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Original Research Article

Neuroprotective Effect of *Clerodendrum serratum* in Amyloid $\beta_{(25-35)}$ Induced Amnesia Mice

Thirupathi G, Babu Rao B, Narsimha Reddy Y*
Department of Pharmacology, University College of pharmaceutical sciences, Kakatiya University, Warangal, Telangana – 506009, India

***Corresponding author**
Narsimha Reddy Y
Email: ynrucpsc@gmail.com

Abstract: The content of the work is to determine the role of methanolic root extract of *Clerodendrum serratum* (CS) in amnesia induced mice by using β-amyloid peptide and compared with normal mice. The CS having anti-cancer, anti-helmenthic activity. But, the activity of root extract of CS as neuroprotective was not reported. Hence, the root extract of CS was tested for neuroprotective activity in neurodegenerative mice. The root extract were subjected to behavioral tests such as conditioned avoidance test, rectangular maze test, water maze test. Further, effect of extract on acetylcholinestarase, peroxidase, MDA levels and antioxidant activity is tested. From the results, it was observed that the root extract of CS exhibited statistically significant anti-amnesic and anti oxidant activity, when compared to normal mice. Therefore, the results conclusively demonstrate that, the chemical constituents of root extract responsible for neuroprotective activity, which might be due to oxidative stress.
Keywords: Neuroprotective action, *Clerodendrum serratum*, amyloid β peptide, behavioral test and biochemical parameters.

INTRODUCTION

Alzheimer's disease (AD), is a devastating neurodegenerative disease with progressive loss in memory [1] and it is associated with a shrinkage of brain tissue, with localized loss of neurons mainly in the hippocampus and basal forebrain [2]. AD is characterized by the deposition of extracellular amyloid plaques and intracellular neurofibrillary tangles (composed of paired helical filaments) in the brain of AD patients [3]. The loss of cholinergic neurons in the hippocampus and frontal cortex is a feature of the disease [4].

The reduction in cholinergic activity is correlated with the degree of cognitive impairment. Several drugs have been designed to enhance cognitive function in AD patients by targeting acetylcholinesterase (AChE), in an attempt to maximize the effect of AChE by increasing its permanence in the synaptic cleft [5]. Acetylcholinesterase inhibitors (ACIs) are the best developed therapy and are used for mild to moderate disease. The mechanism by which ACIs slow progression of disease is thought to be by decreasing levels of amyloid-β protein precursor (AβPP) and production of Aβ and amyloidogenic compounds. Tacrine was the first widely used ACI. Second generation cholinergics; including donepezil, galantamine and rivastigmine have been developed. These drugs have fewer side effects, longer half-lives, and greater efficacy [6].

Current AD treatments have not been shown to prolong life, cure AD, or halt or reverse the pathophysiologic processes of the disorder. Enormous effort is now being devoted to developing drugs that slow neurodegeneration and the drugs that offer neuroprotection in AD. Although several approaches involve new agents for recently discovered targets, many are based on new applications of existing drugs such as antioxidants, statins, NSAIDS, antibiotics [7, 8].

One of the myths claims of the plant *Clerodendrum serratum (CS)* is used as rejuvenator in the treatment of neurodegeneration and in memory. Previously, root extracts of CS possess hepatoprotective activity [9], antioxidant activity [10], anticancer activity [11], antinociceptive activity [12] and anti-inflammatory activity [13]. But, the effect of root extract on neurodegenerative disorder not reported till now. Hence, we attempted to study the role of CS root extract as neuroprotective agent in amnesia induced mice.

Main objective of the study is to assess the pharmacological activity of *Clerodendrum serratum* root extract on β-amyloid peptide induced amnesia in mice. Currently available drugs to treat AD like AChE inhibitors exert symptomatic relief but, do not reduce progression of disease and have side effects. Herbal medicines may provide potential effect as compared to conventional available synthetic drugs with less or no side effects.

MATERIALS AND METHODS

Chemicals

Acetylthiocholine iodide, dithiobisnitro benzoic acid, reduced glutathione, amyloid $\beta_{(25-35)}$ were purchased from Sigma Aldrich, USA. Other reagent solvents including ethyl acetate, methanol used were analytical grade purchased from Himedia laboratories, Hyderabad, India. DPPH, ascorbic acid, thiobarbituric acid, sodiumdodecyl sulphate, tetra ethoxy propane, trichloro acetic acid, donepezil were purchased from Himedia laboratories, Hyderabad, India.

Plant material and extraction

The leaves of *Clerodendrum serratum* were collected from the Kakatiya University, Warangal, Telangana State, India, during month of May and authenticated by Botanist Prof. Mustafa, department of Botany, Kakatiya University, Warangal, Telangana, India. The collected root was shade dried, powdered and extracted with methanolic solvent by using soxhalet apparatus.

Experimental animals

All the animals handling and experimentation were conducted in accordance with the prior approved guidelines from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi and the experimental protocol was approved by Institutional Animal Ethical Committee (IAEC/05/UCPSc/KU/2016). Swiss albino mice (male) weighing 22-25 g at the age of 5-6 weeks, obtained from Sainath agencies, Hyderabad 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 (Vyas Labs, Hyderabad) and water *ad libitum*. The animals were acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into five groups of six animals each.

Grouping and induction of neurotoxicity

Neurotoxicity was induced by intra cerebroventricular (i.c.v.) injection of $A\beta_{(25-35)}$ peptide by identifying bregma point in the skull. Each animal was injected with 10µl which contain 10µg of β-amyloid peptide [14]. Except group I-positive control, received with i.c.v. injection of phosphate buffered saline (PBS), group II-negative control received only with i.c.v. injection of β-amyloid peptide, group III-animals injected with β-amyloid peptide and treated with Donepezil (p.o.) 5mg/kg which is used as standard drug, group IV and V-animals are injected with β-amyloid peptide and treated with 200 mg/kg and 400 mg/kg of methanolic root extract, respectively. Drug treatment started on 14th day of the amyloid-β peptide treatment and continued for six days. On the 7th day of the drug treatment or 21st day after amyloid-β peptide treatment behavioral studies and biochemical parameters were estimated [15, 16].

Jumping box (conditioned avoidance test)

It is done by using medicraft jumping box. Box divided into 2 equal chambers by plexiglass partition, with a gate providing access to adjacent compartment through 14 x17 cm space. In each trial animal is subjected to light for 30 seconds followed by a sound stimulus for 10 seconds. Immediately after sound stimulus, mice receive a single low intensity foot shock (0.5 mA, 3 sec). Each animal received a daily session of 15 trials with an inter trial duration of 15 seconds for 5 days [17].

Rectangular Maze test

Assessment of memory was done using medicraft rectangular maze. The apparatus consisted of three interconnected chambers A, B and C. Chamber B constituted the maze. Food deprived mice were placed in chamber A and challenged to learn and remember the location of C, after travelling through B. Their presence in chamber C was indicated by a pilot light. Chamber C contained the reward which was food for the hungry animal. The animals were trained for consecutive daily sessions, and the time required to traverse the maze was noted. They were considered trained when the maze completion time for 3 consecutive days was more or less constant. Maze traverse time was then recorded for each animal before and after drug treatment [18].

Y-maze test

The Y-maze task was used to measure the spatial working memory in mice. The maze is made of gray plastic. Each arm is 40 cm long, 13 cm high, 3 cmwide at the bottom, 10 cm wide at the top, and converged at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze for 8 min. Mice tend to explore the maze systematically, entering each arm in turn. The ability to alternate requires that the mice know which arm they have already visited. The series of arm entries, including possible returns into the same arm, is recorded by a video-tracking system (VJ Instruments, Washim, Maharastra, India). Alteration is defined as the successive entries into the three arms, on overlapping triplet sets. The percentage of alteration is calculated as the ratio of actual alterations to possible alterations, defined as the total number of arm entries minus two, and multiplied by hundred. Typically, mice exhibit an alteration percentage of 60–70%, and perform 25–35 arm entries within the 8 min session [3].

BIOCHEMICAL ESTIMATIONS

Acetylcholinesterase (AChE) enzyme

Acetylcholinesterase (AChE) enzyme levels are estimated by as per the method described by Ellman *et al.*, [19]. In a Potter-Elvehjem homogenizer 20 mg of brain tissue/ml with Phosphate buffer (pH 8, 0.1 M) was homogenized. 0.4 ml aliquot was added to cuvette contain 2.6 ml of 0.1M phosphate buffer (pH 8). To the photocell 100 µl DTNB solution was added and absorbance was read at 412 nm. Then change of absorbance was recorded after adding 20 µl of the acetylthiocholine iodide and change in absorbance/minute was calculated and activity of enzyme is expressed as µmoles/min/g tissue.

Assay of glutathione peroxidase

GSH was determined by its reaction with 5,5-dithiobis(2-nitrobenzoic acid) DTNB to yield a yellow chromophore which was measured spectrophotometrically [20]. The brain homogenate was mixed with an equal amount of 10% trichloro acetic acid and centrifuged at 2000 g for 10 minutes. The supernatant was used for GSH estimation. To 0.1 ml of processed tissue sample, 2 ml of phosphate buffer (ph 8.4), 0.5ml of DTNB and 0.4 ml of double distilled water were added and the mixture was shaken vigorously. The intensity of color developed was read at 420 nm immediately in spectrophotometer. The activity of GPx is expressed as µmoles/ minutes/ mg protein.

Estimation of MDA

MDA which is a measure of lipid peroxidation was described by Ohkawa *et al*., [21]. Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 sodium phosphate buffer (pH 7.4). the reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulfate (8.1%) were added to 0.1 ml of processed tissue sample. Mixture was then heated at 100 c for 60 minutes. Mixture was then cooled with tap water and 5 ml of n-butanol:pyridine (15:1 % v/v), 1ml of distilled water was added. Mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, organic layer was withdrawn and absorbance was measured at 532 nm using spectrophotometer. 1,1,3,3-tetra ethoxy propane is used as standard.

DPPH radical scavenging activity

The capacity to scavenge the ‘‘stable’’ free radical DPPH by methanol was measured which is based on the reduction of methanolic solution of the coloured free radical of 1, 1- diphenyl-2-picryl hydrazyl (DPPH) [22]. A methanol DPPH solution (0.1 mM, 1 ml) was mixed with serial dilutions (10, 20, 40, 60, 80 µg/ml) of the methanol extract incubated for 30 min at room temperature. For each concentration the assay was run in triplicate and the absorbance was read at 517 nm. Ascorbic acid was used as standard to compare with extract. IC_{50} (the antiradical dose required to cause a 50% inhibition) for ascorbic acid, methanolic extract was determined.

RESULTS AND DISCUSSION

The roots of *Clerodendrum serratum* were shade dried, powdered and extracted with methanol solvent. Yield is noted in terms of w/w of dry material and yield was found to be 21.4% for methanol extract. The test extract was considered as non-toxic, because it did not show any toxic signs or symptoms and mortality in the oral dose of 2000 mg/kg in mice. According to OECD-423 guidelines, the LD_{50} of 2000 mg/kg and above is mentioned as unclassified. So further pharmacological screening is carried out. Hence, two doses i.e., 200mg/kg and 400 mg/kg of methanolic extract was selected for the neuroprotective study.

Jumping box (conditioned avoidance test)

The open field habituation memory was depicted in the Table 1. In jumping box test, there was an in increase in latency period in negative control group (19.8 ± 1.96) when compared to positive control (1.92 ± 0.35), and there is decrease in latency period in groups treated with ME (13.1 ± 1.22 and 22.7 ± 2.14 for 200 mg/kg and 400 mg/kg, respectively).

Table 1: Effect of methanol extract on behavioral activity by Jumping box, rectangular maze and Y-maze test.

Group	Jumping box test	Rectangular maze test	Y-maze test
	Mean ± SD	Mean ± SD	Mean ± SD
Positive control	1.92 ± 0.35	34.65 ± 1.63	1.92 ± 0.35
Negative control (βA)	19.8 ± 1.96	126.49 ± 9.54	19.8 ± 1.96
Standard (dpz)	6.16 ± 1.03***	62.64 ± 2.77	6.16 ± 1.03***
ME (200 mg/kg)	13.1 ± 1.22	96.9 ± 3.76	27.54 ± 1.84***
ME (400 mg/kg)	22.7 ± 2.14**	72.84 ± 1.86	35.80 ± 2.04*

All the values are expressed as mean±SD (n=6). *p<0.05,**p<0.01,***p<0.001 when compared to negative control group. ANOVA (one-way) followed by Bonferroni’s test.

Rectangular maze test

The hippocampal learning of Aβ induced group (negative control) was declined and shown a significant (p<0.001) increase in escape latency while comparing the control group. In the treatment groups, the low dose of methanolic extract (200 mg/kg) depicted a significant (p<0.05) decrease in escape latency duration, the high dose (400 mg/kg) does not significantly (p>0.05) reduced the duration of time taken to escape onto the escape platform but there was a significant (p<0.05) change in high dose treated animals when compared to the low dose treated animals. The

readings were showed in Table 1. From the results, in rectangular maze test there was an increase in maze traverse period in negative control group (126.49 ± 9.54) when compared to positive control (34.65 ± 1.63), and there is a decrease in traverse period in groups treated with methanolic extract (96.9 ± 3.76 for 200 mg/kg and 72.84 ± 1.86 for 400 mg/kg).

Y-maze test

The percentage alteration in the negative control group (II) was significantly (p<0.001) reduced when compared with the positive control group. In the treatment groups, the 400 mg/kg dose treated group exhibited significant improvement when compared with 200 mg/kg dose treated methonalic extract (Table 1).

BIOCHEMICAL ESTIMATIONS

The differences in biochemical estimations in treatment groups were summarized in the Table 2. The i.c.v injection of Aβ peptide in negative control animals showed an extremely significant (*P*<0.001) increase in brain AChE levels. The AChE enzyme levels were significantly decreased in the treatment groups and indicated a difference with p<0.01, p<0.05, *p*<0.001 and p<0.05, respectively when compared with the amnesia induced group.

Table 2: Acetylcholinestarase levels of positive control, negative control, standard (dpz), methanol extract treated (ME) treated groups

Group	AChE (µmole/min/mg)	GSH (µmole/min/mg protein)	MDA (µg/gm wet tissue)
	Mean ± S.E.M	Mean ± S.E.M	Mean ± S.E.M
Positive control	8.64 ± 1.12	23.69 ± 1.53	15.64 ± 0.53
Negative control (βA)	15.78 ± 1.83	13.71 ± 1.05	36.91 ± 0.81
Standard (dpz)	7.72 ± 0.83	21.37 ± 0.93**	20.91 ± 0.66
ME (200 mg/kg)	26.58 ± 1.63***	15.28 ± 1.66*	31.64 ± 0.74*
ME (400 mg/kg)	22.91 ± 2.01***	17.59± 1.54*	29.79 ± 0.93*

All values are expressed as mean±S.E.M (n=6). *p<0.05, **p<0.01, ***p<0.001 when compared to negative control group. ANOVA (one-way) followed by Bonferroni's test

From biochemical parameters, it was found that acetyl cholinesterase levels are increased in negative control group (15.78 ± 1.83), when compared to positive control (8.64 ± 1.12), and decreased levels are observed in methanolic extract at 200 mg/kg (26.58 ± 1.63) and 400 mg/kg (22.91 ± 2.01), respectively. The dose of methonalic extract not showed any statistically significant effect on AChE levels.

Glutathione levels are decreased in negative control group (13.71 ± 1.05) when compared to positive control group (23.69 ± 1.53), and levels are increased in methanolic extract treated groups (15.28 ± 1.66 for 200 mg/kg and 17.59± 1.54 for 400 mg/kg dose).

MDA levels are increased in negative control group (36.91 ± 0.81) when compared to positive control group (15.64 ± 0.53), and decreased levels are observed in methanolic extract treated groups (Table 2).

The evaluation of antioxidant property on AD induced mice expressed the prospective effect of methanolic extract from *CS*. In the negative control group, all antioxidant parameters of brain were significantly (p<0.001) decreased, when compared to phosphate buffered saline treated group i.e., positive control group. There was a significant (p<0.001) increase in all the antioxidant levels were observed in the standard drug treated group. From DPPH scavenging assay, the IC_{50} of methanolic extract at 400 mg/kg was found to be 41.32 µg/mL, and for 200 mg/kg was found to be 28.77 µg/mL. Further, IC_{50} value of ascorbic acid was found to be 3.75 µg/mL.

CONCLUSION

The pharmacological evaluations on methanolic extract of *Clerodendrum serratum* indicated the anti-amnesic effect. The neuroprotective and anti-amnesic effect of these drugs were evidently supported by decrement of neurotransmitter metabolic enzyme (AChE and glutathione) with escalation in antioxidants. Thus to conclude that, the methanolic extract with two doses are containing bioactive molecule and showed the expressed a prospective turnover of biogenic amine for learning and memory process associated with neuroendocrine and neuroimmune bidirectional pathways.

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