ANTI-AMNESIC EFFECTS OF *EVOLVULUS ALSINOIDES* LINN. IN AMYLOID β $_{(25-35)}$ INDUCED NEURODEGENERATION IN MICE.

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Summary

The anti-amnesic potential of the aqueous extract obtained from *Evolvulus alsinoides* Linn. was determined in Alzheimer's type of dementia induced mice. Mice were treated with aqueous extract of *Evolvulus alsinoides* with 200 and 400 mg/kg dose. The animals received intracerebroventricular injection of amyloid β $_{(25-35)}$ neurotoxic peptide on 15th day of treatment with aqueous extract of *Evolvulus alsinoides* and the treatment with extract was continued up to 21 days. Behavioral changes were evaluated using open field exploration and water maze learning task. *In vivo* antioxidant activity and acetylcholinesterase activity was measured in pooled cerebral cortex and hippocampal tissue of brain. The consumption of aqueous extract of *Evolvulus alsinoides* attenuated the cognitive defects induced by amyloid β $_{(25-35)}$ injection. Antioxidant enzymes are noticed with increment and acetylcholinesterase enzyme diminution was indicated in group of animals supplemented with the extract. These evaluations suggest the promising approaches of *Evolvulus alsinoides* on cognition in neurodegeneration induced mice.

Keywords: Alzheimer's disease, intracerebroventricular injection, amyloid β $_{(25-35)}$, amnesia; acetylcholinesterase enzyme; antioxidants

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with complex multifactorial mental illness. The pathological character comprising initial loss of memory and cognitive dysfunction due to the formation of intraneural fibrillary tangles and extraneural amyloid beta (Aβ) plaques.[1] It has referred that 11 amino acid sequence of Aβ $_{(25-35)}$ peptide are neurotoxic for primary neurons[2]. Aβ is a potent neurotoxin both *in vitro* and *in vivo*[3]. These are associated with impairment of central cholinergic neurotransmission[4] and selective loss of cholinergic neurons in the basal fore brain, which project to hippocampus and neocortex[5]. One of the characteristic transform that occurs in AD is the increment of acetylcholinesterase enzyme (AChE) and momoamino oxidase (MAO) around the Aβ plaques[6,7]. AChE has been shown to be promoting the assembly of Aβ plaques into fibrils and it was suggested to play a pathogenic role in AD by formation of a complex which exhibits higher toxic effect than Aβ $_{(25-35)}$ [8]. Oxidative stress signaling is speculated in the pathology of AD and other neurodegenerative disorders[9]. The free radical produced during oxidative stress causes the DNA and RNA oxidation indicated by increased levels of 8-Hydroxyl-2-deoxyguanosine and 8-Hydroxy guanosine[10,11], elevated levels of protein carbonyl residues[12, 13] and the lipid peroxidation (LPO) is marked by higher levels of thiobarbituric acid reacting substances (TBARS), malondialdehyde (MDA), 4-Hydroxy trans neoneal (HNE) and Isoprostrane[14,15]. It is hypothesized that Aβ protein accumulation and diffuse plaque formation is associated with local microglial activation, cytokine release, reactive astrocytosis and a multi-protein inflammatory response. There is considerable evidence that the effects of Aβ $_{(25-35)}$ initiated inflammatory and neurotoxic process include excessive generation of free radicals and peroxidative injury to proteins and other macromolecules in neurons[16].The whole plant of *Evolvulus Alsinoides* Linn. has been used in the Indian traditional medicine for insanity, nervous debility, immunomodulatory and for the management of stress induced neurodegenerative disease[17]. Recent studies had suggested immunomodulatory effect and antioxidant activities[18]. The effect of this plant against scopolamine induced adoptogenic and anti-amnesic activity were studied in rodents[19]. The *in vitro* AChE enzyme inhibitions were also studied[20]. The aim of the present study is to evaluate the anti-amnesic effect of aqueous extracts of *Evolvulus Alsinoides* (AEEA) in Aβ $_{(25-35)}$ induced neurotoxicity in mice.

Materials and Methods

The fresh whole plant of ***Evolvulus alsinoides*** **Linn**, is collected from Kanyakumari district (TN, India) during the month of July. The plant was authenticated by Dr. R. Girija Kumari, Reader and Head, Department of Plant Biology and Plant Biotechnology, Sree Ayappa College, Chunkankadai, Kanyakumari district (India). A herbarium was deposited in the Department of Pharmacognosy, C. L. Baid Metha College of Pharmacy, Chennai (India) for future reference. The shade dried plant was coarsely powdered and extracted with water by Soxhelt extraction method. The extract was collected and defatted with petroleum ether and dried under reduced pressure. The extract was stored at 0-4 ^{o} C.

Experimental Animals

Colony inbreed strains of Swiss albino mice (male) weighing 22-25g at the age of 5-6 weeks, obtained from C. L. Baid Metha College of Pharmacy and Research was used for the pharmacological studies. The animals were kept under standard conditions maintained at 23-25^{o}C, 12h light /dark cycle and given standard pellet diet (Hindustan Lever, Bangalore) provided *ad libitum.* The animals were acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into four groups of each six animals. The experimental protocol was approved by the Institutional Animal Ethics Committee. (REF. NO. 13/15 – CLBMCP / 17/08/2006).

Experimental Design

Grouping and Induction of neurotoxicity

Animals were divided into four groups of each six, viz-Group I (Control) received intracerebroventricular (i.c.v) injection of phosphate buffered saline, Group II (Negative Control) received only i.c.v injection of neurotoxic Aβ $_{(25-35)}$, Group III (AEEA 200) and IV (AEEA 400) animals injected by Aβ $_{(25-35)}$ and treated with 200 and 400 mg/kg (p.o) AEEA. Amnesia is induced by i.c.v injection of Aβ $_{(25-35)}$ by identifying the bregma point in skull[21], each animal was injected with 10 µl which contains 10µg of Aβ $_{(25-35)}$ peptide[22]. Group III and IV received Aβ $_{(25-35)}$ i.c.v injection on the 15th day of the treatment and continued up to 21 days. The negative control animals received only the neurotoxic Aβ $_{(25-35)}$ injection on 15th day without drug treatment.

Behavioral Studies

Open field habituation memory

In order to evaluate the possible effects on locomotor activity, animals were explored twice, with a 24 h interval, to a 40 cm×50 cm×60 cm open field whose brown linoleum floor was divided into 12 equal squares by white lines. In both sessions, the animals were placed in the rear left square and left to explore it freely for 5 min during which time the number of line crossings, rearing and head dippings were counted. [23]

Morris water maze task

The Morris water maze was performed as described previously[24]. The experimental apparatus consisted of a circular water tank (diameter=100 cm; height=35 cm), containing water at 28^{o}C to a depth of 15 cm and rendered opaque by adding powdered milk. A platform (diameter 4.5 cm; height 14.5 cm) was submerged 0.5 cm below the water surface and placed at the midpoint of one quadrant. After several trials, the test was conducted on the 5th day after injection of Aβ $_{(25-35)}$. In each trial, the time required to escape onto the platform was recorded.

Biochemical Estimations

Measurement of AChE activity

AChE activity in the brain was measured using acetylthiocholine iodide as a substrate[25]. The hydrolysis of acetylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoic acid at a wavelength of 412 nm. Protein concentration was determined by using bovine serum albumin and Folin's phenol reagent[26].

Measurement of antioxidant enzyme activity

Superoxide dismutase (SOD) activity was assayed by pyrogallol oxidation[27]. The assay mixture contained 1 ml of pyrogallol-Tris-DETPA, 0.2 ml of suitably diluted tissue homogenate and 0.8 ml of water. The rate of pyrogallol oxidation is taken from the increase in absorbance at 420 nm. Glutathione peroxidase (GPx) activity was measured according to the procedure described below [28]. The reaction mixture consisted of 1.44 ml phosphate buffer (0.05 M, pH 7.0), 0.1 ml of EDTA (1 mM), 0.1 ml of sodium azide (1 mM), 0.05 ml of glutathione reductase (1 eu/ml), 0.1 ml of glutathione (1 mM), 0.1 ml of NADPH (0.2 mM), 0.01 ml of hydrogen peroxide (0.25 mM) and 0.1 ml of PMS (10%, w/v) in a final volume of 2.0 ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nM NADPH oxidized/min/mg protein. Vitamin C was estimated by 2, 4-dinitrophenyl hydrazine reagent and the level of ascorbic acid is expressed as μg / mg protein[29].

Statistical Analysis

The data were analyzed by one way analysis of variance (ANOVA) followed by Dunnet's 't' test. P values lesser than 0.05 ($P < 0.05$) were considered statistically significant. All the data were presented as ± standard error mean (SEM).

Results

Open field habituation test

The amnesia induced (negative control) group without AEEA treatment indicated decrease in head dipings ($P<0.05$), rearings ($P<0.001$) and line crossings ($P<0.001$) with statistical significance when compared with phosphate buffered saline treated animals in control group. There was no significant improvements in head dips and rearings in 200mg/kg treated animals when compared to the control groups, but shown a significant ($P<0.001$) improvements in line crossings. The animals treated with 400mg/kg shown a significant improvements in head dipings ($P<0.05$), rearings ($P<0.05$) and line crossings ($P<0.001$). The graphical representation of various exploratory behavior are shown in figure: 1

Spatial learning in Morris water maze task

Amnesia induced mice without treatment (negative control) taken increased escape latency in Morris water maze task with statistical significance of P<0.001. Treatment of AEEA attenuated the increase in escape latency significantly to normal with P<0.01. But the escape latency was not dose dependent and the significance for both higher dose and lower dose were similar. The effect of AEEA treatment on water maze was shown in figure: 2

AEEA in AChE activity

Injection of $A\beta_{(25-35)}$ without treatment had increased the AChE enzyme level when compared with control group with a statistical significance of P<0.05. The treatment of animal with 200 and 400 mg/kg of AEEA significantly (P<0.05) reduced acetylcholinesterase enzyme. The graphical representation of acetylcholinesterase enzyme on different groups are shown in figure: 3

AEEA in antioxidant enzyme

SOD level in the brain was significantly reduced (P<0.001) in negative control group when compared to the control group. Treatment with AEEA at 200 mg/kg and 400mg/kg dose shown significant increase of SOD (P<0.05 and P<0.001) respectively when compared with the negative control group. The GPx in the amnesia induced mice (Group II) shown significant (P<0.001) reduction in the enzyme activity when compared to the control (Group I). The treatment with AEEA at 200 mg/kg shown the significance (P<0.01) and 400mg/kg dose shown high significance with (P<0.001) respectively when compared with the amnesia induced mice. The amount of Vitamin C present in the amnesia induced group shown the significant (P<0.001) decrease in the activity with comparison of control group. The treatment with AEEA 200 mg/kg does not shown the significance, in contrast 400 mg/kg showed significance with (P<0.001) when compared with the negative control group. All the data are presented in Table: 1

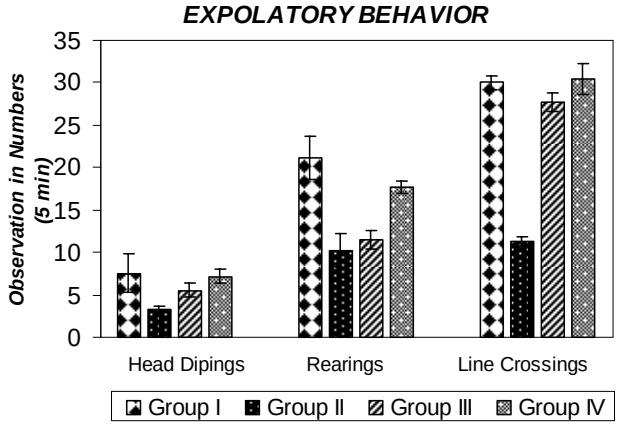

Figure 1: Effect of AEEA on Exploratory behavior.

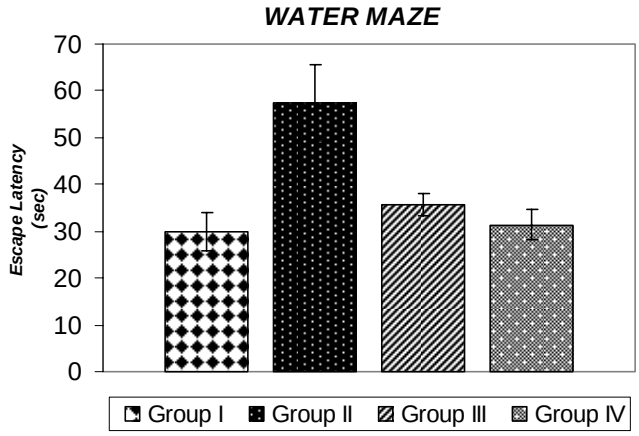

Figure 2: Effect of AEEA on Water Maze.

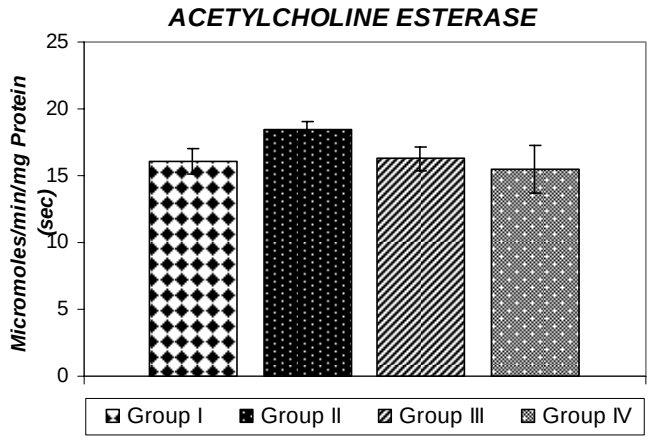

Figure 3: Effect of AEEA on Acetycholinesterase enzyme.

Table 1: Effect of AEEA on SOD, GPx and Vitamin C levels

GROUP	SOD (units/min mg protein)	GPx (nM NADPH oxidized/min/mg protein)	VIT.C µ gm/mg protein.
Control	8.13 ± 0.34	34.14 ± 1.20	0.674± 00
Negative control	5.63 ± 0.14 [a] **	26.16 ± 1.00 [a] **	0.490 ± 0.06 [a] **
AEEA 200 mg/kg oral	6.63 ± 0.37 [b] #	27.26 ± 0.70 [b] *	0.511 ± 0.02 [b] ns
AEEA 400 mg/kg oral	8.02 ± 0.34 [b]**	34.78 ± 1.36 [b]**	0.638 ± 0.01 [b]**

*n=6, Values are expressed as mean ± SEM. Comparisons were made between: a-Group I (control) and II (negative control), b-Group II (negative control), with III (AEEA 200mg/kg) and IV (AEEA 400mg/kg).** P<0.001, * P<0.01, # P< 0.05, ns-Non significant.*

Discussion

The present study revealed the neuroprotective effect of AEEA on Aβ $_{(25-35)}$ induced cognitive deficits in mice. *Evolvulus alsinoides* is a medicinal plant with antioxidant properties which is traditionally used as intellect promoting and for treating neurodegenerative disorders. Previous studies reveled that, oral administration for three days in mice was effective in decreasing scopolamine induced amnesia. There is substantial clinical evidence that muscarinic receptor blockade by drugs like scopolamine results into disruptions of behavioral inhibition, working (short-term) memory, retrieval from reference (long term memory), attention and decisional processes movement, strategy selection and altered sensory processing[30]. Scopolamine induces amnesia only by depleting acetylcholine from the binding sites of cholinergic receptors in brain; in contrast Aβ $_{(25-35)}$ induced amnesia resembles the neurotoxicity in Alzheimer's type of dementia[31]. Thus in our present study the i.c.v injection of Aβ $_{(25-35)}$ induced impairment of memory assessed by open field habituation memory and Morris water-maze tests. Aβ $_{(25-35)}$ has the potential to induce oxidative stress in the brain cholinergic hypofunction and elevation of AChE[32,33]. Moreover, it has been reported that it induces the production of hydrogen peroxide and LPO in hippocampal neurons of the rat brain and the induction of 4-hydroxy-2-nonenal and 8- hydroxy-2-deoxyguanosine (a marker of oxidative damage to DNA). The present study also indicated significant decrease in the level of antioxidant enzymes and the elevated levels of AChE in mice brain after a single i.c.v injection with Aβ $_{(25-35)}$. In other studies, the imbalances in each antioxidant enzyme were observed and also the continuous i.c.v. injection of Aβ $_{(1-42)}$ in rat resulted in a significant decrease of SOD, GPx, and glutathione- S-transferase in rat brain were demonstrated[34]. Glutathione (GSH) is the major non-protein thiol antioxidant in mammalian cells and it is considered to be the main intracellular redox buffer. GSH protects cellular protein-thiols against irreversible loss, thus preserving protein function. One of the most important GSH-dependent detoxifying processes involved is (GPx), which plays a central role in the removal of hydrogen and organic peroxides and leads to the formation of oxidized glutathione (GSSG). GSSG is reduced back to its thiol form (GSH) by the ancillary enzyme glutathione reductase (GR), leading to the consumption of NADPH, which is mainly produced in the pentose phosphate pathway. GSH also takes part in xenobiotic conjugation with the assistance of several glutathione *S*-transferase isoenzymes. GSH conjugates or GSSG can be eliminated from the cell by the family of ATP-dependent transporter pumps. It was suggested that the inhibition of GSH synthesis leads to an increase in β-amyloid induced cell death and intracellular β-amyloid accumulation[35].

These evident features implicated the antioxidants might contribute to the prevention of AD. Antioxidants such as beta-carotene and vitamins C, E and A may protect cells from the type of damage that leads to aging in the brain and other tissues. Both vitamin C and E are antioxidants that are likely reduce oxidative stress and injury in the central nervous system; this may reduce the amyloid plaque deposition in the neuronal cells[36]. Our studies also indicated a considerable increase in antioxidant enzymes in the groups treated with AEEA. An increment in Vitamin C activity was also noticed in group received AEEA at a dose of 400 mg/kg treated animals.

The treatment with AEEA decreased the cognitive deficits in Aβ $_{(25-35)}$ injected mice, especially, the data shown in open-field habituation memory with increase in the number of head dipings, line crossings and rearings. These results are consistent with the favourable effect on open-field habituation memory. In the water-maze test, consumption of AEEA decreased the escape latency almost to normal levels in the dose dependent manner. From these results it's possible to interpret that neuroprotection plays a role in the favorable effect of AEEA on Aβ $_{(25-35)}$ induced cognitive deficits.

The AChE activity has been shown to be increased within and around amyloid plaques in Alzheimer's brain .The calcium influx followed by oxidative stress is involved in the increase in activity of AChE induced by Aβ $_{(25-35)}$ peptide, decreasing cell membrane order and ultimately leading to the exposure of more active sites of the enzyme[37]. The observation in our present study predicted the Aβ $_{(25-35)}$ peptide increases AChE activity and reactive oxygen species production and that it can be possible to decrease cholinergic function. By the treatment of AEEA, AChE activity in the brain was decreased in mice treated with 200 and 400 mg/kg when compared with the negative control.

Conclusion

The traditional uses of *E.alsinoides* for improving memory defects and treatment for neurodegenerative disorders were well supported by these systematic studies. The neuroprotective activity of this plant on Alzheimer's type of dementia due to Aβ $_{(25-35)}$ toxicity is well proved by various behavioral and biochemical studies. Further studies have to be anticipated on the various extracts and isolated principles of the plant for studies with immunomodulatory and proinflammatory changes with the neurotransmitter estimation in CNS associated disorders and should be explored to mechanism based knowledge about *Evolvulus alsinoides.*

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