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Neuroprotective Effect of Phaseoloidin in Scopolamine Induced Amnesia, Neuro-inflammation and Apoptosis in Mice

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ABSTRACT

Background: Alzheimer's disease (AD) is a major form of dementia among neurodegenerative diseases, which results in memory deficits. There is no report of the memory enhancing activity of *Entada phaseoloides*, however we reported its potential as a memory enhancing compound and based on this study, its isolated compound Phaseoloidin is taken up for the present study in scopolamine induced memory impairment in Morris water Maze (MWM).

Methods: Mice (n=6/group) treated with Phaseoloidin (100 and 200 mg/kg) and the standard drug Tacrine (3 mg/kg, i.p.) were pretreated with scopolamine. Animals were sacrificed to evaluate AChE and lipid peroxidase, nitric oxide, reduced glutathione, superoxide dismutase, catalase. Gene (BDNF, TrkB, NF-κB p65, Caspase-3) expression studies by Western Blotting and AChE, PP2A, Tau, Nrf2 and HO-1 by Reverse Transcriptase PCR and Real Time PCR.

Result: Phaseoloidin and Tacrine significantly improved memory dysfunction by decreasing escape latency during training and enhance in time spent in target quadrant. Its treatment reversed the scopolamine induced elevation of AChE, elevated SOD, GSH, Catalase levels and decreased LPO, NO levels in the hippocampus. Expressions of BDNF, TrkB, NF-κB p65 and Caspase-3 were up regulated in the treated group. It suppressed mRNA expression of AChE, Tau and increased Nrf2, HO-1, PP2A. Thus, Phaseoloidin could be a potent neuro-pharmacological compound against amnesia.

Key words: *Entada phaseoloides*, Phaseoloidin, Scopolamine, Tacrine.

INTRODUCTION

The pathogenesis of AD is multifactorial and consists of degeneration of cholinergic neurons, unusual phosphorylation of the protein tau, oxidative stress and altered protein processing resulting in abnormal β-amyloid peptide (Aβ) accumulation (Korczyn and Vakhapova 2007).

Today, the demand for herbal products is growing, exponentially throughout the world. It involves the use of medicinal plants to treat AD and enhances general health and well being. Studies of neurodegenerative diseases in animals have shown strong similarities between cognitive dysfunction in dogs and human AD (Schutt *et al.* 2016). Neurodegenerative diseases do not occur spontaneously in laboratory mice and rats, but do occur in several other mammalian species. Mice could be potentially a very good model for studying novel treatment options for cognitive impairement.

Cholinergic deficit has been regarded as a marker of neurological pathology that is associated with memory dysfunction and consistently correlated with the severity of cognitive impairment in AD (Van der Zee *et al,* 2001). Scopolamine, a muscarinic acetyl choline receptor agonist elevates acetylcholinesterase (AChE) levels in the hippocampus and a suitable agent for experimental dementia or impaired memory (Ahmed *et al*. 2009).

Entada phaseoloides (Linn.) Merr. (Family: Fabaceae) is a remarkable therapeutic plant used for treatment of a wide assortment of diseases, including hemorrhoids, stomachache, toothache, gastritis and lymphadenitis (Mohan and Janardhanan 1993). Various pharmacological properties of *Entada phaseoloides* such as anti-inflammatory and analgesic (Kadam and Bodhankar 2014), anti-arthritic (Dawane *et al*. 2011), anti-diabetic and hypolipidemic (Zheng *et al.* 2012), anti-toxicity (Xiao *et al*. 2010), antiulcer (Ramakrishna *et al*. 2008), anti-complement and antimicrobial (Li *et* al. 2012), hepato-protective (Gupta *et al*. 2011), antioxidant and cytotoxic activities (Garcia *et al*. 1981) were reported by various workers.

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Phaseoloidin, a homogentisic acid glucoside from the seed kernel of *Entada phaseoloides* whose structure has been established as homogentisic acid 2-O-l-D-glucoside (Barua *et al*. 1988) is undertaken in the present study based on our previous findings of its crude methanol extract (Barua *et al*. 2018) in Morris water maze in mice with subsequent biochemical and molecular studies to corroborate the behavioral findings.

MATERIALS AND METHODS

Drugs

Scopolamine hydrobromide, Tacrine hydrochloride (9-Amino-1, 2, 3, 4 tetrahydro-acridine hydrochloride hydrate), Acetylthiocholine iodide, 5,5'-dithio-bis-nitrobenzoic acid (DTNB), Griess reagent and Tetramethylbenzidine (TMB) (Sigma-Aldrich,USA), Enzyme-linked immunosorbent assay (ELISA) Kit (RayBio®, USA), RIPA lysis buffer (Amresco, USA and Canada) were procured. All other chemicals and reagents used in the present work were of analytical grades.

Plant material

The seeds of *E. phaseoloides* were collected from local market during the month of April and May, 2016, India. It was authenticated by taxonomist Dr. Iswar Chandra Barua, Principal Scientist, Department of Agronomy, Assam Agricultural University, Jorhat, Assam; a voucher specimen (AAU-NW-EVM-3) was deposited and kept at the herbarium of the Department of Agronomy, Assam Agricultural University, Jorhat, Assam.

Extraction and isolation

The seeds of *E. phaseoloides* (1 kg) were shade dried, were powdered in a pulvarizer and subjected for extraction with methanol at room temperature for 48 h. The resulting solvent was evaporated to dryness under reduced pressure in a rotary evaporator to obtain 5 g of extract, which was subjected to column chromatography over silica gel (60-120 mesh, 150×15 cm) eluted successively with $CHCl_3$:MeOH (8:2), $CHCl_3$:MeOH (3:2), to give four fractions F_1-F_4. Further purification of F_3 fraction using column chromatography eluting with $CHCl_3$/MeOH (12:1) to give Phaseoloidin as white amorphous powder (15 mg), which was identified on the basis of its NMR and mass spectral data.

Experimental animals

Adult male Albino mice (20-30 g) obtained from the Department of Pharmacology and Toxicology, College of Veterinary Science, Assam Agricultural University, Guwahati, Assam, housed in polypropylene cages, with clean bedding materials, safe drinking water, 12 h light-dark cycle, fed standard laboratory food and water was given *ad libitum*. Experiments were performed (IAEC No.770/ac/CPCSEA/FVSc, AAU/IAEC/11-12/118) and effort was also made to minimize the suffering of the experimental animals throughout the study.

Acute toxicity studies

The acute toxicity studies of Phaseoloidin were performed according to the Organization of Economic Corporation Development (OECD) Guidelines No. 423 using albino mice of either sex (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. Any gross behavioural change, lacrimation, salivation, urination, defecation etc were observed after 1 h, 2 h, 4 h, 6 h and 24 h. The animals were kept under observation for next 14 d for mortality or gross abnormality with the given dose. Based on the acute toxicity study, 100 and 200 mg/kg oral doses were selected.

Study design

The mice were divided into 5 groups (n=6)-Group I: Normal control, only Saline (10 ml/kg body weight, p.o.), Group II: Negative control, with 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: Phaseoloidin 100 mg/kg, p.o. + scopolamine 0.4 mg/kg i.p., Group V: Phaseoloidin 200 mg/kg p. o.+ scopolamine 0.4 mg/kg i.p. Scopolamine was given as a single dose on the 6th day *i.e* the final day of the experiment for the treated animals, following 4 days training period. The duration of the experiment was six days. On the 7th day, the animals were sacrified.

Behavioral study by morris water maze (Morris 1984)

Morris Water Maze was used for behavioral study. 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, animal was positioned in each quadrant during each trial. Animals which failed to reach the platform in 20 s on the 4th 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 60s before the last behavioural test. Data were assessed through a video camera attached to a computerized tracking system (ANY-maze TM software Stoelting Co. Video tracking). Time spent in the target quadrant and escape latency were recorded during retention trials. Further the animals were sacrificed and hippocampus isolated for the biochemical, molecular work.

Estimation of acetylcholinesterase activity

The Acetylcholinesterase activity was measured by the method of Ellman, 1959. Change in absorbance per minute

of the sample was measured spectrophotometrically (MultiscanGo, Themo Fisher) at 420 nm.

Biochemical estimation

Biochemical tests were done 24 h after the last behavioral test. The total protein was estimated by the method of Bradford, 1976. The pro-oxidant markers *i.e*. lipid peroxidation (LPO) (Ohkawa *et al*. 1979) and nitric oxide (NO) (Miranda *et al*. 2001), antioxidant proteins i.e. reduced glutathione (GSH) (Ellman *et al*. 1961), superoxide dismutase (SOD) (Marklund and Marklund, 1974), catalase (CAT) (Sinha *et al*. 1972) 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 (Hoefer Midi Gel apparatus, Harvard Apparatus, Holliston, MA). 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 PCR (RT-PCR)

AChE (F5′-GATCCCTCGCTGAACTACACC-3′, R52-GGTT CTTCCAGTGCACCATGTAGGAG-3′) Nrf2 (F5′-CAGCAT GTTACGTGATGAGG-3′,R5′-GCTCAGAAAA GGCTC CATCC-3′), PP2A (F5′-ATGGACGAGAAG TTGTTCAC-3′, R5′ -GACCACCATGTAGACAGAAG-3′), Tau (F5′-AAGAC AGACCATGGAGCA-3′, R5′-CTCGG CTAACGTG GCAAG-3′) and HO-1 (F5′ -CACGCATATACC CGCTACCT-3′, R5′-CCAGAGTGTTCATTCGAG-3′) mRNA expressions were studied in the hippocampal tissue of mice brain by reverse transcriptase polymerase chain reaction. The PCR products were detected by electrophoresis on a 2% agarose gel containing ethidium bromide and the densities of each band were analyzed by an image analyzer (Image J).

Real time polymerase chain reaction (Q-PCR)

The mRNA expression levels of the genes encoding AChE, HO-1, PP2A, Tau and Nrf2 in the hippocampus were measured by Real time PCR (7500 Real Time PCR system, Applied Biosystems).

Statistical analysis

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). Results are expressed as mean±SEM and were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Phaseoloidin

NMR spectroscopy revealed the following spectra in Phaseoloidin as ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 9.00 (1H, s, 4-OH), 6.94 (1H, d, J = 8.6 Hz, H-3), 6.58 (2H, m, H-5, H-6), 4.99 (2H, brs, H-7), 4.53 (1H, d, J=7.4 Hz), 3.68 (dd, J=11.8, 1.9 Hz, 1H), 3.61 (1H,d, J =15.7) and 3.51(1H,d,J=15.7), 3.46 (dd, J=11.8, 5.6 Hz, 1H),3.20 (td, J=5.7, 3.2 Hz, 4H), 3.14 (1H,dd, J=7.4&8.5). ^{13}C-NMR(DMSO-d_6, 300 MHz) δ: 172.92 (C-8), 152.02 (C-4), 148.51(C-1), 125.88 (C-2), 117.45 (C-6), 117.03 (C-3), 113.97(C-5), 102.91 (C-1'), 76.90 (C-5'), 76.49 (C-2'), 73.38 (C-3'), 69.74 (C-4), 60.80 (C-6'), 34.93 (C-7). HRMS (ESI+) m/z: 329.0875 ([M-H]$^{-}$; $C_{14}H_{18}O_9$ calcd. 329.0873). It is shown in Fig 1.

Escape latency (Sec)

Escape latency is defined as the time of the animals to find the platform and escape the maze. In Morris Water Maze, a significant decline in the escape latency could be observed in phaseoloidin (200 mg/kg) treated groups, followed by Tacrine and Phaseoloidin (100 mg/kg treated animals when compared to scopolamine treated group (Fig 2a).

Time spent in target quadrant (Sec)

During the probe trial, vehicle treated animals spent an average time in the target quadrant (the quadrant where the platform was placed during acquisition trials). Scopolamine-treated animals spent lesser time in the target quadrant than the control group. Phaseoloidin (100 and 200 mg/kg) treated groups showed significant reversal of Scopolamine induced amnesia in the target quadrant indicating improvement of memory function (Fig 2b).

Phaseoloidin and tacrine treatment remarkably decreased the AChE level compared with Scopolamine-treated group (100 and 200 mg/kg, p.o.) (Fig 3). Scopolamine treatment increases acetyl cholinesterase activity in the brain affecting memory and our treatment could reverse scopolamine-induced increase in acetyl cholinesterase level in the brain. Antioxidant enzymes, GSH, SOD and Catalase, showed significant decline in Scopolamine treated group and the levels were elevated significantly in Phaseoloidin 200 mg/kg, p.o. treated group, presumably due to its antioxidant property (Fig 4). A complete reversal in LPO and NO levels, which were elevated following scopolamine administration, but in Tacrine and Phaseoloidin (100 and

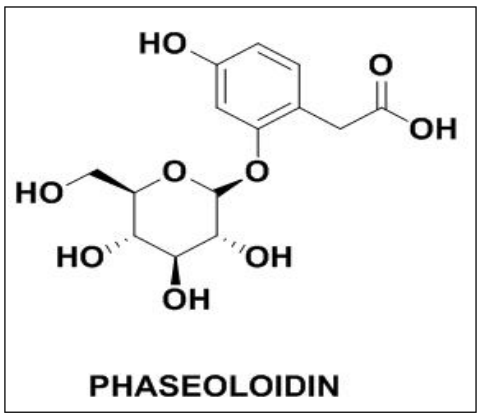

Fig 1: Structure of phaseoloidin.

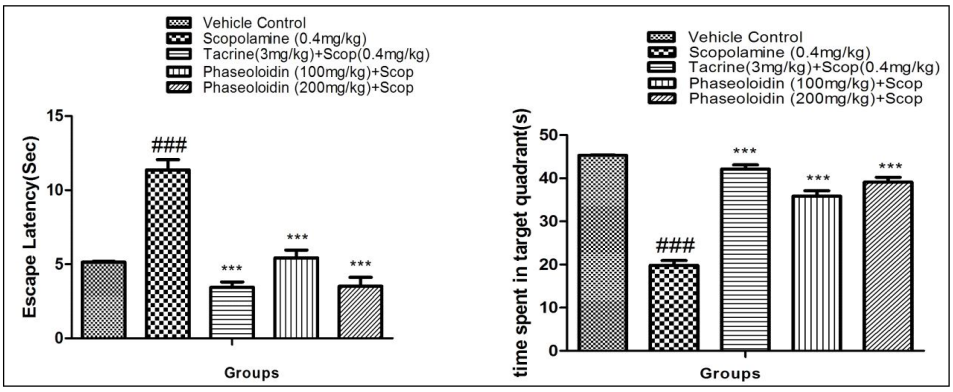

Fig 2: Effect of phaseoloidin on Morris water maze showing a) Escape latency (Sec) and b): Time spent in target quadrant (Sec). All values represent mean±standard error of mean (n=4). Statistical significance was determined by one way ANOVA followed by Tukey's post hoc test, ***p<0.001, **p<0.01, *p<0.05 when compared to Scopolamine group, ###p<0.001, ##p<0.01, #p<0.05 when compared to control group.

200 mg/kg) treated groups significant decline in their levels were observed. These results ascertained the antioxidant property of phaseoloidin.

The protein levels of TrkB and BDNF were down regulated and NFκB p65 and Caspase-3 were up regulated in the hippocampal tissue of scopolamine induced mice compared to control group was prominent. Conversely, pre-treatment with Phaseoloidin (200 mg/kg) up regulated TrkB, BDNF and down-regulated in NFκB p65 and Caspase-3 protein expression in the hippocampal tissues of mice analogous to Tacrine as compared to scopolamine treated mice (Fig 5).

The mRNA expression levels of AChE and Tau were up-regulated in the hippocampal tissues of scopolamine induced mice, but pre-treatment with Phaseoloidin significantly down regulated the mRNA expression of AChE and Tau expression compared to the scopolamine treated mice. On the other hand, mRNA expression levels of PP2A, HO-1 and Nrf2 were significantly down-regulated in the hippocampal tissues of scopolamine induced mice, respectively, compared to the vehicle control group. Conversely, pre-treatment with Phaseoloidin and tacrine significantly up regulated the mRNA expression of PP2A, HO-1 and Nrf2 expression, than scopolamine treated mice (Fig 6, 7).

Scopolamine alters the gene expression of various candidate molecules in mice hippocampus, which indicates association of cholinergic framework in long haul potential (Brouillete and Defranco 2007).

In the Morris water-maze test, Phaseoloidin (200 mg/kg) shortened the escape latency time from days 2nd to 4th. At the probe trial session, Phaseoloidin (200 mg/kg) increased the time spent within the target quadrant.

Phaseoloidin antagonizes acetylcholinesterase (AChE) activity, thus retaining the levels of ACh in the encoding of new memories. The boost in cholinergic activity and inhibition of AChE enzyme is a promising therapy to treat a cognitive defect in Alzheimer's disease (Sutar *et al*. 2014). Priyadarshini *et al.* (2022) reported that chronic exposure to EMR (Electromagnetic radiation) decreased cognitive

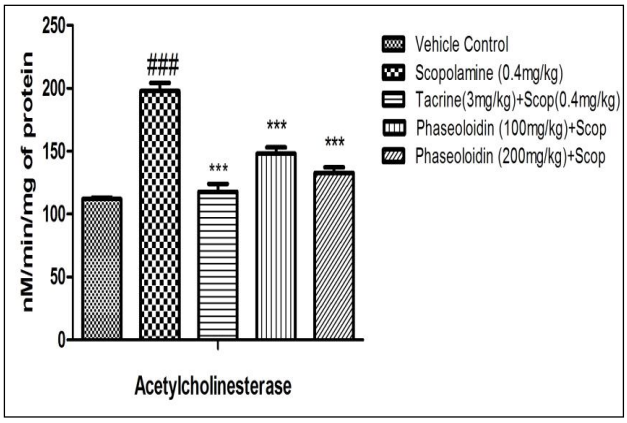

Fig 3: Effect of phaseoloidin on Morris water maze showing acetylcholinesterase activity (nM/min/mg of protein). All values represent mean ± standard error of mean (n=4). Statistical significance was determined by one way ANOVA followed by Tukey's post hoc test, ***p<0.001, **p<0.01, *p<0.05 when compared to Scopolamine group, ###p<0.001, ##p<0.01, #p<0.05 when compared to control group.

characteristics in rats as revealed by significant changes in their behaviour as well as neurotransmitters such as GABA, ACh and dopamine. Treatment with *Lorenthus longiferous* et hanolic extract and melatonin reversed these changes to near normal values indicating the efficacy of the plant extract in combating the neuronal changes in animals. A normal expression of tyrosine hydroxylase in *Lorenthus longiferous* ethanolic extract treated animals as compared to melatonin group was also recorded that further confirmed reversal of brain activity. Wang *et al.* (2018) explored the effect of *Ginkgo biloba* extract on the cognitive function and neurotransmitter levels in rats with vascular dementia (VD) and its mechanism of action. Morris water maze was used to evaluate the cognitive function of rats. After behavioral observation, these rats were sacrificed for detecting the level of acetylcholine (ACh), dopamine (DA) and

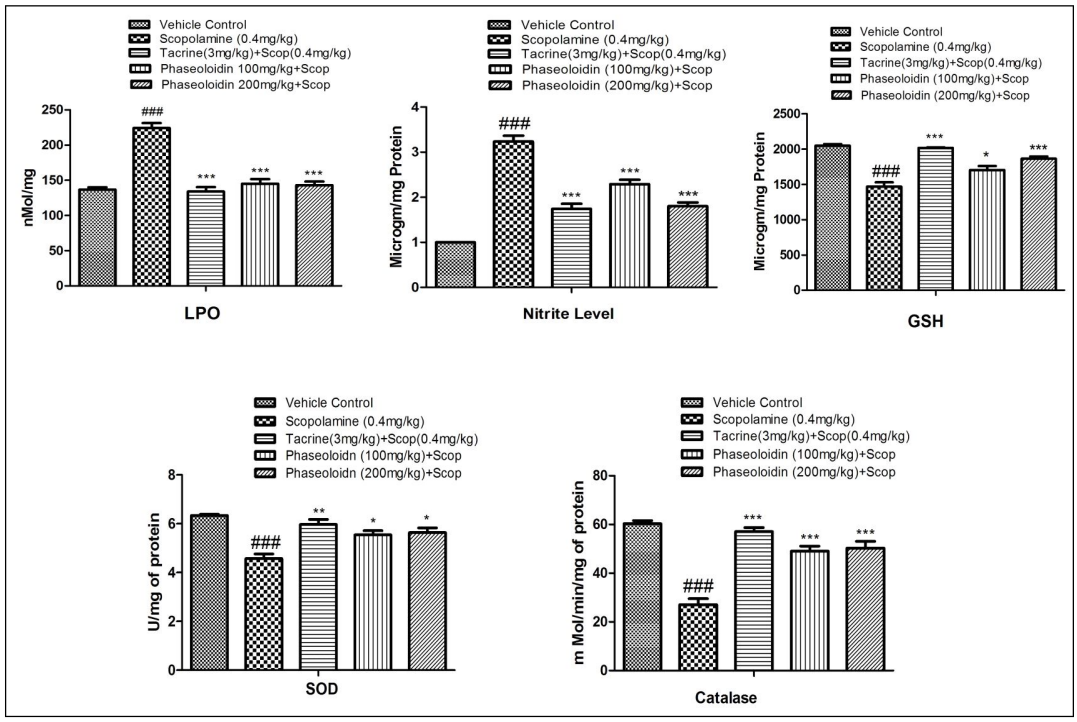

Fig 4: Effect of Phaseoloidin (100 and 200 mg/kg) pre-treatment on LPO, NO, GSH, SOD and catalase level in scopolamine induced mice. Values represent the Mean±SEM of six animals for each group. ###p<0.001, ##p<0.01, #p<0.05 compared with normal control. *p<0.05; **p<0.01; ***p<0.001 compared with scopolamine group.

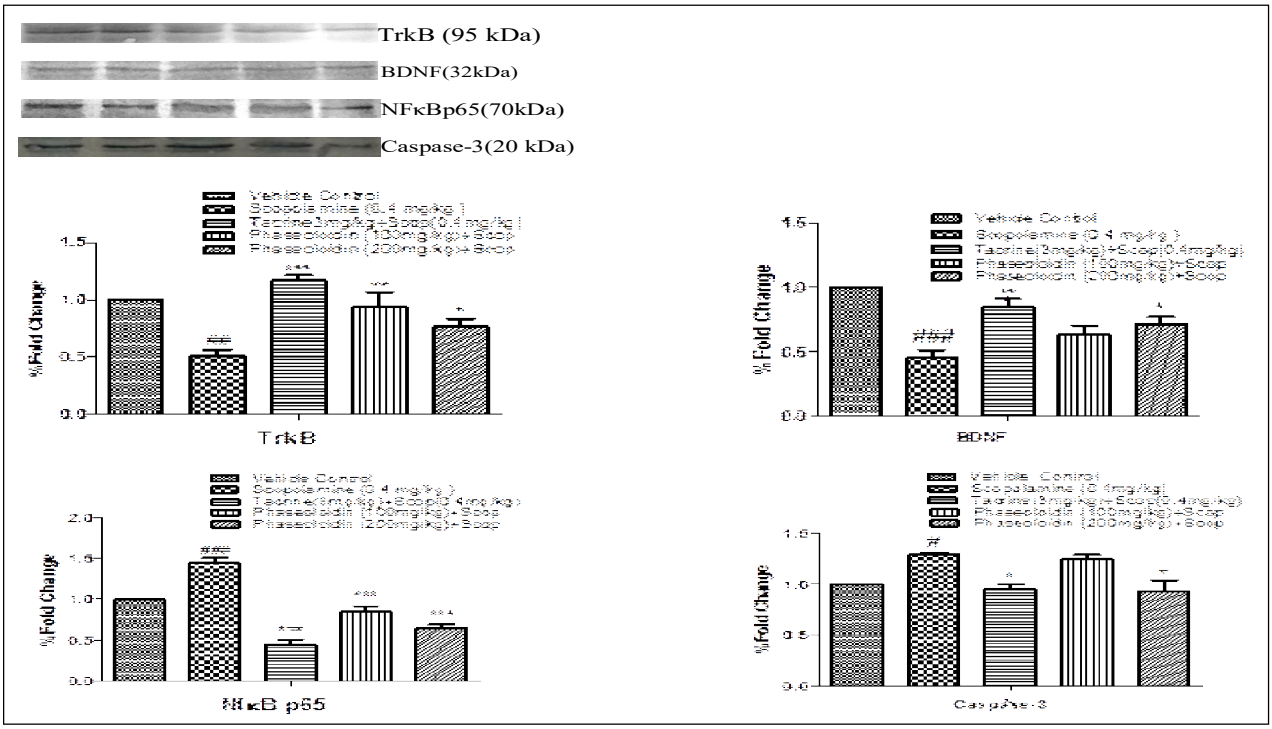

Fig 5: Quantitative expression of TrkB, BDNF, NFκB p65 and Caspase-3 genes by Western blotting in different treatment groups *viz*. Lane 1-control, Lane 2-Scopolamine, Lane 3-Tacrine, Lane 4-Phaseoloidin (100 mg), Lane 5-Phaseoloidin (200 mg) in mice hippocampus with Scopolamine induced memory loss. Values are expressed as per cent fold change represented as mean±SE (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test; ***p<0.001, **p<0.01, *p<0.05 when compared to Scopolamine group, ###p<0.001, ##p<0.01, #p<0.05 when compared to control group.

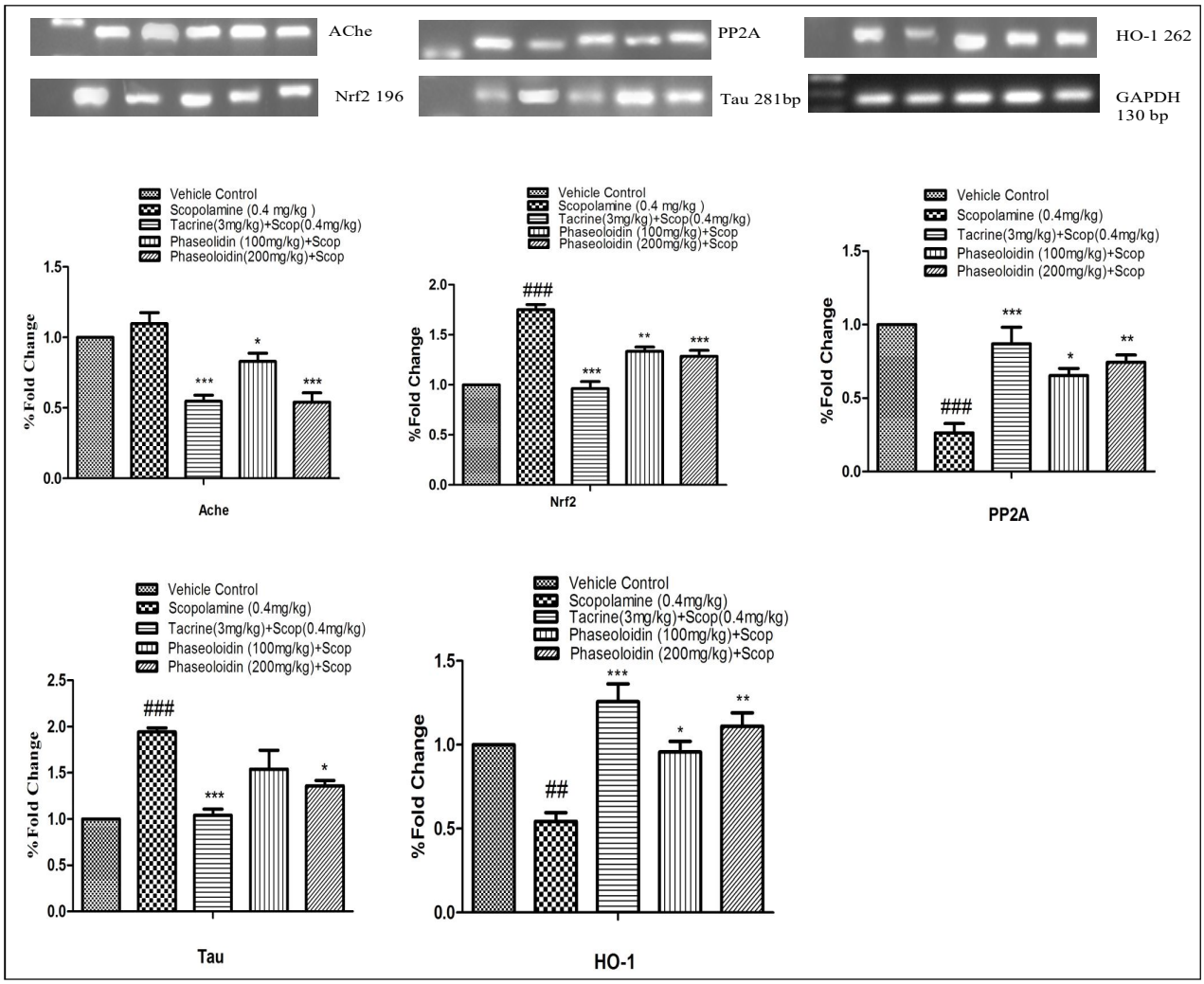

Fig 6: Quantitative expression of AChE, Nrf2, PP2A, Tau and HO-1 genes by RT-PCR in different treatment groups *viz*. Lane 1- DNA Ladder, Lane 2- Vehicle control, Lane 3-Scopolamine, Lane 4-Tacrine, Lane 5-Phaseoloidin (100 mg), Lane 6-Phaseoloidin (200 mg) in mice hippocampus with Scopolamine induced memory loss. Values are expressed as per cent fold change represented as Mean±SE (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey post hoc test. ***p<0.001, **p<0.01, *p<0.05 when compared to Scopolamine group, ###p<0.001, ##p<0.01, #p<0.05 when compared to control group.

5-hydroxytryptamine (5-HT) in brain tissue. *Ginkgo biloba* extract could considerably improve the cognitive function of rats with vascular dementia, the mechanism may be putatively associated with the extract to elevate the levels of ACh, 5-HT and DA and inhibit the activity of AChE.

The brain is susceptible to oxidative stress because it consumes huge amounts of oxygen, has abundant lipid content and a low antioxidant level compared than other organs. Besides, it is well known that the hippocampus located within the brain is vital for learning and memory and the arrangement of spatial memory (Huang *et al*. 2015).

BDNF, is an indicative biomarker in patients with early Alzheimer's illness and gentle cognitive impairment (O'Bryant *et al*. 2009). We have observed that hippocampal BDNF and TrKB were recognizably diminished due to scopolamine infusion and pretreatment with Phaseoloidin just reversed it favorably.

Reactive oxygen species under oxidative stress may start and exaggerate the inflammatory reaction due to their capability to invigorate and direct the inflammatory-signaling cascades qualities like NF-κB p65 (Rosales Coral *et al*. 2010). Pretreatment with Phaseoloidin led to a critical reduction within the NF-κB p65 movement. Apoptosis in excess is related to cellular degeneration by oxidative push, habitually related with maturing and pathogenesis of neurodegenerative conditions (Chandra *et al*. 2000). Preventive treatment with phaseoloidin led to diminished oxidative stress resulting in diminished pro-apoptotic Caspase-3 qualities demonstrating neuro-protection.

During oxidative stress, endogenous PP2A is explicitly and reversibly restrained. The balance between the phosphorylation and dephosphorylation of Tau is controlled by numerous sorts of proteinases, for example, PP2A (Levinthal and Defranco 2005). In our study, scopolamine treated mice confirmed significantly decreased PP2A activity/mRNA expression and up-regulated Tau mRNA expression in hippocampus region. Pretreatment with Phaseoloidin restored PP2A activity/mRNA expression and in the end reduced Tau hyper phosphorylation which could prevent neurofibrillary tangle formation and deposition.

During oxidative stress, Nrf2 translocates into the core to initiate the expression of HO-1 (Johnson *et al*. 2008)which plays an essential role in keeping up cell redox homeostasis against responsive oxygen species (ROS) age and oxidative stress (Min *et al*. 2011). Pretreatment with Phaseoloidin prompted significant augmentation in the Nrf2 level alongside HO-1 gene expression.

Hence, these findings suggest that the pretreatment with Phaseoloidin may prevent retardation of memory and learning in scopolamine induced amnesia model via multiple mechanisms including reversing the oxidative stress, decreasing the AChE level, increasing BDNF and TrkB levels in hippocampus in treated mice, which may possibly be the apparent mode of actions for its beneficial effect.

CONCLUSION

From the above behavioral, biochemical and molecular data, it can be concluded that Phaseoloidin has an ability to improve or enhance spatial long-term memory and short-term memory. Thus, it can be exploited as a useful natural agent for cure and/or prevention for amnesia and neurodegenerative disorders associated with learning, memory and cognitive dysfunctions in animals.

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Conflict of interest: None.

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