Research Article

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NEUROPROTECTIVE EFFECT OF HYDROALCOHOLIC EXTRACT OF *ARECA CATECHU* LINN ON β-AMYLOID (25-35) INDUCED COGNITIVE DYSFUNCTION IN MICE

R. Kannan*, D. Sivaraman, P. Muralidharan, N. Deepakvenkataraman
Department of Pharmacology and Toxicology, C.L. Baid Metha College of Pharmacy, Chennai, India

*Corresponding author
E-mail: kannanramalingam@yahoo.in

ABSTRACT
Alzheimer's disease is the most common form of dementia in elderly. There is currently no cure for Alzheimer's disease. But some category of drugs like AchE inhibitors and NMDA antagonists were used along with some antioxidants and some other supportive therapy. There is a possibility to slow down the brain's degeneration caused by Alzheimer's with natural treatments. In the present study animals were divided randomly into five groups of six animals each. Group I animals were given 0.1 % w/v CMC orally by using intra-gastric catheter at dose (10 ml/kg), Group III and Group IV animals were pretreated with hydroalcoholic extract of *Areca catechu* Linn (HAEAC) for a period of 3 weeks (200 and 400 mg/ kg b.w) and Group V animals were treated with donepezil (1.5 mg/kg/b.w i.p) and were kept in light/dark cycle. During this period the animals were trained in water-maze, Y-maze, exploratory behaviour and passive avoidance apparatus for memory. Amnesia is induced by intra cerebro ventricular injection (I.C.V) of β-amyloid (25-35). I.C.V injection for the 2nd, 3rd, 4th and 5th groups were performed on the 21st day of the pretreated animals and continued for 5 days. The last dose was given 60 minutes prior to behavioral testing and on 30th day scarification of animals was done for *in-vitro* studies. Hydroalcoholic extract of *Areca catechu* Linn showed significant protective effect on neurodegeneration and also showed improvement on memory retention activity when compared with β-amyloid (25-35) induced animals (Group II).
Keywords: Alzheimer's disease (AD), β-amyloid (25-35), Ubiquitinated proteins, Intra cerebro ventricular injection (I.C.V), Areca *catechu* Linn.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by a gradual decline in memory associated with shrinkage of brain tissue, with localized loss of neurons mainly in the hippocampus and basal forebrain, with diminished level of central cholinergic neurotransmitter-acetylcholine and also reported to be associated with accumulation of ubiquitinated proteins in neuronal inclusions and also with signs of inflammation[1]. Alzheimer's disease is the most prevalent form of dementia which does not have any previous cause such as stroke, brain trauma or alcohol toxicity. It is also distinct from vascular dementia, which is associated with brain infarction. There is a disturbance of higher cortical functions such as memory, speech, learning, thought, orientation and judgment. Consciousness is not affected. The disease is associated with degeneration of cholinergic neurons, particularly those extending from subcortical areas such as nucleus basalis of meynert. There is also a deficit of acetyl choline transferase, the enzyme responsible for the formation of acetyl choline. This results in decreased central cholinergic transmission[2]. Alzheimer's disease affects about 5 % population at the age of 65 - 80 years, 10 % between 80 - 95 years and 90 % above 95 years.[3]Alzheimer disease (AD) is the seventh most prevalent cause of death in the US and the leading cause of dementia, affecting more than 5 million Americans and 26 million worldwide. Without an effective therapy it is estimated that the number of patients with AD will duplicate by the year 2050[4]. The fundamental abnormality in AD is the deposition of Aβ peptides, which are derived through processing of APP. The Aβ portion of the protein extends from the extracellular region into the

transmembrane domain. Processing of APP begins with cleavage in the extracellular domain, followed by an intramembranous cleavage. Once generated, Aβ is highly prone to aggregation-first into small oligomers (which may be the toxic form responsible for neuronal dysfunction), and eventually into large aggregates and fibrils. The Aβ peptides readily aggregate and can be directly neurotoxic. There are various lines of evidence indicating that the small aggregates of Aβ can result in synaptic dysfunction, such as blocking of long-term potentiation and changes in other membrane properties.[5] *Areca catechu* is the areca palm or areca nut palm betel palm, a species of palm which grows in much of the tropical Pacific, Asia, and parts of east Africa.[6] The seed of this palm ("areca nut") is used in the preparation of betel quid, generally by combining it with slaked lime and the leaf of *Piper betle* (betel leaf). Areca palm seed is now among the most important stimulant products in the world used by around 200 to 600 million people globally.[7-9] The seed contains alkaloids such as arecaidine and arecoline which, when chewed, are intoxicating and slightly addictive.[10] In this present study the effect of *Areca catechu* Linn on β-amyloid induced cognitive dysfunction is studied using mice models.

MATERIALS AND METHODS
Collection of plant material
The plant material was collected from Kerala of India and authenticated at Dept. of Medical Botany, National Institute of Siddha, Chennai, India (Voucher No: NIS/MB/68/2012).

Preparation of extract
The nuts of *Areca catechu* Linn were cleaned and the adherent sand and dust particles were removed. It was dried and made into a coarse powder with the help of electric grinder. About 500 g of grinded plant material was subjected to Soxhlet extraction (50-60^{0}C) using hydro alcoholic (water and ethanol in the ratio of 1:1) solvent. The solvent was evaporated at 40^0 C to obtain a viscous mass. The percentage yield of the extract was 20.1 % w/w.[11]

Animals
Colony inbred strains of Swiss albino male mice weighing 22-25 g and 2-4 weeks old mice were used in the study. Animals were obtained from C. L. Baid Metha College of Pharmacy which was used for pharmacological studies. The animals were kept under standard conditions at 23-25^{0}C, 12 h. light/dark cycle and given standard pellet diet and water *ad libitum*. The animals were acclimatized to the laboratory conditions for a week prior to the experiment and randomly divided into four groups of each ten animals. Principles of animal handling were strictly adhered and the handling of animals was made under the supervision of animal ethics committee of this institute. The experimental protocol was approved (Approval. No: IAEC/I/03/CLBMCP/2012 dated 28.08.2012) by Institutional Animal Ethics Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experimental Animals).

Experimental design
The animals were divided randomly into five groups of six animals each.
Group I treated with CMC (0.1 %w/v)
Group II injected with beta amyloid (25-35) (10 μl i.c.v)
Group III injected with beta amyloid (25-35) (10 μl i.c.v) and treated with 200 mg/kg of HAEAC (p.o)
Group IV injected with beta amyloid (25-35) (10 μl i.c.v) and treated with 400 mg/kg of HAEAC (p.o)
Group V injected with beta amyloid (25-35) (10 μl i.c.v) and treated with donepezil (1.5 mg/kg/b.w i.p)

Amnesia was induced by intra cerebro-ventricular injection (I.C.V) of beta amyloid. I.C.V injection for the pre treated 2nd, 3rd, 4th and 5th groups which was done on the 21st day and was continued for 5 days. Control animals were given 0.1 % w/v CMC orally using intra-gastric catheter (10 ml/kg), where the last dose was given 60 minutes prior to behavioral testing and on 30th day sacrification of animal was done for *in-vitro* studies.

Assessment of Habituation Behavior
Open Field Test (Hole board apparatus)
Exploratory behavior was evaluated in an open-field paradigm. The open field was made up of plywood and comprises of 40 X 50 X 60 cm dimension. The entire apparatus was painted black and was divided into 16 squares with white lines on the floor. Each animal was placed at one corner of the apparatus and for the next 5 minutes they were observed for their ambulation such as line crossings and head dippings.[12]

Closed Field Activity (Actophotometer)
The locomotor activity was measured by using an Actophotometer. The actophotometer consists of a square arena (30 × 30 × 25 cm) with wire mesh bottom, in which the animal moves. Six lights and six photocells were placed in the outer periphery of the bottom in such a way that a single mouse can block only one beam. The movement of the animal interrupts a beam of light falling on a photocell, during which a count was recorded and displayed digitally. The locomotor activity was measured for a period of 10 minutes[13-14].

Assessment of Memory and Retention
Passive Shock Avoidance Test
Passive avoidance behavior based on negative reinforcement was used to examine the long-term memory. The apparatus consists of a box (27 X 27 X 27 cm) having three walls of wood and one wall of Plexiglas, featuring a grid floor (3 mm stainless steel rods set 8 mm apart) with a wooden platform (10 X 7 X 1.7 cm) in the centre of the grid floor. Electric shock (20 V, AC) was delivered to the grid floor. During Training session, each mouse was gently placed on the wooden platform set in the centre of the grid floor, when the mouse stepped down and placed all its paw on the grid floor, shock was delivered for 15 seconds and the Step-Down Latency (SDL) was recorded. SDL is defined as the time taken by the mouse to step down from the wooden platform to grid floor with its entire paw on the grid floor. Animals showing SDL in the range of 2-15 seconds during the first test were used for the second session and the retention test. The second-session was carried out 90 minutes after the first test. During second session, if the animals stepped down before 60 seconds, electric shocks were delivered once again for 15 seconds. During the second test, animals were removed from shock free zone, if they did not step down for a period of 60 seconds and were subjected to retention test. On the 29th day, 90 minutes after the treatment of last dose training was given and memory retention was examined after 24 h (i.e. on 30th day) in a similar manner, except that the electric shocks were not applied to the grid floor observing an upper cut-off time of 300 seconds.[15-16]

Morris Water Maze Test
The Morris water maze test is preformed to evaluate spatial working and reference memory. In this model the animals were placed into a large circular pool of water and they can escape onto a hidden platform. The platform is hidden by its placement just below the water surface and by opaque water. Therefore the platform offers no local cues to guide escape behavior. The animal can escape from swimming by climbing onto the platform and with time the animal apparently learns the spatial location of the platform from any starting position at the circumference of the pool. Morris water maze consists of a large circular tank made of black opaque polyvinyl chloride or hard board coated with fiberglass and resin and then surface painted white (1.8-2.0 meter in diameter and 0.4-0.6 meter high). The pool was filled up to a height of 30 cm with water maintained at around 25^{0}C and rendered opaque by addition of a small quantity of milk or

nontoxic white color. The pool was provided with filling and draining facilities and was mounted at waist level. The tank was hypothetically divided into four equal quadrants and a platform (11cm^2) of 29 cm height was located in the centre of one of these four quadrants. The platform remains fixed in the position during the training session. Each animal was subjected to four consecutive trials for four days (from 21st to 24th day) during which they were allowed to escape on to the hidden platform and allowed to remain there for 20 seconds. Escape latency time to locate the hidden platform in water maze was noted as an index of acquisition or learning. In case the animal was unable to locate the hidden platform within 120 seconds, it was gently guided by hand to the platform and allowed to remain there for 20 seconds. On the 29th day, 60 minutes after last dose, platform was removed and time spent by each animal in target quadrant searching for the hidden platform was noted as an index of retrieval and measured.[17-18]

Estimation of Brain Neurotransmitters
Estimation of Acetylcholineserase enzyme (AchE)
20 mg of brain tissue per ml of phosphate buffer (pH 8, 0.1 m) was homogenized in a potter-elvehjem homogenizer. 0.4 ml aliquot of brain homogenate was added to a cuvette containing 2.6 ml of 0.1 m phosphate buffer (pH 8). 100 µl of the DTNB reagent was added to the photo cell. The absorbance was measured at 412 nm then acetylthiocholine iodide was added. Changes in absorbance were recorded and the change in absorbance per minute was calculated. The enzyme activity is expressed as µmoles/minute/mg tissue.

Estimation of Mono amino oxidase enzyme (MAO)
MAO activity was assessed spectrometrically. 250 µl solution of the mitochondrial fraction and 100 mm sodium phosphate buffer (pH 7.4) were added up to the final volume of 1 ml, the reaction was allowed to proceed at 37ºC for 20 minutes and stopped by adding 1 m HCl (200 µl). The reaction product was extracted with 5 ml of butyl acetate, the organic phase was measured at wavelength of 280 nm in a spectrometer. Blank samples were prepared by adding 1 m HCl (200 µl) prior to the reaction and worked subsequently in the same manner.[19]

Estimation of Antioxidant Enzyme
Estimation of super oxide dismutase (SOD)
The assay mixture should contain 1 ml of pyrogallol-tris-DEPTA, 0.2 ml of suitably diluted tissue and 0.8 ml of water. The absorbance was measured at 420 nm. The rate of pyrogallol autoxidation was taken from the increase in absorbance. The activity of SOD was expressed as units/minutes/mg protein. One unit of the enzyme is defined as the amount of enzyme, which inhibits the rate of pyrogallol auto oxidation by 50 %. The SOD is expressed as units/min/mg protein.[20]

Estimation of glutathione peroxidase (GPX)
The reaction mixture consisting of 0.2 ml each of EDTA, sodium azide and 0.4 ml of phosphate buffer, 0.1 ml of suitably diluted tissue was incubated at 37ºC at different time intervals. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. To 0.5 ml of supernatant, 4 ml of Disodium hydrogen phosphate and 0.5 ml DTNB were added and the color developed was read at 420 nm immediately. The activity of GPx was expressed as µmoles of glutathione oxidized/minutes/mg protein.[21]

Estimation of glutathione reductase (GRD)
The reaction mixture is containing 1 ml of phosphate buffer and 0.5 ml of EDTA. 0.5 ml of oxidized glutathione and 0.2 ml of NADPH was made up to 3 ml with water. After the addition of suitably diluted tissue, the change in optical density at 340 nm was monitored for 2 minutes at 30 seconds intervals. The activity of GRD was expressed as nmoles of NADPH oxidized/minute/mg protein.[22]

RESULTS
Effect of HAEAC on Open field activity
The Group II animals showed significant decrease in head dippings and line crossings when compared with the Group I animals ($p < 0.001$). Treatment with HAEAC (200 and 400 mg/kg) and standard drug Donepezil 1.5 mg/kg (i.p) increased the head dippings and line crossings which were statistically significant ($p < 0.01$, $p < 0.001$ and $p < 0.01$ for Group III, IV and V respectively) when compared with Group II. Results are plotted in histogram-1.

Effect of HAEAC on Closed field activity
There was a significant ($p < 0.001$) decrease in the activity scores produced by Group II animals when compared with Group I animals. Treatment with HAEAC (200 and 400 mg/kg) and standard drug Donepezil 1.5 mg/kg (i.p) showed significant ($p < 0.001$, $p < 0.001$ and $p < 0.001$ for Group III, IV and V respectively) increase in the activity scores when compared with group II animals. Results are plotted in histogram -2.

### Effect of HAEAC on Step down Passive Shock avoidance test
The Step down Latency (SDL) of Group II animals were significantly decreased ($p < 0.001$) when compared with Group I animals. Treatment with HAEAC (200 and 400 mg/kg) and standard drug Donepezil 1.5 mg/kg (i.p) ($p < 0.001$, $p < 0.001$ and $p < 0.001$ for Group III, IV and V respectively) showed significant increase in the step down latency when compared with Group II. The increase in SDL indicates enhancement in short term memory (STM). Results are plotted in histogram -3.

Effect of HAEAC on Morris water maze task
The escape latency of Group II animals was significantly increased when compared with Group I animals ($p < 0.001$). Treatment with HAEAC (200 and 400 mg/kg) and standard drug Donepezil 1.5 mg/kg (i.p) significantly decreased ($p < 0.001$, $p < 0.001$ and $p < 0.05$ for Group III, IV and V respectively) the escape latency, when compared with the Group II animals. The decrease in escape latency indicates enhancement of memory retention and non-spatial working memory. Results are plotted in histogram -4.

Effect of HAEAC on Open field Activity HEAD DIPPING
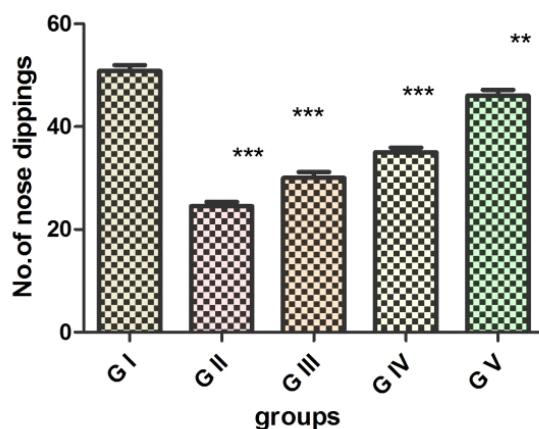
Effect of HAEAC on Open field Activity LINE CROSSING
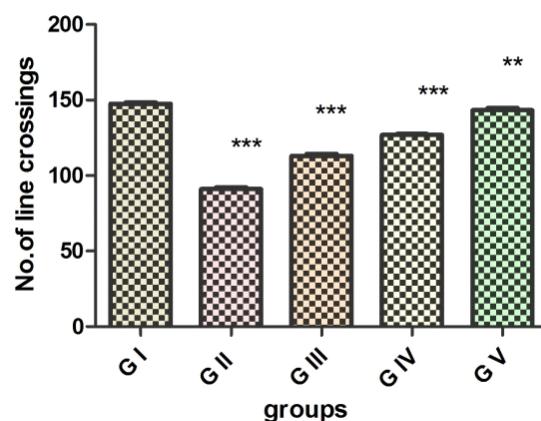

Histogram 1: Effect of HAEAC on Open field Activity

Effect of HAEAC on Closed field Activity
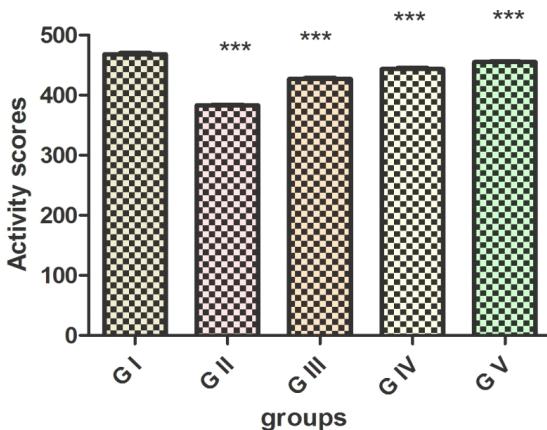
Histogram 2: Effect of HAEAC on Closed field Activity

## Effect of HAEAC on Step down Passive Shock avoidance test
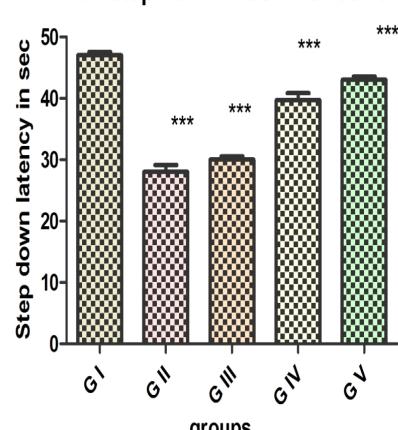
Histogram 3: Effect of HAEAC on Step down Passive Shock avoidance test

Effect of HAEAC on Morris water maze task
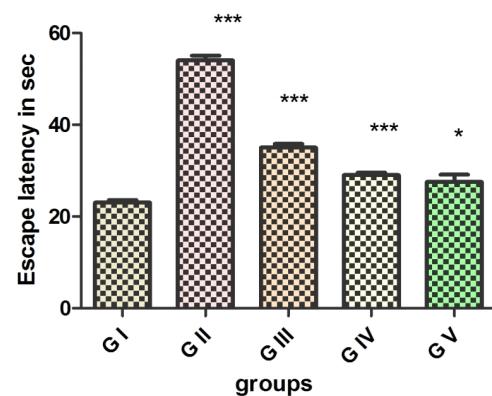
Histogram 4: Effect of HAEAC on Morris water maze task

Effect of HAEAC on AChE Activity
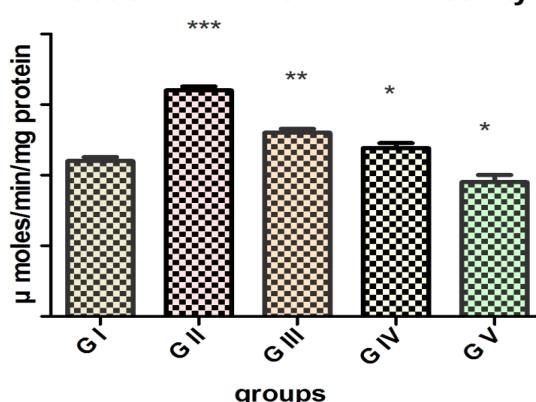
Histogram 5: Effect of HAEAC on AChE Activity

**Effect of HAEAC on Mono amine oxidase A**
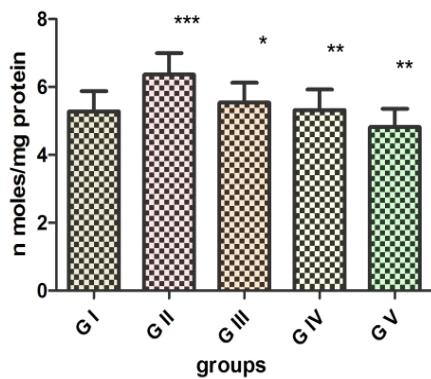
Effect of HAEAC on Mono amine oxidase B
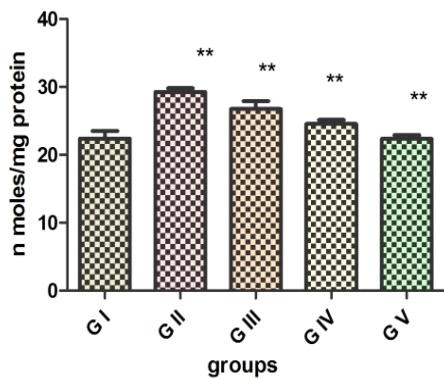
**Histogram 6: Effect of HAEAC on MAO-A and MAO-B**
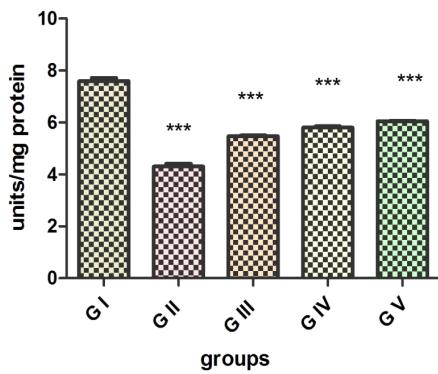
Effect of HAEAC on Superoxide dismutase (SOD)

Effect of HAEAC on Glutathione peroxidase (GPx)

Histogram 7: Effect of HAEAC on Superoxide dismutase (SOD)
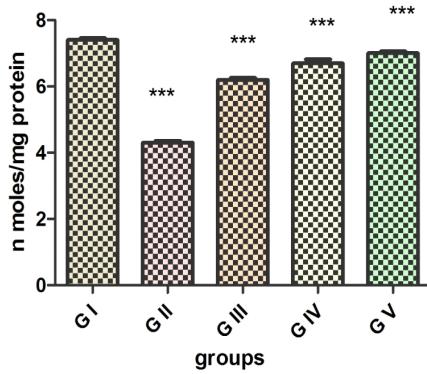
Histogram 8: Effect of HAEAC on Glutathione peroxidase (Gpx)

Effect of HAEAC on Glutathione reductase (GRD)
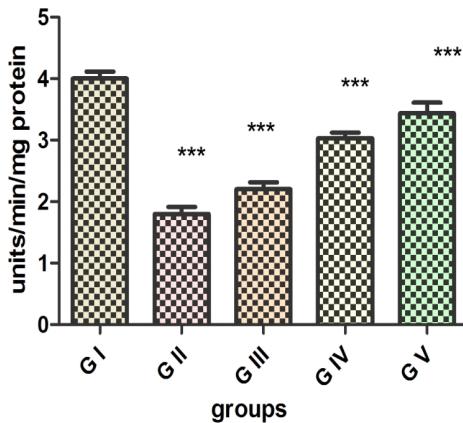
Histogram 9: Effect of HAEAC on Glutathione reductase (GRD)

Effect of HAEAC on Acetyl cholinesterase level

The level of AchE in group II animals showed ($p < 0.001$) significant increase when compared with Group I animals. Treatment with HAEAC significantly decreased ($p < 0.01$ and $p < 0.01$ for Group III and Group IV -200 mg/kg and 400 mg/kg respectively) the AchE level when compared with Group II animals. Standard drug Donepezil 1.5 mg/kg (i.p) (Group V, $p<0.05$) showed significant reduction in AchE activity; when compared with Group II animals. Results are plotted in histogram -5.

Effect of HAEAC on Mono Amine Oxidase A and B

The level of MAO in group II animals was ($p < 0.001$) significantly increased when compared with Group I animals. Treatment with HAEAC 200 mg/kg significantly ($p < 0.05$) decreased MAO level when compared with Group II animals. Treatment with HAEAC 400 mg/kg and Donepezil 1.5 mg/kg significantly ($p < 0.01$) decreased the MAO level compared with Group II animals. Results are plotted in histogram -6.

Effect of HAEAC on Superoxide dismutase

The SOD in the brain of Group II animals were decreased significantly ($P < 0.001$) when compared with Group I animals. Treatment with HAEAC (200 and 400 mg/kg) and standard drug Donepezil 1.5 mg/kg (i.p) (Group III, IV and V respectively) showed significant ($p < 0.001$, $p < 0.001$ and $p < 0.001$ for Group III, IV and V respectively) increase of SOD level when compared with Group II animals. Results are plotted in histogram -7.

Effect of HAEAC on Glutathione peroxidase

The Glutathione peroxidase in the brain of Group II animals were decreased significantly ($P < 0.001$) when compared with Group I animals. Treatment with HAEAC (200 and 400 mg/kg) and standard drug Donepezil 1.5 mg/kg (i.p) (Group III, IV and V respectively) showed significant ($p < 0.001$, $p < 0.001$ and $p < 0.001$ for Group III, IV and V respectively) increase of Glutathione peroxidase level when compared with Group II animals. Results are plotted in histogram -8.

Effect of HAEAC on Glutathione reductase

The Glutathione reductase in the brain of Group II animals were decreased significantly ($P < 0.001$) when compared with Group I animals. Treatment with HAEAC (200 and 400 mg/kg) and standard drug Donepezil 1.5 mg/kg (i.p) (Group III, IV and V respectively) showed significant ($p < 0.001$, $p < 0.001$ and $p < 0.001$ for Group III, IV and V respectively) increase of Glutathione reductase level when compared with Group II animals. Results are plotted in histogram -9.

DISCUSSION

The results from the behavior studies like open and closed field activities revealed the there was a significant improvement in memory and learning activity in animals after treatment with HAEAC at both the dose levels employed. The negative reinforcement on memory due to Beta amyloid protein was restored by the administration of the extract. This is evident from increased latency period in the shock avoidance paradigm. Further it was observed that spatial working memory (water maze) was enhanced by HAEAC, which emphasizes the positive effect of the extract on memory. A marked reduction in the enzyme level such as AchE, MAO activity was noticed in the brain samples of HAEAC treated animals, which was responsible for regeneration of acetylcholine (Ach) and Dopamine levels. Similar effect was observed in the animal treated with standard drug Donepezil. The antioxidants defense in the brain tissues were estimated in terms of SOD, GPx, GRD level by UV spectrophotometric method. From this study it was observed that there was a marked increase in SOD, GPx and GRD antioxidant enzyme levels in the extract treated groups. This clearly indicates the potential of the extract to delay the generation of free radicals, peroxy radicals leading to neuronal damage and memory impairment. It is also noticed that standard drug Donepezil has a potential role in restoring the antioxidant enzyme almost to the normal level.

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