EVOLVULUS ALSINOIDES (EAE) PLANT EXTRACT AS A MODULATOR OF CHOLINERGIC SYSTEM WITH REFERENCE TO ALZHEIMER’S DISEASE

K. Yellamma

Department of Zoology, Sri Venkateswara University, Tirupati - 517502, Andhra Pradesh, India.

ABSTRACT: Alzheimer’s disease is a form of dementia, in which nerve cells in memory areas of brain and eventually other areas begin to die at accelerated rate resulting in serious deterioration in several mental functions such as memory loss, language, orientation and judgment. The main objective of the present study was to investigate and compare the neuro-pharmacological effects of *Evolvulus alsinoides* plant extract on the cholinergic system and Histopathological aspects in AD-induced rat model. Ethanolic extract of *Evolvulus alsinoides* Linn (EAE) was prepared and administered to rats orally at a dose of 200 mg/kg body weight. To evaluate the anti-cholinergic potential of the plant extract, selected regions of the brain viz. Cerebral Cortex, Hippocampus, Pons medulla and cerebellum were used. As a corollary to these, Histopathological experiments only on two selected regions of the brain viz. cerebral cortex and hippocampus were conducted in all groups of rats on 60th day of experimentation in order to assess the extent of cytoarchitectural changes in neurons brought out by AD induction and the reversal efficiency of EAE. A common observation was made i.e., *Evolvulus alsinoides* (EAE) extract exhibited a positive stimulatory effect on the cholinergic system in all groups of rats in general and AD-induced rats in particular. This was further reiterated by Histopathological experiments changes in the neurons of cerebral cortex and hippocampus of AD-induced rat brains have been restored back to the near normal condition after administration of EAE to AD-induced rats. In view of this, it was concluded that EAE has neuroprotective effect on cholinergic system which would pave new vistas in the discovery of safe and novel anti-Alzheimer’s compounds.

INTRODUCTION: Alzheimer’s disease, a serious form of dementia could have a devastating impact on developing countries, whose populations are aging most rapidly. Alzheimer’s disease is the sixth leading cause of death in the United States and the fifth leading cause of death for people aged 65 years and over. The risk of people dying from Alzheimer’s disease increases significantly with age. In 2010, the Population aged 85 years and over was 50 times more likely to die from Alzheimer’s disease than the age group 65–74 years. In overall, women had a 30 percent higher risk of dying from Alzheimer’s disease than men 1. It is reported that by the year 2020, approximately 70% of the world’s population aged 60 years will be located in developing countries and it was noted that 37 lakhs of Indians were already affected by Alzheimer’s disease between 2002 to 2011.

According to Cholinergic hypothesis, damage to the Cholinergic system results in reduced synthesis of the neurotransmitter ACh and elevation in AChE levels due to accelerated rate of damage of neurons resulting in serious deterioration in several mental functions, such as cognitive impairments, language, orientation and judgment etc found in
Alzheimer’s disease. Apart from this, AD is caused by a number of other changes such as extracellular deposits of β-amyloid senile plaques, intracellular formation of neurofibrillary tangles and loss of neuronal synapses and pyramidal neurons culminating in further deterioration of neuronal functions. Further, Cholinergic neurotransmission may be a specific target for β-amyloid, as it has been shown to reduce both choline uptake and ACh release in vitro. So, it is of immense interest here that disease-related changes in the Alzheimer’s disease brain are focused on pyramidal neurons which are lost in the disease condition due to tangle formation and thus represent a major source of APP regulated by a neurotransmitter (ACh).

Acetylcholine is hydrolytically destroyed in the brain by two cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) both associated with amyloid plaques. Although AChE is found in higher concentrations than BuChE in the brain tissue of patients with AD, there is evidence that BuChE is active in all hippocampal and cortical areas known to receive cholinergic innervations and also that BuChE may accelerate maturation of benign plaques into plaques associated with neuronal degeneration and AD.

Hence, therapies designed to reverse the cholinergic deficit are in large measure based on the importance of cholinergic function in cognition. In fact, reduced cholinergic activity in the brains of individuals with AD provides the rationale for the development of acetyl cholinesterase (AChE) inhibitors to treat the dementia associated with AD. The commonly prescribed AChE inhibitors, such as Donepezil®, Rivastigmine, and Galantamine were developed on this basis. Since these drugs exerted several side effects, formulation of synthetic drugs and herbal products are moving from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects and thus gaining popularity in the world market. In this context, Indian traditional medical system, Ayurveda assumes lot of significance since it is believed to be safe for all chronic diseases in general.

*Evolvulus alsinoides*, is commonly known as Shankhpushpi and in traditional systems of medicine, Ayurvedas “Medhya Rasayana” having Nootropic effects. Shankhpushpi is considered as a potential “Nervine tonic” recommended for treating several nervous disorders such as stress, anxiety, mental fatigue, and insomnia. Pre-clinical investigations have demonstrated its memory enhancement, anti-epileptic and immune-modulatory, anti-amyloidic, anti-stress (adaptogenic), anti-microbial and gastroprotective properties as well. Therefore, it is worthwhile to explore the application of traditional medicines for the treatment of various cognitive disorders.

In view of this link between the cholinergic system and neuronal damage in AD progression, in the present study the main emphasis was laid on assessment of anti-cholinergic potential of *E. alsinoides* plant extract in AD-induced rat model. Based on the results, it might be possible to suggest *E. alsinoides* plant extract as one of the best compounds for treating Alzheimer’s disease.

**MATERIALS AND METHODS:**

**Procurement and maintenance of experimental rats:** Male albino Rat, *Rattus norvegicus* of three months old, weighing 150 ± 10 grams was used as the experimental model in the present study. The rats, obtained from Sri Venkateswara enterprises, Bangalore were housed in polypropylene cages under the controlled conditions of 28 ± 2°C temperature with photoperiod of 12 hours light and 12 hours dark and 75% relative humidity maintained in the animal house of the Department according to the ethical guidelines for animal protection and welfare bearing the Resolution No. 05/(i) /a/CPCSEA/ IAEC/ SVU/ KY-DV/ dt.28/03/2011. The rats were fed with standard pellet diet and water *ad libitum*.

**Collection and Preparation of plant extract:**

**Evolvulus alsinoides** (Shankhpushpi):

**Scientific Classification:**

- Kingdom : Plantae
- Phylum : Magnoliophyta
- Class : Magnoliopsida
- Order : Solanales
- Family : Convolvulaceae
- Genus : Evolvulus
- Species : *E. alsinoides*

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Synonyms: Dwarf Morning Glory, Shankhapushpi, Vishnukranthi

A bulk amount of fresh whole, *Evolvulus alsinoides* plant material of around 5-6 kgs was collected from in and around Tirumala hills, washed with water and shade-dried to avoid evaporation of active compounds. Then, it was made in to coarse powder with motor and pestle. Then it was soaked in 95% ethanol for 10 days at room temperature and the solvent was filtered. This process was repeated 3 to 4 times until the extract gave no coloration. The extract was distilled and concentrated under reduced pressure in the Hahn vapor Rotary Evaporator HS-2005V. The resulting ethanol crude extract was air-dried and used in the present study.

**Induction of Alzheimer’s disease:** Memory impairment was induced by intraperitoneal (i.p.) injection of D-Galactose (120mg/kg body weight) to rats.

**Administration of test substance:** The selected dose of plant extract of *E. alsinoides* (200 mg/kg body weight) was dissolved in distilled water and given to the rats through a gavage tube by oral route, which is clinically accepted route for administration of EA. The volume of plant extract administered was kept at 0.2 ml per animal.

**Grouping of animals:** After the Rats were acclimated to the laboratory conditions for 10 days, they were randomly divided in to four main groups and all animals in each Group were administered with the following compounds only once in the morning hours between 8 to 9 AM, keeping in view the altered activity of rat during the nights compared to the day time.

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<thead>
<tr>
<th>Group I</th>
<th>Control (C)</th>
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<tr>
<td>Group II</td>
<td>Rats administered with <em>Evolvulus alsinoides</em> plant extract (EAE) for 60 days</td>
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<td>Group III</td>
<td>AD-induced Rats (administered with D-Galactose (D-Gal) for 60 days</td>
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<td>AD-induced Rats simultaneously treated with <em>Evolvulus alsinoides</em> plant extract (EAE) from 10th day to 60th day</td>
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In the present study, to evaluate the neuroprotective properties of the plant extract, *Evolvulus alsinoides*, the main emphasis was laid on the cholinergic system which was supported by histopathological experiments since cholinergic neurons seem to be the target cells that were affected during the progression and finally for manifestation of Alzheimer’s Disease.

**1. Cholinergic system:**

**Isolation of tissues:** For cholinergic estimations, all the four groups of Rats were sacrificed on selected time intervals i.e., on 20th, 40th and 60th days by cervical dislocation. The brain was isolated immediately, placed on a chilled glass plate and different regions of brain such as, Cerebral Cortex, Hippocampus, Pons medulla and cerebellum were separated by following standard anatomical marks frozen in liquid nitrogen (180°C) and then stored at -70°C until further use. At the time of assay of ACh and AChE, the tissues were thawed and used. The results obtained were analyzed statistically.

i) **Acetylcholine (ACh):** Selected areas of brain as mentioned above were weighed accurately, transferred to test tubes and placed in a boiling water bath for 5 minutes to terminate the Acetylcholinesterase enzyme activity and also to release the bound ACh. Then the tissues were homogenized in 1ml of distilled water. To the homogenate, 1ml of alkaline hydroxylamine hydrochloride was added followed by 1ml of 50% hydrochloric acid solution. The contents were mixed thoroughly and centrifuged. To the supernatant 0.5ml of 0.37M ferric chloride solution was added and the brown colour developed was read at 540nm against a reagent blank (1ml of
alkaline hydroxylamine hydrochloride, 1ml of 50% hydrochloride, and 1ml of distilled water and 0.5ml of 0.37M ferric chloride solution) in a spectrophotometer. The Acetylcholine content was expressed as μ moles of ACh/gm wet weight of tissue.

i. Acetylcholinesterase (AChE): 10% homogenates of selected regions of rat brain were prepared in 0.25M ice cold sucrose solution. The reaction was started with the addition of 100 μ liters of homogenate to the reaction mixture containing 3.0ml of phosphate buffer (PH 8.0), 20 μ moles of substrate (0.075M) and 100 μ moles of dithiobis trinitrobenzene (DTNB,0.01M). The contents were incubated at 37°C for 15 minutes. The developed colour was read at 412 nm in a spectrophotometer against a reagent blank containing 3.0ml of phosphate buffer (PH8.0), 20 μ moles of substrate (0.075M) and 100 μ moles of dithiobis trinitrobenzene (DTNB,0.01). The enzyme activity was expressed as μ moles of ACh hydrolysed /mg protein/hour.

2. Histopathology: For histological studies, only two selected regions of the brain viz. Cerebral cortex and Hippocampus from all groups of rats were used.

Light Microscopy: On the selected days of experimentation, rats were sacrificed by cervical dislocation and the selected brain regions such as Cerebral cortex (CC) and Hippocampus (HC) were isolated, gently rinsed with a physiological saline solution (0.9% NaCl) to remove blood and debris adhering to the tissues, fixed in 5% formalin for 24 hrs. The fixative was removed by washing through running tap water overnight. After dehydrating through a graded series of alcohols, the tissues were cleared in methyl benzoate, embedded in paraffin wax. Sections were cut at 6µ thickness and stained with hematoxylin and counter stained with eosin (dissolved in 95% alcohol). After dehydration and clearing, sections were mounted with DPX. The stained sections were observed under microscope and the histological changes were recorded with the help of a pathologist.

RESULTS: A common observation made in the present study was that Evolvulus alsinoides (EAE) extract exhibited a positive stimulatory effect on the cholinergic system in all groups of rats in general and AD-induced rats in particular. Further, our observations on the Histopathological experiments wherein the cytoarchitectural changes in the neurons of cerebral cortex and hippocampus regions of AD-induced rat brains have been reversed back to the near normal condition after administration of EAE to AD-induced rats.

Acetylcholine (ACh) content: (Fig. 1) In control rats, the ACh content was found to be highest in ponsmedulla (5.14) followed by Hippocampus (4.48), Cerebral Cortex (3.41) and Cerebellum (3.03). When compared to the control rats, ACh levels in all the experimental groups showed considerable changes in all brain regions of albino rats. A significant elevation was noticed in EAE-treated rats at all time periods and the percentage of elevation was increasing from 20th day to 60th day. Maximum percent change was noticed in Cerebellum (24.83%) followed by Cerebral cortex (23.18%), Ponsmedulla (22.74%) and Hippocampus (18.90%).

Contrary to this, in AD-induced rats, there was a remarkable inhibition in ACh content in all brain regions of rats at selected time intervals with maximum inhibition in Hippocampus (-26.96%) followed by Cerebral cortex (-24.39%), Cerebellum (-17.90%) and Ponsmedulla (-17.63%). Intrestingly, the AD-induced rats simultaneously treated with EAE showed a significant elevation in ACh content from 20th day to 60th day and the maximum elevation was recorded on 60th day in Hippocampus (-8.05%) followed by Cerebral Cortex (-7.95%), Cerebellum (-4.42%) and Ponsmedulla (-3.95%).

A note worthy observation in the present study was that on all selected days of experimentation, rats which were administered with EAE recorded a significant elevation in ACh content, while AD-induced ones showed considerate decrease in ACh content. However, in case of AD-induced rats which were simultaneously administered with EAE, the ACh content has been brought back to almost control levels thus demonstrating that EAE has reversed the AD-induced effects with particular reference to ACh.
Acetylcholinesterase (AChE) activity: (Fig. 2) As was noticed in case of ACh content, changes in the specific activity AChE were determined in selected regions of brain in control as well as all experimental groups of rats on chosen days. From the results, it was clear that in control rats, the AChE was highest in Hippocampus (13.79) followed by Ponsmedulla (11.72), Cerebral cortex (10.24) and Cerebellum (9.78). Contrary to the ACh content, the AChE activity was declined significantly in all brain regions of rats treated with EAE at selected time intervals. The percent of inhibition was gradually increased from 20th day to 60th day and the maximum percent change was noticed in Ponsmedulla (-20.72%) followed by Cerebellum (-16.34%) and Hippocampus (-12.11%). When compared with the control group, the activity of AChE in AD-induced rats increased significantly in all regions of brain, with maximum elevation in Cerebellum (14.16%) followed by Cerebral cortex (11.82%), Ponsmedulla (10.02%) and Hippocampus (9.41%). As in the case of ACh content, the AChE levels in AD-induced rats which were simultaneously treated with EAE was decreased significantly from 20th day to 60th day in all brain regions. Maximum inhibition was recorded on 60th day in Ponsmedulla (10.25%) followed by Cerebellum (8.88%), Cerebral cortex (5.46%) and Hippocampus (3.84%).
FIG. 2: GRAPHICAL REPRESENTATION OF PERCENT CHANGES IN THE ACTIVITY OF ACETYLCHOLINESTERASE (IN VIVO) IN CEREBRAL CORTEX (CC), CEREBELLUM (CB), HIPPOCAMPUS (HC), PONSMEDULLA (PM), REGIONS OF EXPERIMENTAL GROUPS OF RATS TREATED WITH EAE, D-GALACTOSE AND D-GALACTOSE + EAE

Histopathology: (Photographs 1 and 2.): In the present investigation, Histological studies were conducted only on two important regions viz. Cerebral cortex and Hippocampus since these two regions are involved in perception and processing of higher mental faculties such as learning and memory. The selected day for these studies was 60th day after inducing AD and treatment of rats with EAE, since remarkable cytoarchitectural changes were observed on this day.

From the observations, it was obvious that both the regions viz. the cerebral Cortex and hippocampus of control rat showed neuronal cells with all subcellular structures in intact condition, while in AD-induced rat the neuronal cells have undergone maximum damage and formation of plaque which is the characteristic feature of AD was obvious. It is of interest here that disease related changes in the Alzheimer’s disease brain are focused on pyramidal neurons in that these cells are lost in the disease, subject to tangle formation, represent a major source of APP and are regulated by a neurotransmitter (ACh), affected early in the disease.

Interestingly, in AD-induced rats simultaneously treated EAE, the number of plaques was minimized and the cellular damage caused by AD induction was reversed to near normal condition thus demonstrating the positive effect of EAE on the cytoarchitectural of the neurons.
PHOTOGRAPH 1: HISTOPATHOLOGICAL CHANGES IN THE CEREBRAL CORTEX REGION OF CONTROL AND EXPERIMENTAL GROUPS OF RAT ON 60TH DAY OF TREATMENT

PHOTOGRAPH 2: HISTOPATHOLOGICAL CHANGES IN THE HIPPOCAMPUS REGION OF CONTROL AND EXPERIMENTAL GROUPS OF RAT ON 60TH DAY OF TREATMENT
DISCUSSION: In the present study, D-Galactose treated rats were used as AD model for all experiments. From the results on the cholinergic system, it was obvious that oral administration of EAE caused significant elevation in ACh and declined the AChE activity in all selected regions of brain, whereas in AD-induced rats, a reverse trend was noticed i.e. ACh content was inhibited and AChE activity was elevated. In general, the present investigation on cholinergic system in different regions of rat brain following the oral administration of EAE has shown the neuroprotective effect on cholinergic system by increasing the levels of ACh content and by inhibiting the AChE activity in both normal and D-Galactose treated rats.

These observations in our study derive strong support from several earlier findings wherein it was reported that *E. alsinooides* by virtue of having a compound namely, MENTAT potentially inhibits AChE and acts as a brain tonic in the management of many neuronal disorders viz. mania, epilepsy insomnia etc. Further, my observations on the enzyme, AChE activity in AD-induced rat revealing that chronic administration of D-galactose caused a marked increase in acetylcholinesterase (AChE) was well supported by similar findings by 5.

Cholinergic neurotransmission may be a specific target for β-amyloid, as it has been shown to reduce both choline uptake and ACh release in vitro. Similar studies have revealed 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. A large number of studies have shown that the cholinergic system plays an important role in learning and memory.

Central cholinergic dysfunction causes the cognitive symptoms of various neurological diseases. Loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and AD. There is considerable evidence implicating acetylcholine (ACh) in higher brain functions such as attention, learning and memory. Alzheimer disease was represented by the degeneration of the cholinergic connection from the nucleus of Meynert to the cortex and hippocampus.

The activity of the enzyme responsible for the synthesis of acetylcholine, choline acetyltransferase, a reliable marker of cholinergic neurons and synapses, was found to be remarkably decreased, sometimes in rather severe way, in pathological samples from the cortex and hippocampus of Alzheimer’s patients. The parallel observation of a substantially decreased number of the cholinergic neurons led to conclude that the pathogenesis of Alzheimer disease was represented by the degeneration of the cholinergic connection from the nucleus of Meynert to the cortex and hippocampus. As memory impairment and dementia are primary symptoms of Alzheimer’s disease, the then emerging role of acetylcholine transmission in cognitive functions supported this conclusion.

Our observations on the histological aspects demonstrated that almost all cell types have undergone changes in their cytoarchitectural in AD-induced rat brain which were, of course reversed by EAE treatment. An interesting finding was that most of the cortical AChE activity present in AD brain is predominantly associated to the amyloid core of senile plaques rather than with the neuritic component found in the periphery. This was supported by the earlier report where in double AChE-APP transgenic mouse presents more and larger plaques than the control animals, as well as some behavioural deterioration, as demonstrated by a working memory test. Indeed, injection of the complex into the rat hippocampus produces neuronal cell loss an astrocyte hypertrophy. Similarly, changes in AChE molecular forms in Cerebro Spinal Fluid (CSF) reflect changes in the brain and also AD plasma 6.

Changes in the central cholinergic system in AD may also contribute to a variety of adverse behavioral symptoms (i.e., in addition to cognitive deficits) such as depression, aggressive behavior, psychosis, and over activity.
An increase in AChE levels around amyloid plaques and NFT is a common feature of AD neuropathology, and although the significance of this increase remains to be determined. There is evidence that acetylcholinesterase (AChE) accelerates assembly of amyloid-b-peptides into the amyloid fibrils that form the senile plaques characteristic of AD. A study on a double transgenic mouse over expressing both the human APP containing the Swedish mutation and the human AChE started to form amyloid plaques around 3 months, earlier than mice expressing only the APP transgene. Indeed, injection of the complex into the rat hippocampus produces neuronal cell loss and astrocyte hypertrophy. Brain regions that are associated with higher mental functions, particularly the neocortex and hippocampus, are those most affected by the characteristic pathology of Alzheimer’s disease. Loss of cortical pyramidal neurons, synapse loss, and reduced glutamate concentration, together with the formation of neurofibrillary tangles, all correlate with the severity of dementia.

Observations on the Histological aspects over the four groups of rats i.e. Control, EAE-treated, AD-induced, AD-induced+simultaneously treated with EAE provided further substantial evidences showed that administration of D-galactose induced production of reactive oxygen species (ROS) and inflammatory response resulting in neurodegenerative changes. D-gal is a reducing sugar and can be metabolized at normal concentration. However, at high levels, it induces the production of reactive oxygen species (ROS) and advanced glycation end products (AGEs) 7-8. Recent findings show that ROS and AGES induced by continuous injection of D-gal in rodent lead to the decreased expression of memory-related protein, deterioration of learning and memory function, and pathological alterations of astrocytes which might be associated with the increased expression of inflammation related gene 7, 9-10. Further, it was demonstrated that both ROS and AGES have been implicated in the pathological processes of age-related disease such as diabetes, arteriosclerosis, nephropathy, infection, and Alzheimer’s disease 11.

D-gal-induced senescence acceleration has been widely used as a aging model for studying aging mechanisms and screening drugs. As with the onset of cognitive decline, the synaptic plasticity deficits manifest prior to the accumulation of the hallmark pathological lesions and best correlate with intra neuronal Aβ. Overall, findings suggest that synaptic dysfunction and cognitive decline are early events in the pathogenesis of AD. These findings are also consistent with results from another transgenic model in which synaptic deficits were found to occur independently of plaque accumulation. By contrast, transgenic mice that overexpress Ab1–42 show fairly robust cell loss. Notably, some of the tau transgenic mice show cell loss, suggesting that neurofibrillary pathology might be are quite for neuronal loss. The neurotransmitter-protein interactions that occur in the Alzheimer’s disease brain, may further contribute to increased tau phosphorylation and β- amyloid production and hence neurodegeneration in selectively vulnerable regions. Furthermore, it is possible that AChE inhibitors may reduce the histopathological features of disease progression.

CONCLUSIONS: The present study clearly demonstrated that consecutive i.p. injection of D-gal (120 mg/kg/d) for 2 months produced AD-like disorders, including neurodegenerative evidence, Cortex & hippocampus neuron abnormalities etc, which can be nullified with simultaneous treatment of AD-induced with EAE. The biochemical studies on the cholinergic system estimations indicate the neuroprotective effect of EAE. Histopathological studies stand as perfect evidence for the above results by reverting the cell damages with oral administration of EAE.

REFERENCES: