0.05$, ##$p<0.01$, ###$p<0.001$ compared with scopolamine treated group.

the other hand, antioxidants enzymes viz. GSH, SOD and Catalase significantly declined (1368±5.77 µg/mg, 4.42±0.04 U/mg, 29.00±0.57, p<0.001) in scopolamine treated group as compared to control; while, the levels were significantly (p<0.001) elevated in Tacrine (2012±4.66 µg/mg; 6.007±0.05 U/mg; 55.67±0.88), MEEP 100 mg/kg (1708±2.60 µg/mg; 5.727±0.01 U/mg; 40.00±2.08) and 200 mg/kg (1891±3.05 µg/mg; 5.827±0.02 U/mg; 52.33±0.88) p.o. dose treated groups than compared to scopolamine treated group (Fig. 4).

**Immunoblotting assay of TrkB, BDNF, NF-κB p65and Caspase-3**

The Immunoblotting assay of TrkB, BDNF, NF-κB p65 and Caspase-3 showed significant (p<0.001, p<0.01) up regulation in the expression of TrkB, BDNF, NF-κB p65 and Caspase-3 in the hippocampal tissue of scopolamine induced mice compared to the control group. Pre-treatment with MEEP significantly down regulated NF-κB p65 (200 mg/kg; 0.65±0.04, p<0.001) and Caspase-3 (200 mg/kg; 0.82±0.04, p<0.001) protein in the hippocampal tissues of mice similar to that of standard drug tacrine when compared with scopolamine alone treated group. On the other hand, down regulation of TrkB (p<0.001) and BDNF (p<0.001) protein in the hippocampal tissue of scopolamine induced mice compared to control group was prominent. Conversely, pre-treatment with MEEP (100 and 200 mg/kg) up regulated TrkB and BDNF protein expression in the hippocampal tissues of mice analogous to Tacrine (1.16±0.04, p<0.001; 0.75±0.07, p<0.05) as compared to scopolamine

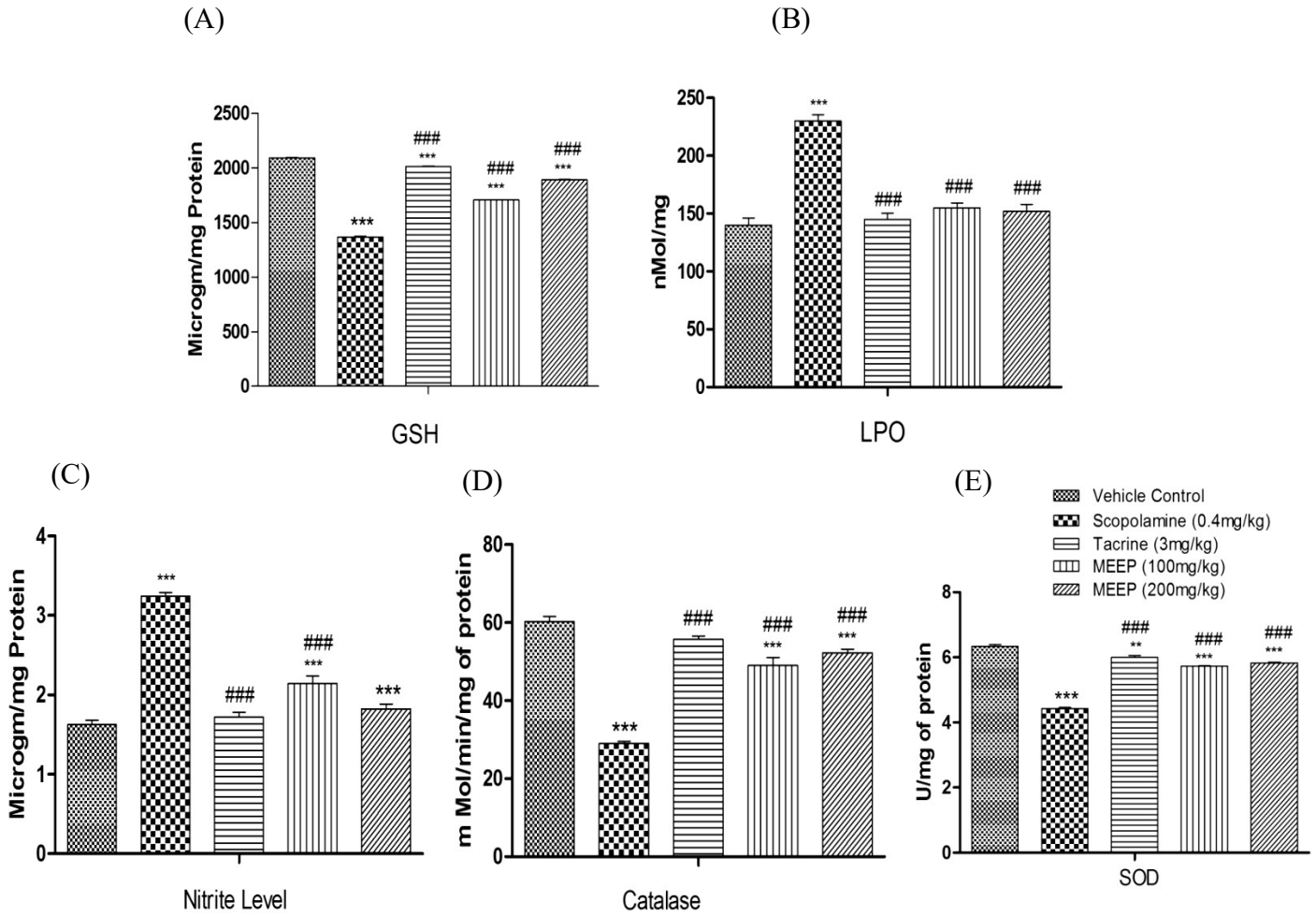



Fig. 4 : Estimation of in-vivo Antioxidants A) GSH B) LPO C) NO D) Catalase and E) SOD in mice hippocampus with Scopolamine induced memory loss in Morris Water Maze model. Values are expressed as percent fold change compared with control. Values represented as Mean±SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 compared with vehicle control group; #p<0.05, ##p<0.01, ###p<0.001 compared with scopolamine treated group.

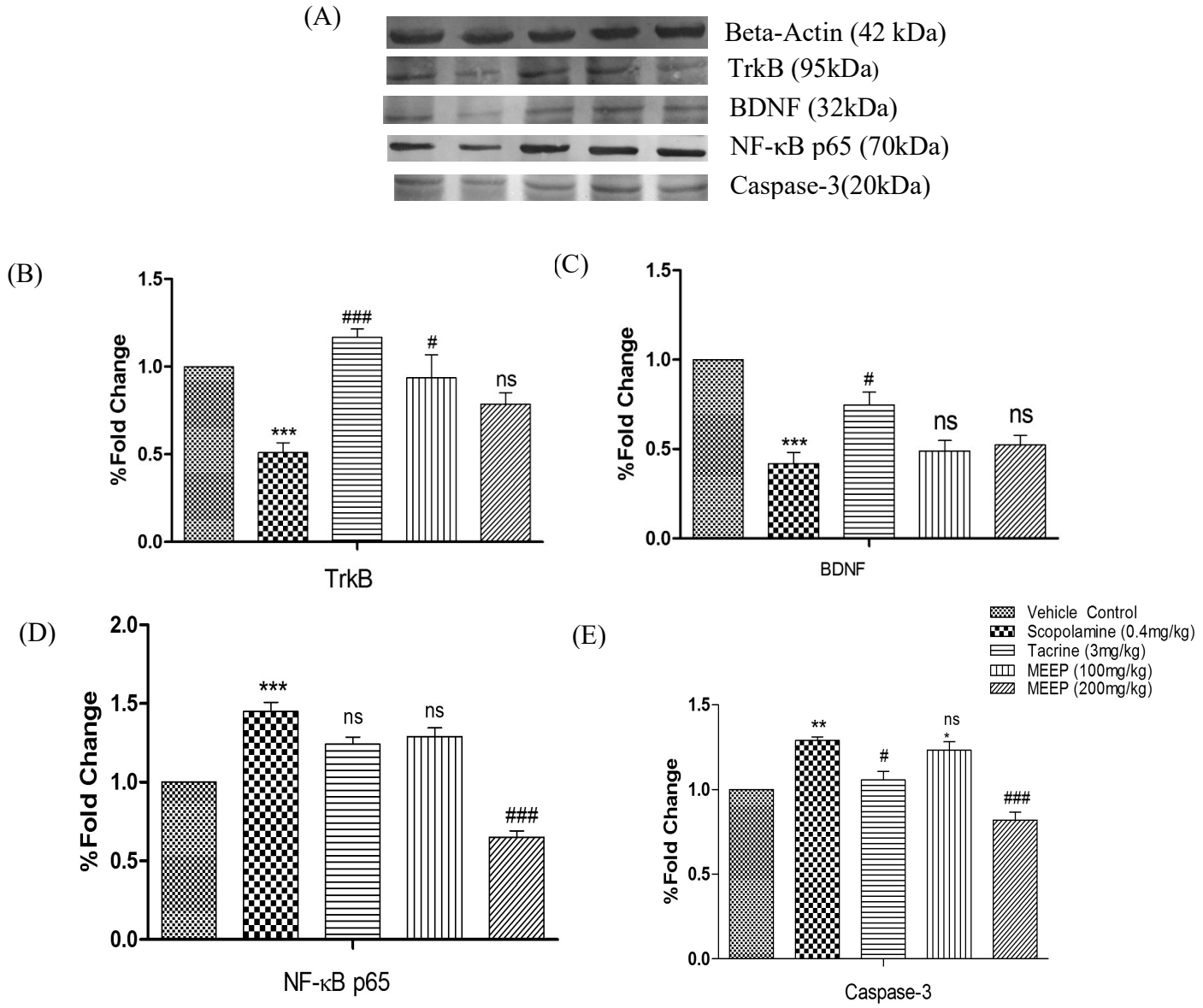



Fig. 5 : Effect of MEEP pre-treatment on scopolamine-induced amnesia on TrkB, BDNF, NF-κB p65 and Caspase-3 proteins expression in mice brain. (A) Lane a-vehicle control, lane b-scopolamine (0.4 mg/kg), lane c- tacrine (3 mg/kg), lane d- MEEP (100 mg/kg), lane e-MEEP (200 mg/kg). β actin was used as internal control to assess the equal loading of sample. Quantitative data expression of (B) TrkB, (C) BDNF, (D) NF-κB p65 and (E) Caspase-3 proteins levels. Values are expressed as percent fold change compared with control. Values represented as Mean±SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 compared with vehicle control group; #p<0.05, ##p<0.01, ###p<0.001 compared with scopolamine treated group.

treated mice. It is shown graphically in Fig. 5.

**Effects of MEEP on AChE and Nrf2 gene expression by RT-PCR.**

The RT-PCR analysis of AChE and Nrf2 gene expression is presented in Fig. 6, the mRNA expression levels of AChE (1.26±0.03 fold change, p<0.05) was significantly up-regulated and Nrf2 (0.346±0.06 fold change, p<0.001) was significantly down-regulated in the hippocampal tissues of scopolamine induced mice, respectively, compared to the vehicle control group. However, pre-treatment with MEEP significantly down regulated the mRNA expression of AChE (MEEP 100 mg/kg; 0.74±0.05 fold change, p<0.05, 200 mg/kg; 0.43±0.06 fold change, p<0.001) and up regulation of Nrf2 gene (MEEP 100 mg/kg; 0.76±0.09 fold change, p<0.05, 200 mg/kg; 1.03±0.11 fold change, p<0.001) expression in the hippocampal tissues of mice,

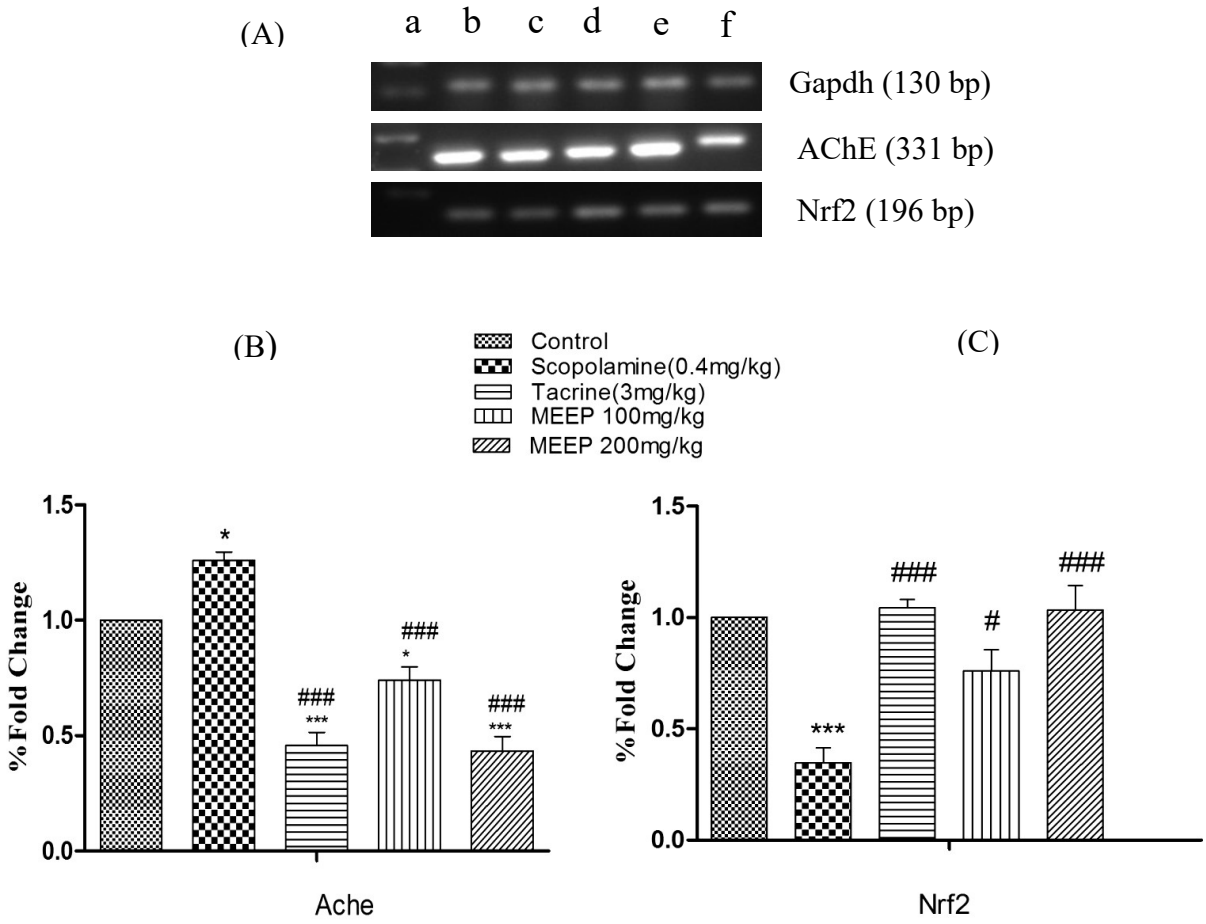



Fig. 6 : Effect of MEEP pre-treatment on scopolamine-induced amnesia on AChE and Nrf2 mRNA expression in the hippocampus of mice. (A) Lane a- DNA marker, lane b- vehicle control, lane c- scopolamine (0.4 mg/kg), lane d- tacrine (3 mg/kg), lane e- MEEP (100 mg/kg), lane f- MEEP (200 mg/kg). GAPDH was used as positive control to assess the equal loading of sample. Quantitative data expression of (B) AChE and (C) Nrf2 mRNA levels were assessed using densitometry as expressed as fold change as compared with vehicle control. Values are expressed as Mean±SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 compared with vehicle control group; #p<0.05, ##p<0.01, ###p<0.001 compared with scopolamine treated group.

similar to that of standard drug Tacrine, as compared to the scopolamine treated mice.

**Effects of MEEP on DA and 5-HT levels in the hippocampus in Scopolamine induced mice.**

Scopolamine significantly reduced DA and 5-HT concentrations in the hippocampus compared to normal animals. Administration of MEEP (100 and 200 mg/kg, p.o.) increased the levels of DA (68.08±0.02 ng/gm, 66.27±0.06 ng/gm, p<0.001) and 5-HT (90.23±0.06 ng/gm, 80.35±0.09 ng/gm, p<0.001) concentrations. Likewise, the standard drug tacrine reversed the effects of scopolamine on DA (9.32±0.12 ng/gm, p<0.001) and 5-HT (50.24±0.05 ng/gm) from lower to higher levels (Fig. 7).

## Discussion

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and a major and increasing public health concern. The characteristic pathological features of the central nervous system (CNS) in Alzheimer's disease are senile plaque, neurofibrillary tangle formation, aberrant oxidative and inflammatory processes, and neurotransmitter disturbances (25). Moreover, cholinergic deficit is a consistent neuropathological symptom associated with memory loss, and has been correlated with the severity of Alzheimer's disease (26). Experimental and clinical studies indicate that acetylcholine plays a major role in the regulation of cognitive functions

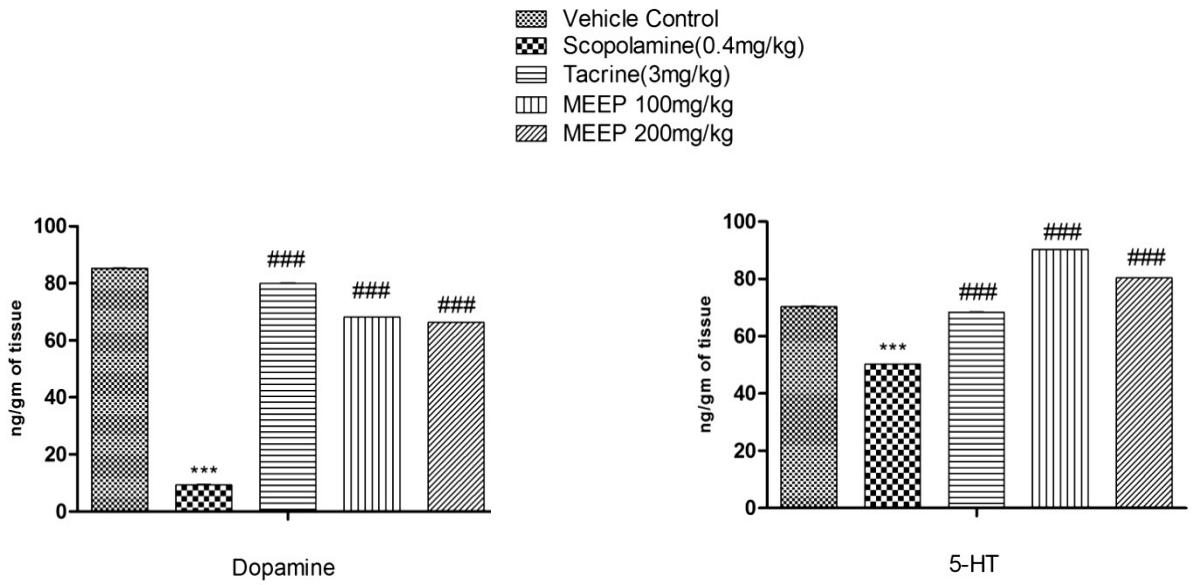



Fig. 7 : Catecholamine (DA and 5HT) levels in the hippocampus of mice in different treatment groups viz. Vehicle control, Scopolamine, Tacrine, MEEP (100 mg/kg), MEEP (200 mg/kg) in mice hippocampus with Scopolamine induced memory loss. Values are expressed as Mean±SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 compared with vehicle control group; #p<0.05, ##p<0.01, ###p<0.001 compared with scopolamine treated group.

(27). Animal and human studies indicate that disruption of the cholinergic nervous system is a major factor in the early state of Alzheimer's disease (28).

We evaluated the effect of *Entada phaseoloides* seed extract on scopolamine-induced learning and memory deficits using the Morris water maze. This test is designed to assess spatial memory and learning function (Morris, 1984). Scopolamine, a nonselective and competitive muscarinic cholinergic receptor antagonist, is extensively used in mouse behavior tests of cognition and memory (29). Scopolamine induces learning and memory impairment by blocking cholinergic signaling (30).

Escape latency of repeated trial tests for 4 days and the time spent in the target quadrant in the probe test were investigated in the Morris water maze test. These results indicated that *Entada phaseoloides* attenuated scopolamine induced spatial memory impairment and improved long term memory in the Morris water maze test.

Acetylcholinesterase (AChE) is the primary enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) to choline and acetate. By rapid hydrolysis of the neurotransmitter, acetylcholine, AChE terminates neurotransmission at cholinergic synapses (31). Increased AChE activity leads to a lack of ACh and thus memory deficits, as observed in the brains of AD patients (32). Scopolamine increases AChE activity and we found *Entada phaseoloides* seed extract could restrain AChE activity in the hippocampus of mice. These results indicated that *Entada phaseoloides* extract increases cholinergic activity through the inhibition of AChE.

Aggregation of β amyloid protein in brain tissue lead to cognition impairment which is characterized by plaque deposition in extracellular spaces, and β amyloid$_{1-42}$ is predominant in brain. Pre-treatment with *Entada phaseoloides* seed extract (MEEP) at the given dose, resulted in significant decrease in the amount of β amyloid$_{1-42}$ in the mice hippocampus.

The scopolamine induced memory deficit model exhibits prominent oxidative stress and memory deficits, although the mechanism remains unclear (33). The hippocampus plays a crucial role in short and long term memory and highly susceptible to oxidative stress (34). Our result shows that

scopolamine induction increased the levels of LPO and NO; following administration of MEEP or tacrine, significant attenuation of their levels was visible.

To protect tissues against oxidative damage, cellular organisms maintain an antioxidant system containing enzymatic and non-enzymatic components. SOD and catalase play an important role in the detoxification of superoxide anion and hydrogen peroxide respectively, and protects the cells against oxidative damage induced by free radicals (35). Our study shows that scopolamine significantly decreased antioxidant capacity of SOD, Catalase and GSH in the hippocampus of mice brain, whereas, pre-treatment with MEEP significantly ameliorated these level to restore neuronal plasticity and memory function.

BDNF could be a diagnostic biomarker in patients with early Alzheimer's disease and mild cognitive impairment (36). In the present study, the MEEP treatment almost restored the levels of the BDNF proteins, and its receptor, TrkB in the hippocampus, considerably down regulated by scopolamine. We observed that hippocampal BDNF level was significantly suppressed in scopolamine treated animal as compared to normal control group. Our study showed an up regulation in hippocampal BDNF expression in the groups treated with MEEP and tacrine.

Under oxidative stress, Reactive Oxygen Species (ROS) may initiate and exaggerate the inflammatory response due to their capability to stimulate and regulate the inflammatory signalling cascades genes like NF-κB p65 (37). In our study, scopolamine treated mice showed increased NF-κB p65 activity. Pre-treatment with MEEP down regulates NF-κB p65 activity decreasing in neuro-inflammation.

Apoptosis in excess is related to cellular degeneration by oxidative stress, frequently associated with aging and pathogenesis of neurodegenerative conditions (38). Expression of Caspase-3 is a frequently activated death protease catalyzing the specific cleavage of many key cellular proteins. In our study, scopolamine induced apoptotic neuronal cell death by increasing Caspase-3 (apoptotic proteins) activity. Pre-treatment with MEEP lead to decreased Caspase-3 activity indicating neuro-protection. MEEP protected neuronal injury modulating the apoptotic enzymes Caspase-3 in scopolamine induced Caspase-3 activation and neuronal apoptosis.

Nrf2 is an upstream transcription factor modulating phase II enzyme activity, which interacts with the antioxidant response element (ARE) in the nucleus to induce ARE dependent gene expression. During oxidative stress, Nrf2 translocates into the nucleus to induce the expression of hemeoxygenase-1 (HO-1) (39), which plays an essential role in maintaining cellular redox homeostasis against ROS generation and oxidative stress (40). Pre-treatment with MEEP and tacrine led to significant increase in the Nrf2 level decreasing oxidative stress and strengthen endogenous antioxidant.

In stressful conditions, change in dopamine level is associated with a transient change in behavioural aberrations, memory learning disorders (41). Administration of MEEP increased dopamine concentration in scopolamine induced amnesic model in hippocampus, following 14 days of pre-treatment, revealed in HPLC analysis. The extract might show cognition enhancement by increasing dopamine level, which is more prominent than other catecholamines, namely 5HT observed in our experiment.

Bioactivity-guided phytochemical analysis of methanolic extract led to the isolation and identification of four compounds (1-4). In this paper, we have presented anti amnesic activity of the crude extract along with isolation and structure elucidation of the isolated constituents. Oleic acid, an isolated constituent of *Entada phaseoloides* has many positive effects on health. Few study reported its usefulness for proper brain function. A study found a 6.2% decline in oleic acid in the postmortem brains of patients who had been suffering from major depressive disorder when compared to a normal brain (42). Another study reported that a diet rich in oleic acid reduces age-related changes in the brain's mitochondria (43). One more finding suggests that

the isolated constituent Entadamide A may be useful to treat inflammatory diseases (44).

**Conclusion**

From the above behavioural and biochemical data, it can be concluded that *Entada phaseoloides* has an ability to improve or ameliorate spatial long-term memory and short-term memory, in part, via enhancement of cholinergic nervous system, possibly due to its two important phytochemicals, i.e. oleic acid and entadamide A, combined with its inherent antioxidant property. Upregulation of BDNF gene expression shows that there is improvement of cognitive performance due to the treatment. These data suggests that *Entada phaseoloides* possesses memory enhancing activity in impaired memory function.

**Conflict of Interest**

There is no conflict of interest involved in this study.

## Acknowledgements

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