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Neuroprotective Activity of *Asparagus racemosus* Linn. Against Ethanol-Induced Cognitive Impairment and Oxidative Stress in Rats Brain: Auspicious for Controlling the Risk of Alzheimer's Disease

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Abstract

Background: Medicinal plants are superior gift of nature to human lives to support disease free healthy life. Neurodegenerative diseases especially Alzheimer's disease (AD) affects the central nervous system causing progressive degeneration of neurons, which affect cognitive function. The plant *Asparagus racemosus* (AR) Linn. has been used traditionally by Ayurvedic practitioners for nervous disorders. In this consequence, the intention of this study was to evaluate the neuroprotective effects of ethanolic extract of *Asparagus racemosus* (EEAR) Linn. roots in ethanol-induced cognitive impairment and oxidative stress in rats brain.

Methods: The learning and memory enhancing activity of EEAR roots extract were investigated in Swiss albino male rats for 21 days and its effects on learning and memory were examined using various behavioral studies such as elevated plus maze (EPM) test, passive avoidance (PA) test, morris water maze (MWM) test, novel object recognition (NOR) test and biochemical studies such as lipid peroxidation (TBARS) contents and acetylcholinesterase (AChE) activity.

Results: In the EPM test, administration of EEAR (i.e., 100 and 200 mg/kg b.w.) significantly ($P<0.05$, $P<0.01$) decreased retention transfer latency (RTL) on 21st day with respect to the disease control group. EEAR at 200 mg/kg b.w. markedly ($P<0.05$, $P<0.01$) increased the retention latency (RL) on 11th and 21st day compared to disease control group for PA test. In the NOR test administration of EEAR (i.e., 200 mg/kg b.w.) considerably ($P<0.05$) increased DI of rats on 21st day with respect to disease control group. Both doses of EEAR (i.e., 100 and 200 mg/kg b.w.) markedly ($P<0.05$, $P<0.01$) decreased escape latency (EL) and highest dose of EEAR (i.e., 200 mg/kg b.w.) noticeably ($P<0.05$, $P<0.001$) increased time spent in the target quadrant (TSTQ) on successive days for acquisition trial of MWM test. In case of probe trial administration of EEAR (i.e., 200 mg/kg b.w.) considerably ($P<0.05$, $P<0.01$) increased TSTQ and TSA (time spent in the annuli) of rats as compared to that of disease control group. EEAR administration (i.e., 100 and 200 mg/kg b.w.) significantly ($P<0.01$) decreased the TBARS level in the brain tissue of rats with respect to disease control group. The lowest and highest dose of EEAR (i.e., 100 and 200 mg/kg b.w.) significantly ($P<0.05$, $P<0.01$) decreases the AChE activity in the brain tissue of rats as compared to disease control group.

Conclusion: The existing study displays that EEAR roots possesses an outstanding source for natural nootropic and confirming the traditional uses of this plant which could be industrialized for enhancing learning and memory impairment associated with neurodegenerative disorders particularly AD.

Keywords: Neuroprotective; *Asparagus racemosus*; Cognitive impairment; Oxidative stress; Alzheimer's disease

Abbreviations: AD: Alzheimer's disease; AR: *Asparagus racemosus*; EEAR: Ethanolic extract of *Asparagus racemosus*; EPM: Elevated plus maze; PA: Passive avoidance; MWM: Morris water maze; NOR: Novel object recognition;TBARS: Thiobarbituric acid reactive species (TBARS); AChE: Acetylcholinesterase; ITL: Initial transfer latency; RTL: Retention transfer latency; EL: Escape latency; RL: Retention latency; TSTQ: Time spent in the target quadrant; TSA: Time spent in the annuli; DI: Discrimination index; PUFA: Polyunsaturated fatty acids; CNS: Central nervous system; Aβ: amyloid beta; WHO: World Health Organization; TCA: Trichloroacetic acid; TBA: Thiobarbituric acid; ATCI: Acetyl thiocholine iodide; DTNB: 5,5-dithiobis-2-nitrobenzoate ion; Tris-HCl: Trisfamino methane hydrochloride; BSA: Bovine serum albumin; NIH: National Institutes of Health; OECD: Organisation for Economic Cooperation and Development .

Introduction

Alzheimer's disease (AD) is the most common form of dementia that results in memory impairment and cognitive dysfunction due to progressive neurodegeneration [1]. Worldwide at present 35 million people are affected by AD, including 5.5 million Americans and it is projected that in 2050 more than 115 million people will have dementia [2,3]. AD is currently ranked as the 6th leading cause of death in the United States, but recent estimates indicate that the disorder may rank 3rd, just behind heart disease and cancer, as a cause of death for older people [4]. This disease is characterized by deposition of amyloid β (Aβ)

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plaques, development of neurofibrillary tangles (NFTs), inflammation and neuronal loss in specific regions of the forebrain that slowly destroys memory and thinking skills and eventually the ability to carry out the simplest tasks. The most notable symptom of AD is progressive neurodegenerative disease in which dementia gradually worsen over time followed by an overall cognitive decline [5]. Alzheimer's is not a normal part of aging, although the greatest known risk factor is increasing age and the majority of people with Alzheimer's are 65 years and older [6]. But Alzheimer's is not just a disease of old age. Up to 5% of people with the disease have early onset Alzheimer's also known as younger-onset, which often appears when someone is in their 40s or 50s [7].

Ethanol is neurotoxic that able to alter behavioral and cognitive performance in experimental animals in addition to humans. It mainly impairs hippocampal-dependent learning and memory functions [8]. The mechanism of ethanol-induced neurotoxicity is not well understood. Several studies show that free-radical mediated oxidative stress play an imperative role [9]. The brain is extremely susceptible to oxidative stress due to high level of polyunsaturated fatty acids (PUFAs) and catecholamines, large amounts of oxygen (O_2) in relatively small mass and in conjunction with low antioxidant activities. Furthermore, certain regions of the central nervous system (CNS), especially hippocampus and cerebellum, may be more sensitive to oxidative stress because of their low endogenous antioxidant, in relation to other brain regions [10]. Study showed that acetaldehyde dehydrogenase is responsible for the generation of reactive oxygen species (ROS) by converting cytotoxic acetaldehyde produced from oxidation of ethanol to acetate. It has been confirmed that ethanol induces the synthesis of CYP2E1 that lead to oxidative stress. It also increases the ratio of NADH/NAD, responsible for reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which causes lipid peroxidation by generating hydroxyl radical [11].

Increasing evidence highlights the role played by oxidative stress in AD. It has been shown that free radical and oxidative stress induce memory deficits and enhance behavioral impairments in AD patients [12]. Several studies have suggested that Aβ induced the oxidative stress observed in neurodegenerative AD brain [13]. Alzheimer's has no current cure. Although current Alzheimer's treatments cannot stop Alzheimer's from progressing, they can temporarily slow the worsening of dementia symptoms and improve quality of life for patients with Alzheimer's [14]. Currently, the treatment for AD is acetylcholinesterase (AChE) inhibitors these are donepezil, galantamine, rivastigmine and tacrine. However, the resulting adverse effects associated with nootropic agents, such as piracetam, pramiracetam, aniracetam and donepezil have restricted their use for improving memory, mood and behavior [15]. The connection of oxidative stress within the forebrain cholinergic system has been recommended by several studies [16]. The drugs with antioxidant effects might be beneficial for preserving brain function. Today, there is a worldwide effort under way to find better ways to treat the disease, delay its onset and prevent it from developing [17].

Nature is the best combinatorial chemist and possibly has used to treat almost any medical problem of mankind. It is estimated that about 75% of useful bioactive plant derive pharmaceuticals used globally are discovered by systemic investigation of leads from traditional medicines [18]. The World Health Organization (WHO) estimates that 80% of the population of some Asian and African countries presently uses herbal medicine for some aspect of primary health care [19]. Complementary and alternative medicine, especially Ayurvedic, Unani and Siddha provide health care facility of more than 70% of people exist in the rural areas [20]. Studies in the United States and Europe have shown that their use is less common in clinical settings, but has become increasingly more common in recent years as scientific evidence about the effectiveness of herbal medicine has become more widely available [21]. Herbal medicine treatment for AD becomes very popular nowadays because of their activities against AD and slowing down the progression of it. Many herbal medicines have been researched and the benefits derived from using these medicines for AD and dementias are promising. Also, these herbs are inexpensive and can be easily available. The results observed for the treatment with herbal medicines are promising also with fewer adverse effects [22]. A variety of medicinal plants such as *Ginkgo biloba, Panax ginseng, Withania somnifera, Huperzia serrata, Rhodiola rosea, Vinca minor, Bacopa monnieri, Centella asiatica, Eruca sativa, Marsilea quadrifolia, Terminalia chebula, Aloe vera, Alpinia galanga, Andrographis paniculata, Arnica montana* and *Bupleurum falcatum* have been reported to exhibit anti-Alzheimer activity [23].

The plant *Asparagus racemosus* (AR) Linn. is known in Bengali as Shatamull belongs to Asparagaceae family [23]. This plant is common throughout Nepal, Sri Lanka, India and also widely distributed throughout the villages of the Bangladesh [24]. The plant is a spinous under-shrub, with tuberous, short rootstock bearing numerous succulent tuberous roots (30-100 cm long and 1-2 cm thick) that are silvery white or ash colored externally and white internally [25]. It has folk usage as a treatment for dyspepsia, gastric ulcers, constipating, galactogogue, aphrodisiac, diuretic, rejuvenating, carminative, immunostimulant, gastroprotective, nerve tonic and antiseptic effects [26]. The plant has phytochemical constituents such as alkaloids, triterpenes, saponins, glycosides, diosgenin, cytoesterol, stigmaesterol, isoflavonoids etc [27]. The important pharmacological actions of this plant are adaptogenic, hypolipidemic, immunomodulatory, antibacterial, antidepressant, antiulcerant, antidiabetic, antioxidant, cardio protective and memory enhancing activity [28].

A previous preliminary study has shown that the roots extract of this plant has memory enhancing activity in mice [29]. Therefore, the purpose of this study was to investigate the neuroprotective effect of ethanolic extract of *Asparagus racemosus* (EEAR) Linn. roots in ethanol-induced rats of cognitive impairment and oxidative stress by behavioral studies such as elevated plus maze (EPM) test, passive avoidance (PA) test, novel object recognition (NOR) test, morris water maze (MWM) test as well as the activity of antioxidant enzymes by biochemical studies such as estimation of lipid peroxidation (TBARS) and acetylcholinesterase (AChE) activity in rat brain tissue homogenates.

Materials and Methods

Chemicals and Drugs

Ethanol (98%); trichloroacetic acid (TCA); thiobarbituric acid (TBA); acetyl thiocholine iodide (ATCI); 5,5-dithiobis-2-nitrobenzoate ion (DTNB); trisfamino methane hydrochloride (Tris-HCl) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, USA. Unless otherwise stated, all other chemicals were of analytical grade and purchased from native sources. Donepezil hydrochloride powder was achieved from Incepta Pharmaceuticals Ltd. Dhaka, Bangladesh as gift.

Collection and Identification of Plant Materials

The fresh roots of AR were collected from Kurigram Sadar, Kurigram, Bangladesh, in the month of February, 2015. After collection

the roots were identified by expert of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. Accession number: DACB-39527 for AR.

Drying and Grinding of Plant Materials

To remove adhering dirty materials from the collected roots of the plant these were washed properly. Then these were shade dried for 7 days with irregular sun drying. For facilitating grinding after that root were dried in an oven for 24 hrs at 50°C. The roots were crushed into the coarse powder with the help of a suitable grinder and stored in an airtight clean glass container for extraction.

Extraction of Plant Materials

For extraction about 500 g powdered plant materials were taken in an amber colored glass bottle and subjected to extraction by 2 liter of 98% ethanol for 7 days with occasional shaking. After that, the whole mixture was filtered through cotton and finally by Whatman No.1 filter paper. The collected filtrate after filtration was concentrated with a rotary evaporator under reduced pressure at 50°C temperature. The sticky concentrate was designated as crude extract (8.65 g) of ethanol and stored at 4°C for further tests.

Experimental Animals

Fifty six healthy Swiss albino male rats weighing between 170-220 g were obtained from the animal resources outlet of ICDDR,B, Dhaka, Bangladesh. The obtained rats were housed 3 per cage and placed under standard environmental condition with a 12:12 hrs light-dark cycle. The rats were timely fed with standard laboratory food and water. As per the guide for laboratory animals of the National Institutes of Health (NIH) the care and usage of entire rats were completed [30]. The protocol of the study was approved by the animal ethics committee of the Department of Pharmacy, Southeast University, Dhaka, Bangladesh.

Administration of Drugs and Test Compounds

The solution of the standard drug donepezil hydrochloride was prepared by using normal saline (pH 7.4) and ingested orally to rats at 2.5 mg/kg body weight (b.w.). Ethanol was prepared as a 20% solution in sterile normal saline and treated subcutaneously at a dose of 4.5 g/kg b.w. Normal saline (pH 7.4) was used to prepare the suspension of the EEAR and administered orally at 100 and 200 mg/kg b.w. to rats. According to the result of the literature searches the duration of the study and the doses of the donepezil hydrochloride, ethanol and EEAR were adjusted [31,32,33]. The standard drug, ethanol and the suspension of the extract were prepared newly everyday and ingested 30 min before the experiment.

Experimental Design

Rats were divided randomly into eight groups with 6 rats in each as follows:

Group 1: In case of this group standard laboratory food and water were administered for 21 days to rats (Con)

Group 2: In case of this group donepezil hydrochloride at a dose of 2.5 mg/kg b.w. was administered orally for 21 days to rats (Don)

Group 3: In case of this group ethanol at a dose of 4.5 g/kg b.w. was administered subcutaneously for 21 days to rats (Eth)

Group 4: In case of this group plant extract at a dose of 100 mg/kg b.w. was administered orally for 21 days to rats (EEAR 100)

Group 5: In case of this group plant extract at a dose of 200 mg/kg bw was administered orally for 21 days to rats (EEAR 200)

Group 6: In case of this group ethanol at a dose of 4.5 g/kg b.w and plant extract at a dose of 100 mg/kg b.w. were administered subcutaneously and orally respectively for 21 days to rats (Eth + EEAR 100)

Group 7: In case of this group ethanol at a dose of 4.5 g/kg b.w. and plant extract at a dose of 200 mg/kg b.w. were administered subcutaneously and orally respectively for 21 days to rats (Eth + EEAR 200)

Group 8: In case of this group only ethanol at a dose of 4.5 g/kg b.w. and donepezil hydrochloride at a dose of 2.5 mg/kg b.w. were administered subcutaneously and orally respectively for 21 days to rats (Eth+ Don)

Acute Toxicity Study

The acute toxicity of EEAR roots was determined by using 24 Swiss albino male rats were divided into 4 groups, with 6 rats per groups. The suspension of the extracts were prepared by using normal saline and administered to rats only once at a dose of 5, 50, 300 and 2000 mg/kg b.w. The rats were observed for 24 hrs for signs of toxicity and 14 days for mortality. This test was performed as per the guidelines of the Organisation for Economic Cooperation and Development (OECD) [34].

Behavioral Study

Before starting the behavioral studies 1 week training was conducted. Only food and water was administered during this period. All the experiments were carried out between 10.00 am and 3.00 pm.

Elevated Plus Maze (EPM) Test

The apparatus of EPM test was consisted of two open arms (length 500 mm × width 100 mm) and two close arms (length 500 mm × width 100 mm × height of side walls 400 mm), with an open roof used for the measurement of spatial long-term memory of rats [35]. The arms were connected by a central square in the middle of the maze. The maze was elevated to a height of 500 mm from the floor [36]. At the start of the trial each rat was placed at the end of an open arm and the time it took to move from the end of open arm to either of the closed arms was recorded using a stopwatch as initial transfer latency (ITL). If the rat did not enter into one of the closed arm within 300 sec, was eliminated from the experiments. After that rat was allowed to explore the apparatus for 30 sec and returned to its home cage. Retention transfer latency (RTL) of this learned task (memory) was examined 24 hrs after the first day trial [37]. The apparatus was cleaned after each test with 70% ethanol to remove any olfactory clue [38].

Passive Avoidance (PA) Test

The apparatus of PA test was consisted of light compartment (depth 270 mm × width 370 mm × height 360 mm) and dark compartment (depth 270 mm × width 370 mm × height 360 mm) used for the measurement of emotional memory of rats [39,40]. The two compartments of this apparatus were connected by a sliding door having 90 mm of diameter in the middle part. The floor of this apparatus was consisted of a metal grid with spaced 0.9 cm apart and connected to a shock generator, able to generate shock in the range of 0.5 mA [40,41]. Each test consists of two distinct trials such as acquisition trial and retention trial. For the acquisition trial, each rat was placed in the light compartment and when the rat entered into the dark compartment, an electrical foot shock of 0.5 mA was administered for 3 sec. By the help of stopwatch, the latency times once the rat had entered the dark

compartment was recorded as escape latency (EL). After that the rat was returned to its home cage. A retention trial was performed after 24 hrs of the acquisition trial, in which no shock was given when the rat entered the dark compartment and latency times to re-enter the dark chamber was measured as retention latency (RL) up to 300 sec [42]. The apparatus was cleaned after each test with 70% ethanol to remove any olfactory clue [38].

Novel Object Recognition (NOR) Test

The apparatus of NOR test was consisted of a box shaped (depth 70 cm × width 50 cm × height 40 cm) used for the measurement of the object recognition memory of rats [43]. Each test consists of two distinct trials such as training session and test session. In the beginning of the trial two identical objects were introduced into the apparatus. As object metal triangular (8.5 × 5 × 14 cm) and rectangular prisms (5 × 5 × 14 cm) were used. In the training sessions, a rat was placed in the apparatus and allows exploring the objects for a period of ≥ 10 sec. Object exploration was determined when the rat sniffed and or touched the object < 2 cm from its nose. Later the rat was returned to its home cage. After 24 hrs a test session was performed and before the test session one of the identical objects was replaced randomly by a novel object. In the test session each rat was allowed to explore the novel as well as familiar object for 4 min. Based on the investigation times of novel and familiar object discrimination index (DI) was determined [44]. The apparatus was cleaned after each test with 70% ethanol to remove any olfactory clue [38].

Morris Water Maze (MWM) Test

The apparatus of MWM test was consisted of tank shaped (diameter 1700 mm × depth 450 mm) used for the measurement of the spatial learning and memory of rats [40,45]. The water maze was divided into four quadrants in which a submerged platform (diameter 110 mm × height 250 mm) was placed in one compartment. During testing, the tank was filled to a depth of 260 mm with opaque water so that the platform was situated approximately 1 cm below the water surface and also hidden [46]. In acquisition trial, a rat was placed in the middle of a compartment of the tank and the time it took to mounted onto the submerged platform was recorded using a stopwatch as escape latency (EL). If the rat failed to find the platform within 300 sec, it was directed toward the platform. In both of this case a rat was allowed to stay on the platform for 30 sec [47]. Each day each rat was subjected to four consecutive trials remaining fixed the platform (East) with a different starting point, in random order as specified in (Table 1). The time spent in the target quadrant (TSTQ) was also recorded using a stopwatch. After the end of the fourth trial, the rat was gently dried with a soft cloth and kept warm under a bulb within the home case [48].

After the fourth trial of the last day, a probe trial was performed in which the platform was removed and the time the rat spent in the target quadrant (TSTQ) and time spent in the annuli (TSA) were measured for 60 sec. In the probe trial, all rats started from the same start position [49].

Table 1: The sequence of trials during the study period of WMM test.

1th Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
Q1	Q2	Q3	Q4	Q1	Q2	Q3
Q2	Q3	Q4	Q1	Q2	Q3	Q4
Q3	Q4	Q1	Q2	Q3	Q4	Q1
Q4	Q1	Q2	Q3	Q4	Q1	Q2

Biochemical Study

The rats were sacrificed on 22nd day by using light anesthesia and whole brain was collected. From the whole brain the cerebellum and brainstem were removed and the remaining brain (i.e., portion without cerebellum and brainstem) was washed with ice cold sterile NaCl solution (0.9%). Then sodium phosphate buffer (30 mM, pH 7.4) was used to prepare 10% brain homogenate in a homogenizer. Finally the homogenate was subjected to centrifugation at 20,000 RPM for 2 hrs at 4°C to eliminate cellular debris followed by collection of supernatant for further studies. The protein concentration was measured using bovine serum albumin [50].

Lipid Peroxidation (TBARS) Activity

The oxidative damage of lipid was estimated by the method of Iqbal *et al.*, to measure the formation of thiobarbituric acid reactive species (TBARS) [51]. The total volume of the reaction mixture of this test was 1.0 ml includes 0.58 ml of 0.1 M phosphate buffer having pH 7.4, 0.2 ml of 100 mM ascorbic acid, 0.02 ml of 100 mM ferric chloride and 0.2 ml of 10% brain homogenate incubated at 37°C in a shaking water bath for 1 hrs. To stop the reaction 1.0 ml of 10% TCA was added after that 1.0 ml 0.67% TBA was added, followed by boiling in a water-bath for 20 min. Then the test tubes were placing in a ice-bath followed by centrifuging at 2500 × g for 10 min. The amount of TBARS was measured at 535 nm by the help of spectrophotometer. The results of this test were expressed as nM TBARS/min/mg protein at 37°C by means of a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Acetylcholinesterase (AChE) activity

The cholinergic marker, acetylcholinesterase was estimated by the method of Ellman *et al.*, [52]. In this test, 25 μl of 15 mM ATCI, 75 μl of 3 mM DTNB and 75 μl of 50 mM Tris–HCl having pH 8.0, containing 0.1% BSA were added in the 96 well plates. After that the absorbance of this reaction mixture was measured at 405 nm by the help of spectrophotometer. Then to the aforementioned reaction mixture 25 μl of 10% brain homogenates was added followed by incubation for 5 min at 25°C. After that again absorbance was measured at 405 nm by the help of spectrophotometer. The results of this test were expressed as M/min/g protein.

Statistical Analysis

Results were expressed as mean ± SEM and analyzed using one-way analysis of variance (ANOVA). For behavioral studies Tukey's post hoc test were carried out and in case of biochemical studies the least significant difference (LSD) was determined using post hoc testing for inter group comparisons at a probability level of 0.05% and 0.01%. Microsoft Excel 2010 (Roselle, IL, USA) was used for the statistical and graphical evaluations. A probability of P<0.05 was considered as significant with respect to disease control group.

Results

Determination of Acute Toxicity

The doses of EEAR did not exert any lethality consequently there were no behavioral, neurological or physical changes. In addition, to this body weight, skin, fur and eyes of the rats remained unchanged and no mortality was observed at the test doses. Thus, the doses of this study were within safe margin.

Neuroprotective Effect of EEAR on ITL and RTL of Rats using EPM test

In EPM test the ITL was measured on 10th and 20th days and RTL

was measured on 11th (after 24 hrs of ITL) and 21st day (after 24 hrs of ITL) respectively presented in Figure 1. Donepezil significantly (P<0.05, P<0.01) reduced RTL of rats on 11th and 21st day as compared to disease control group on 10th and 20th day ITR. Ethanol-treated group exhibited increased in RTL with respect to other groups on successive days. Administration of EEAR resulted in decrease in RTL on 11th and 21st day when compared to disease control rats. However, administration of 100 and 200 mg/kg b.w. EEAR markedly (P<0.05, P<0.01) reversed ethanol-induced memory deficits, indicating considerable improvement of learning and memory of rats.
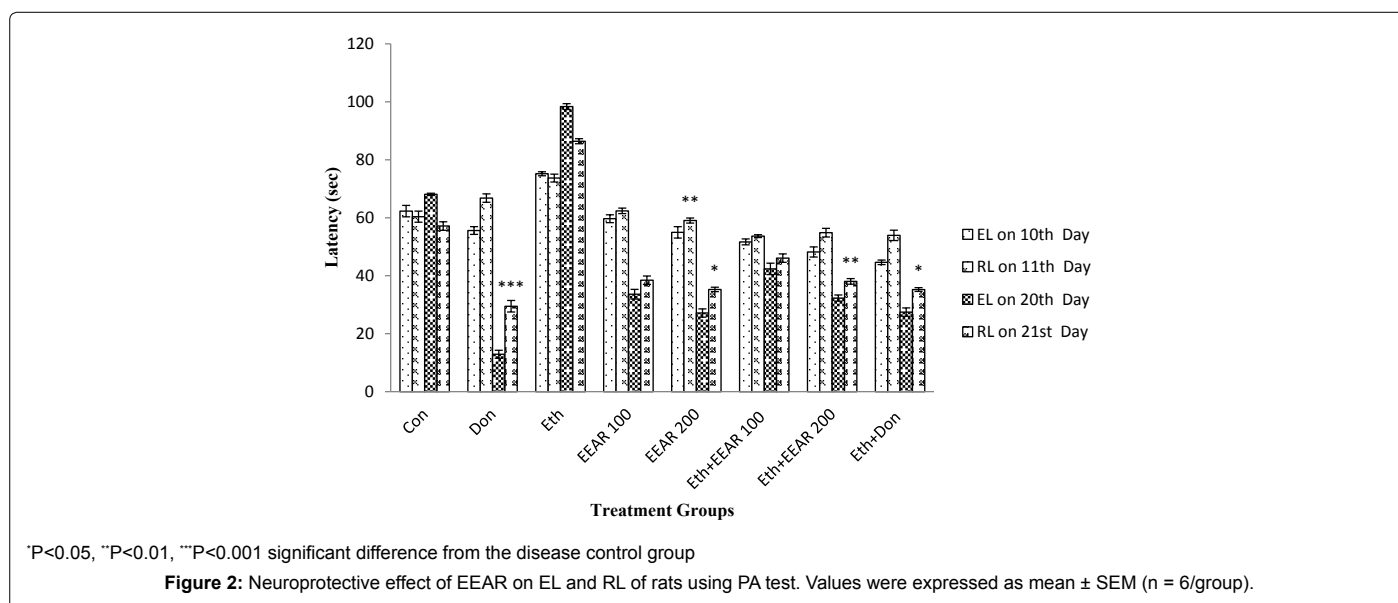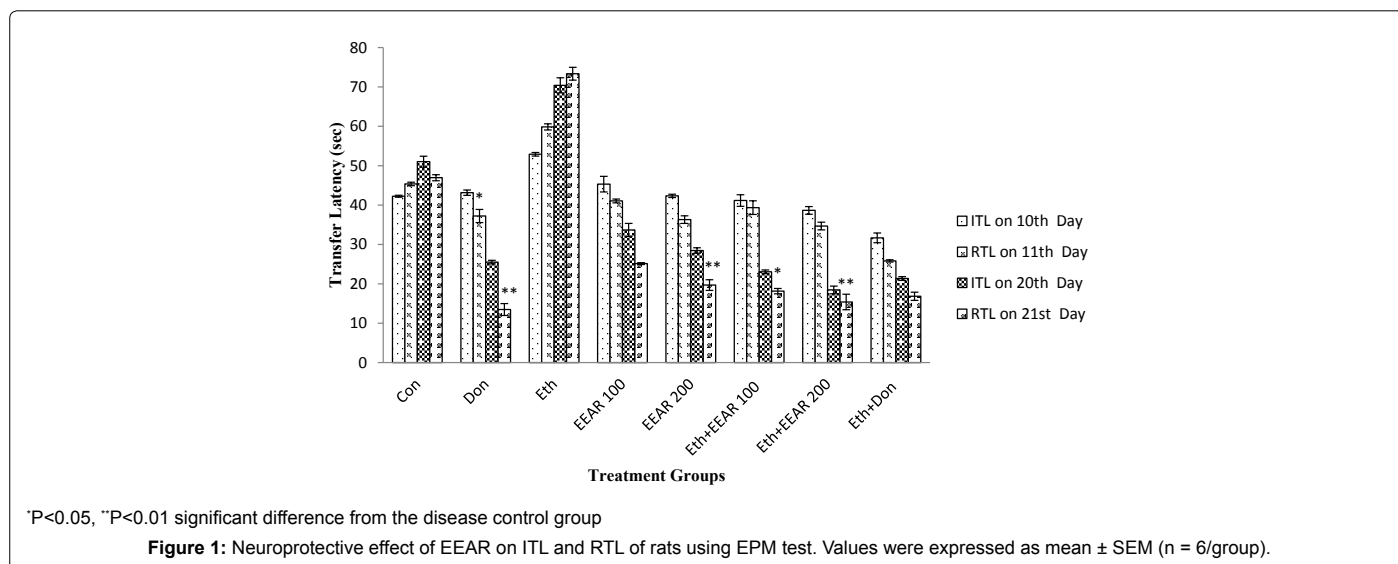
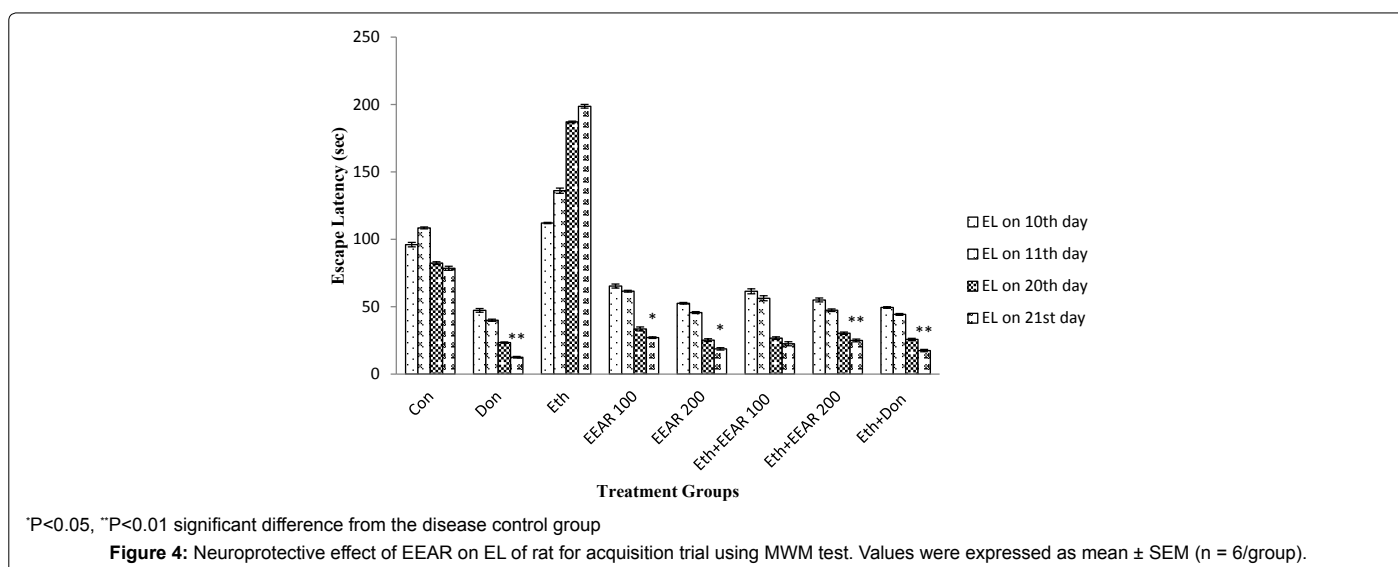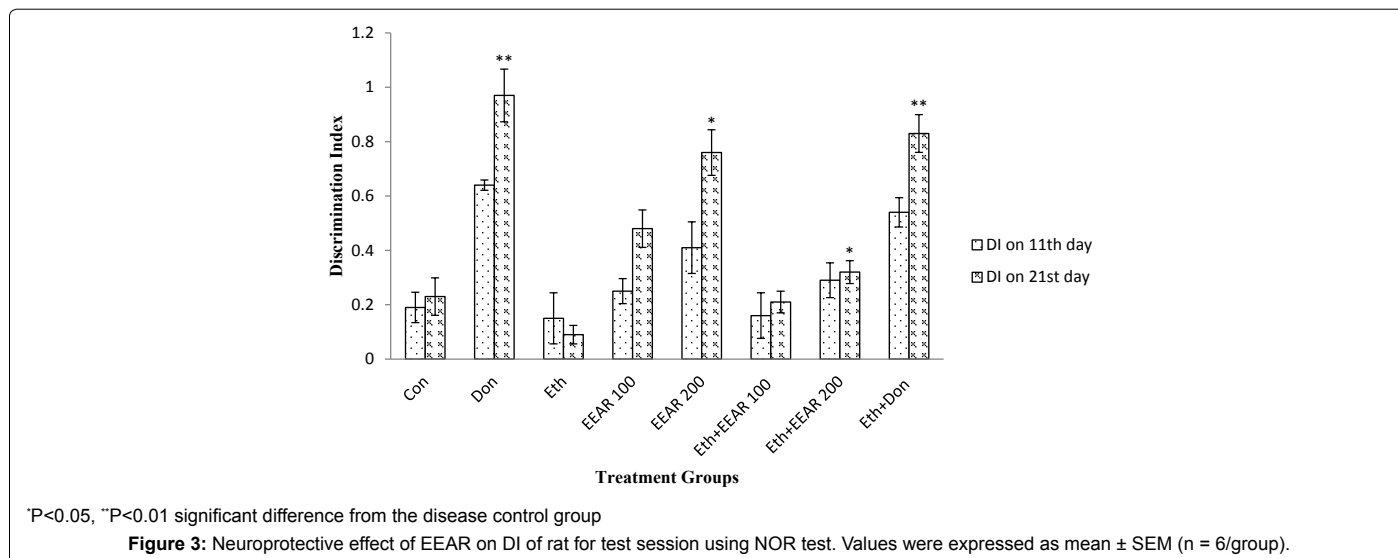## Neuroprotective Effect of EEAR on EL and RL of Rats using PA test

In PA test the EL was measured on 10th and 20th days and RL was measured on 11th (after 24 hrs of EL) and 21st day (after 24 hrs of EL) respectively given in Figure 2. In the ethanol treated group, significant decreased in RL was reported compared to other groups on successive days. Treatment with donepezil significantly (P<0.05, P<0.001)

increased RL of rats on 21st day compared with disease control group. The highest and lowest doses of EEAR increased RL of rats as compared to that of disease control group on successive days. Particularly administration of 200 mg/kg b.w. EEAR markedly (P<0.05, P<0.01) increased the value of RL on 11th and 21st day thus showed significant cognition enhancing activity.

Neuroprotective Effect of EEAR on DL of Rats using NOR test

In NOR test the DI was measured on 11th (after 24 hrs of training session) and 21st (after 24 hrs of training session) day respectively assumed in Figure 3. The standard drug, donepezil hydrochloride significantly (P<0.01) increased the DI of rats on 21st day with respect to disease control group. Ethanol-treated rats showed a reduced in DI on 11th and 21st day as compared to other groups. This outcome was altered by the highest dose of EEAR. EEAR (i.e., 200 mg/kg b.w.) treated rats exhibited a significantly (P<0.05) increased in DI on 21st day compared with that of disease control rats.

*P<0.05, **P<0.01 significant difference from the disease control group

Figure 1: Neuroprotective effect of EEAR on ITL and RTL of rats using EPM test. Values were expressed as mean ± SEM (n = 6/group).

*P<0.05, **P<0.01, ***P<0.001 significant difference from the disease control group

Figure 2: Neuroprotective effect of EEAR on EL and RL of rats using PA test. Values were expressed as mean ± SEM (n = 6/group).

*P<0.05, **P<0.01 significant difference from the disease control group

Figure 3: Neuroprotective effect of EEAR on DI of rat for test session using NOR test. Values were expressed as mean ± SEM (n = 6/group).

*P<0.05, **P<0.01 significant difference from the disease control group

Figure 4: Neuroprotective effect of EEAR on EL of rat for acquisition trial using MWM test. Values were expressed as mean ± SEM (n = 6/group).

Neuroprotective Effect of EEAR on EL, TSTQ and TSA of Rats using MWM test

In MWM test the EL and TSTQ for acquisition trial was measured on 10th, 11th, 20th and 21st day (Figure 4 and Figure 5). In acquisition trial, ethanol-treated rats showed markedly increased EL and decreased TSTQ on successive days as compared to other groups. Donepezil administered rats for 21 days showed significantly (P<0.01) decreased in EL and markedly (P<0.05, P<0.01) increased in TSTQ of rats as compared to that of ethanol-induced group. Administration of EEAR decreases the EL and increased the TSTQ on successive days. Administration of EEAR (i.e., 100 and 200 mg/kg b.w.) resulted in markedly (P<0.05, P<0.01) decreased in EL on 21st day as compared to that of disease control group. Out of two doses highest dose of EEAR (i.e., 200 mg/kg b.w.) markedly (P<0.01, P<0.001) increased the TSTQ of rats on 11th and 21st day as compared to that of disease control group. In case of probe trial, like acquisition trial EEAR (i.e., 200 mg/kg b.w.) significantly (P<0.05, P<0.01) increased TSTQ and TSA of rats as compared to that of disease control group on 21st day thus showed significant improvement of learning and memory given in Figure 6 and Figure 7.
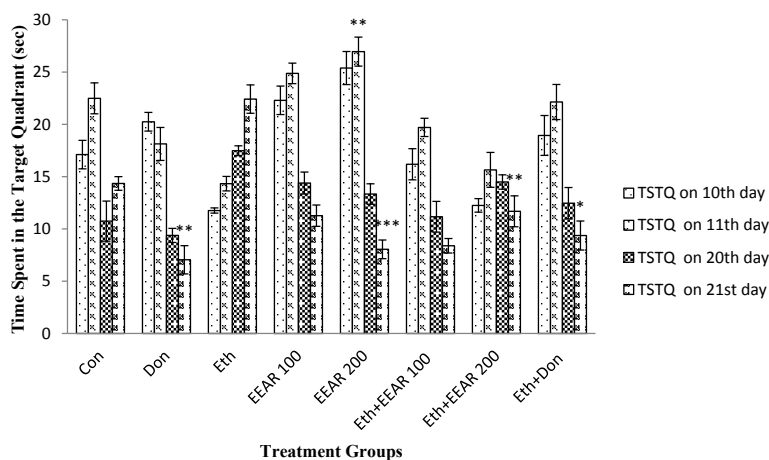
Table 2: Neuroprotective effect of EEAR on TBARS status of rat brain.

Treatment	TBARS (nM/min/mg protein)
Con	394.05 ± 5.36
Don	158.29 ± 4.69***
Eth	457.95 ± 6.92
EEAR 100	216.25 ± 3.65
EEAR 200	198.57 ± 4.68**
Eth+EEAR 100	274.39 ± 6.73**
Eth+EEAR 200	241.67 ± 5.67
Eth+Don	186.49 ± 6.95**

The rats brain TBARS status was expressed as mean ± SEM values (n = 6/group). **P<0.01, ***P<0.001 significant difference from the disease control group
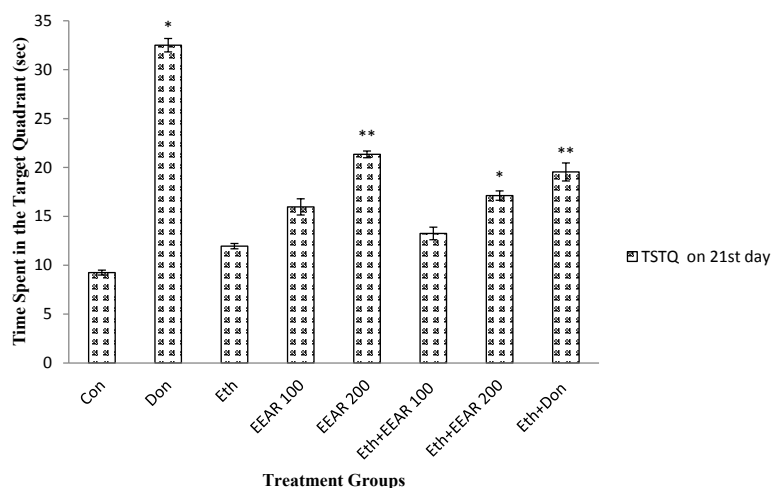
Neuroprotective Effect of EEAR on TBARS Status in Rat's Brain

Treatment with ethanol increased the level of TBARS as compared to other groups specified in Table 2. Administration of both doses of EEAR (i.e., 100 and 200 mg/kg b.w.) significantly (P<0.01) decreased the TBARS level with respect to disease control group. The rats treated

*P<0.05, **P<0.01, ***P<0.001 significant difference from the disease control group

Figure 5: Neuroprotective effect of EEAR on TSTQ of rat for acquisition trial using MWM test. Values were expressed as mean ± SEM (n = 6/group).

*P<0.05, **P<0.01 significant difference from the disease control group

Figure 6: Neuroprotective effect of EEAR on TSTQ of rat for probe trial using MWM test. Values were expressed as mean ± SEM (n = 6/group).

with donepezil markedly (P<0.01, P<0.001) decreased the TBARS level to that of disease control group.

Neuroprotective Effect of EEAR on AChE Activity in Rat's Brain

Ethanol-treated rats showed a decreased in the brain AChE activity as compared to other groups. Donepezil treatment significantly (P<0.01) inhibited the brain AChE level compared to their corresponding disease control group. Brain AChE activity was considerably decreased in rats owing to EEAR administration. The lowest and highest dose of EEAR (i.e., 100 and 200 mg/kg b.w.) significantly (P<0.05, P<0.01) decreased the AChE activity in the brain tissue of rats as compared to disease control group (Table 3).
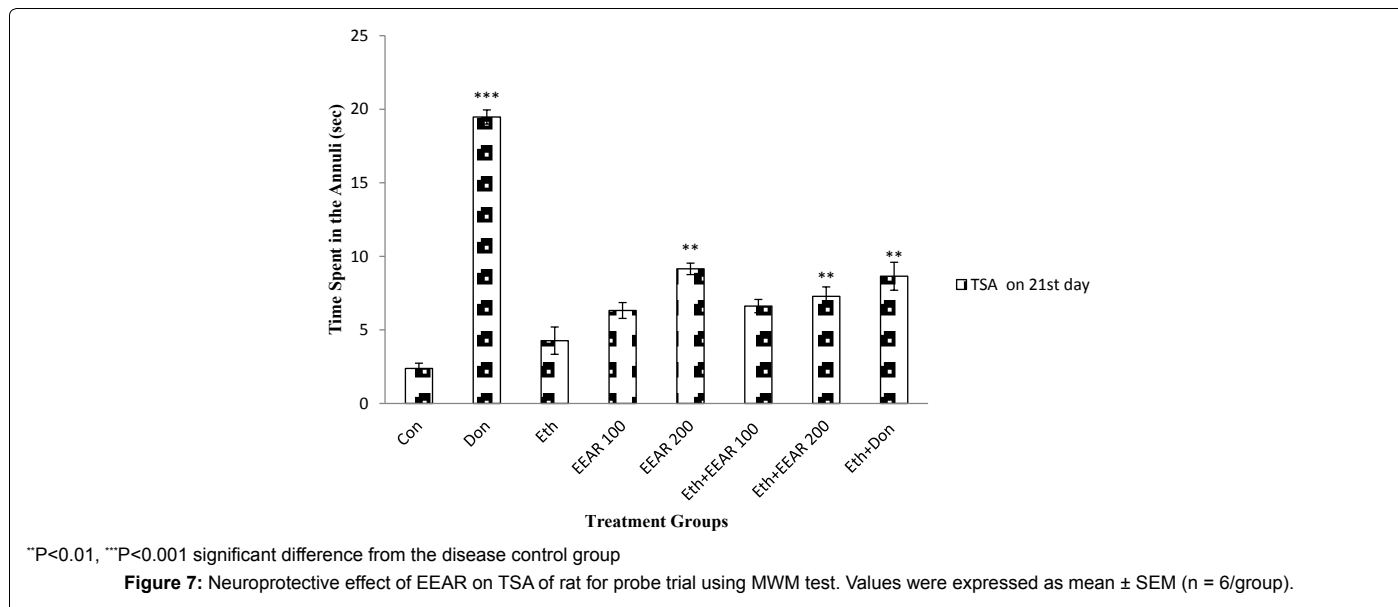
Discussion

No cure for Alzheimer's exists and the drugs currently available to treat the disease have limited effectiveness. The uses of complementary medicines such as plant extracts exert a substantial strategy for the

Table 3: Neuroprotective effect of EEAR on AChE activity in rat brain.

Treatment	AChE (M/min/g of tissue protein)
Con	0.194 ± 0.001
Don	0.049 ± 0.004**
Eth	0.307 ± 0.005
EEAR 100	0.166 ± 0.003*
EEAR 200	0.129 ± 0.005**
Eth+EEAR 100	0.244 ± 0.006
Eth+EEAR 200	0.201 ± 0.004**
Eth+Don	0.102 ± 0.004**

The rats brain AChE activity was expressed as mean ± SEM values (n = 6/group). *P<0.05, **P<0.01 significant difference from the disease control group

treatment of neurological disorders such as AD [53]. Indeed, several scientific studies have described the use of various medicinal plants and their bioactive constituents for the treatment of AD [54]. Although the exact mechanism of their action is still not clear, phytochemical studies of the different parts of the plants have shown the presence of many

$^{**}P<0.01$, $^{***}P<0.001$ significant difference from the disease control group

Figure 7: Neuroprotective effect of EEAR on TSA of rat for probe trial using MWM test. Values were expressed as mean ± SEM (n = 6/group).

valuable compounds, such as lignans, flavonoids, tannins, polyphenols, triterpenes, sterols and alkaloids, that show a wide spectrum of pharmacological activities, including anti-inflammatory, anti-amyloidogenic, anti-cholinesterase, hypolipidemic and antioxidant effects [55]. In this study, EEAR roots extract were administration for 21 days showed significant neuroprotective activity in ethanol-induced rats of cognitive impairment and oxidative stress analyzed by using various behavioral and biochemical studies.

The EPM is a commonly used test for the determination of spatial long term memory by measuring ITL and RTL. In this test, after 24 hrs of ITL determination RTL was measured in which a decrease in RTL after the ILT determination indicated improvement of spatial long-term memory of rats. In this study, EEAR administration significantly reduced RTL of rats as compared to disease control group. In the study of protective effects of *Punica granatum* seeds extract against aging and scopolamine induced cognitive impairments in mice Kumar *et al.,* reported analogous result in transfer latency of animals in this test [56]. The PA is a used test for the determination of emotional memory by measuring EL and RL. During EL determination an electric shock was given and after 24 hrs of electric shock RL was measured. An increased in RL was observed in rats treated by EEAR as compared to disease control rats. In this test an increase in RL after the EL determination indicated improvement of emotional memory of rats as compared to disease control rats. By using this test Bera *et al.,* observed brain tonic activity of *Shankhavali churna* in Wistar albino rats [57]. The NOR test is used for the determination of recognition memory by measuring DI based on the exploration times of novel and familiar object. In this test, an increase in DI after the training session indicated improvement of recognition memory, observed in EEAR treated rats as compared to disease control rats. In term of this test Une *et al.,* in the study of *Ziziphus mauritiana* leaves extract stated nootropic effect [58]. The MWM test is the most commonly test used to evaluate cognitive functions related to spatial learning and memory by measuring EL, TSTQ and TSA. EL is the time required to find the submerged platform. TSTQ and TSA are the time spends to stay in the target quarantine and annuli respectively. Herein in case of acquisition trial a decrease in EL and an increase in TSTQ indicated improvement of spatial learning and memory of rats. For probe trial an increase in TSTQ and TSA also means enhancement

of spatial learning and memory of rats. In this test EEAR administration considerably decrease EL and increase TSTQ and TSA of rats with respect to disease control rats. In the study of *Nyctanthes arbor-tristis* leaves extract against scopolamine-induced cognitive impairment in rats, neuroprotective effect was obserbed by Raju et al., [59].

The main target for lipid peroxidation is the CNS due to high lipid content (PUFA), high consumes of the body's oxygen (20%) and high in redox transition metal ions. Lipid peroxidation is the hallmark of AD [60]. Increasing evidence demonstrates abnormal lipid peroxidation, cerebrospinal fluid and plasma in the brain of AD patients. In addition, lipoproteins in the AD patient's brain are more susceptible to oxidation damage [61]. As per this study EEAR roots administration in rat's markedly decreased brain TBARS activity. For the study of neuroprotective effect of *Phyllanthus acidus* fruits extract on learning and memory impairment in scopolamine-induced animal model of dementia and oxidative stress, Uddin *et al.,* reported similar finding [62]. The foremost enzyme of the cholinergic nervous system is the AChE. Higher level of AChE is found in the brain of AD patients [63]. Study suggested that AChE is linked with the formation of Aβ plaques and NFTs, both are pathological features of AD [64]. Numerous studies recommend that in the expression of AChE, Aβ and atypically hyperphosphorylated tau (P-tau) can impact [65]. In this study treatment with EEAR roots widely decreased AChE level. In the study on nootropic effects of aerial part of *Persicaria flaccida* on cognitive performance, brain antioxidant markers and acetylcholinesterase activity in rats by Uddin et al., showed beneficial effects of this plant extracts for the management of AD [66].

The outcome of the aforementioned studies recommend that EEAR roots extract has neuroprotective activity and gifted to improve learning, memory impairments and hippocampus, cerebellum oxidative stress.

Conclusion

From the present study it was clearly demonstrated that EEAR root has significant neuroprotective activity and showed marked constructive effects for improving the learning and memory. Therefore, this root extract can be used for the treatment of various cognitive dysfunctions

connected with neurodegenerative disorders predominantly AD. Regardless of these findings, further investigations will be necessary to illustrate the active nootropic compound(s).

Ethical Approval

The protocol of the experiment was approved by the animal ethics committee of the Department of Pharmacy, Southeast University, Dhaka, Bangladesh.

Authors' Contributions

This work was carried out in collaboration among all authors. MSU and MA designed the study, wrote the protocol, managed the analyses of the study and prepared the draft of the manuscript. MSU, AAM and MAI performed the laboratory experiments. FW participated in literature searches. RKR participated in data analysis and interpretation. All authors read and approved the final manuscript.

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Competing Interests

The authors proclaim that they have no competing interests.

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