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Original Research Article (Experimental)

Protective role of hydroalcoholic extract of *Cajanus cajan* Linn leaves against memory impairment in sleep deprived experimental rats

Layeeq Ahmad, Md Mujahid, Anuradha Mishra*, Md Azizur Rahman

Bioactive Research Laboratory, Faculty of Pharmacy, Integral University, Lucknow, Uttar Pradesh, 226026, India

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ABSTRACT

Background: The plant *Cajanus cajan* had earlier shown protective effect against hypoxic-ischemic brain damage in rats.
Objective: Hence, hydroalcoholic extract of *C. cajan* Linn leaves (HECC) was evaluated for its protective role against memory impairment in sleep-deprived Sprague Dawley rats.
Materials and methods: Adult rats were divided into five groups each consisting of 5 rats ($n = 5$). Groups I, II, III, IV and V received 1 mL/kg 1% CMC, 1 mL/kg 1% CMC, 200 mg/kg HECC, 400 mg/kg HECC and 200 mg/kg piracetam respectively as per b.wt. orally everyday for 14 days. Animals of every groups except group-I were subjected to sleep-deprivation from 15th to 19th day for induction of memory impairment. Behavioral activities i.e., elevated plus maze test and locomotor activity were evaluated. Afterwards, brain was isolated from the sacrificed animals for biochemical investigation of acetylcholinesterase (AChE); antioxidant activities i.e., catalase (CAT), superoxide dismutase (SOD), lipid peroxide; and histopathological changes.
Results: The percent number of entries, number of entries in open arm, AChE activity, lipid peroxide activity of HECC-treated group-III and group-IV were significantly ($p < 0.01$) decreased while, their CAT and SOD activities were significantly ($p < 0.01$) increased in dose-dependent manner as compared to sleep-deprived group-II. The activities of group-IV were almost significantly equivalent to that of piracetam-treated group-V. Protective effect of HECC was well supported with brain's histopathology.
Conclusion: HECC possesses a protective effect against memory impairment indicating its therapeutic efficacy against memory loss as in Alzheimer's disease. Probable underlying mechanisms may be brain's AChE inhibition and increased antioxidant potential by HECC.

1. Introduction

Amnesia, impaired memory, is one of the symptoms of some degenerative brain diseases, such as Alzheimer's disease (AD) caused by brain damage through injury or by the use of particular drugs usually sedatives [1]. More fundamental pathological abnormalities in AD are neurofibrillary tangles, amyloid plaques and neuronal cell death. Disorders of several neurotransmitters to different degrees occur in AD patients where level of acetylcholine (ACh) is decreased [2]. Among the various acetylcholinesterase (AChE) inhibitors; huperzine-A and galantamine, isolated from plant's extracts, have been used to treat the early symptoms of AD where elevation of ACh leads to modification of amyloid precursor protein, improvement of central cholinergic synapses, improved synthesis of neurotrophics and protection of neuronal degeneration [3,4].

The plant *Cajanus cajan* Linn (family Leguminosae, subfamily Rapiolanaceae), commonly known as Pigeon pea, is widely cultivated and used in most parts of India as rich source of protein [5,6]. Leaves of *C. cajan* are rich in flavonoids (flavones, flavonones, isoflavones and chalcone) and stilbenes [7–10]. It has shown protective effects against alcohol-induced liver damage, carbon tetrachloride-induced hepatotoxicity and hypoxic-ischemic brain damage in rats [11–13]. It also possesses antimicrobial, hepatoprotective and centrally acting analgesic activities [14,15]. It has been used as hepatoprotective, antidiabetic, antiulcer, antiinflammatory, antimicrobial, CNS depressant, anticancer, analgesic

* Corresponding author.
E-mail: anuradha@iul.ac.in
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and anthelmentic agent in the traditional systems of medicine [16–18]. Despite the brutality and high incidence of the memory impairment as in Alzheimer's disease, the allopathic system of medicine is yet to provide a suitable drug for its treatment. Hence, the present study was undertaken to investigate protective role of hydroalcoholic extract of *C. cajan* Linn leaves (HECC) against memory impairment as in Alzheimer's disease on sleep deprived rats.

2. Materials and methods

2.1. Drugs, chemicals and equipments

All the chemicals used in present research experiments were of analytical grade. Some of the drugs, chemicals and equipments used in the experiments were piracetam (UCB India Pvt. Ltd), 5,5-dithiobis (2-nitro benzoic acid) (DTNB, Himedia chemicals), ethanol (Changshu Yangyuan chemical, China), sucrose (SD fine chem. Ltd), anhydrous potassium dihydrogen phosphate (Chemikabiochemika reagent), disodium monohydrogen phosphate (SD fine chem. Ltd.), monosodium dihydrogen phosphate (SD fine chem. Ltd), standard pellet diet (Dayal animal feed Unnao, India), micropipette (10–100 μL & 100–1000 μL) (Superfit), centrifuge (Spin win), UV-spectrophotometer (PharmaSpec UV-1700 Shimatzu), digital balance (Shimatzu AUX220 Unibloc (PAT 1987)) and refrigerator (Intello cool LG).

2.2. Procurement and authentication of the plant materials

Leaves of *C. cajan* Linn were collected from the nearby region of Kukrel forest located in Lucknow, Uttar Pradesh (India) in the month of March. It was taxonomically identified and authenticated by the botanists, authentication office, Faculty of Pharmacy, Integral University, Lucknow, India (authentication reference number: *IU/PHAR/HRB/16/25*).

2.3. Preparation of plant extract

Leaves of *C. cajan* Linn were shade dried, subjected to coarse powder with the help of mechanical grinder and then, extracted with 70% hydroalcoholic solvent by cold maceration process for 48 h with intermittent agitation. The obtained extract was filtered and concentrated under reduced pressure below 40 °C using rotary evaporator (Buchi Rotavapor-R, Labco, India) to dryness to get a constant weight. Its extractive value was calculated and then, the dried extract (HECC) was stored below 10 °C for further research studies [19].

2.4. Experimental animal

Adult rats, *Rattus norvegicus* strain Sprague Dawley (SD) of either sex (150 ± 20 g) were procured from Central Drug Research Institute (CDRI), Lucknow and kept in departmental animal house. They were housed separately in several polypropylene cages for acclimatization at a temperature of 23 ± 2 °C and relative humidity of 50–60% with a 12 h dark/light cycle one week before and during the commencement of the study period. They were kept on standard pellet diet and drinking water *ad libitum* throughout the study. Animal experimentation study protocol was approved by Institutional Animal Ethics Committee (IAEC), Faculty of Pharmacy, Integral University (IU), Lucknow, Uttar Pradesh, India (Approval number: *IU/IAEC/15/04*).

2.5. Experimental study protocol and treatment schedule

Protective effect of hydro-alcoholic extract of *C. cajan* Linn leaves (HECC) was evaluated against memory impairment in AD using five groups of adult SD rats each consisting of 5 rats ($n = 5$). Animals were housed as 5 SD rats per cage for one week in the departmental animal house at 23 ± 2 °C temperature with appropriate feeding prior to the experimentation. Group I served as Sham control and received 1% CMC (1 mL/kg b. wt., *po*) once a day for 14 days. Group II served as stress control and received 1% CMC (1 mL/kg b. wt., *po*) once a day for 14 days. Groups III and IV served as test drug treated groups and received HECC (200 and 400 mg/kg b. wt. *po*, respectively) once a day for 14 days [20]. Group V served as standard drug-treated group and received standard drug piracetam (200 mg/kg b. wt., *po*) once a day for 14 days. Then, rats of all the groups except group I were subjected to sleep deprivation from 15th to 19th day [21]. Food and water were availed properly during these 5 days of sleep deprivation for induction of memory impairment as in AD. All the behavioral activities such as elevated plus maze test and locomotor activity were evaluated. Rats were sacrificed by instant decapitation after 2 h of drug treatments. The brain was quickly removed and kept in an ice bath. It was isolated for biochemical investigation i.e., AChE activity and evaluation of antioxidant activities i.e., catalase (CAT) and superoxide dismutase (SOD) activities [22].

2.6. Evaluation of effect of hydroalcoholic extract of Cajanus cajan Linn leaves on behavioral activity in SD rats

2.6.1. Evaluation of effect of hydroalcoholic extract of Cajanus cajan Linn leaves on elevated plus maze test for spatial memory in SD rats

Plus maze apparatus was consisted of two open arms (50 cm × 10 cm) crossed with two closed arms of same dimensions. It was elevated to a height of 25 cm above the floor and a fine line was drawn in the middle of the floor of each enclosed arm. The arms were joined by central area (5 cm × 5 cm) to furnish the apparatus a plus sign (+) appearance. All the rats of each group were given a single trial on the apparatus. Each rat was placed individually at the end of open arm facing away from central platform. Time taken by the rats to enter from open arm with all four legs into enclosed arm was taken as transfer latency time (TLT). It was gently pushed into enclosed arm in case it did not enter the enclosed arm within 90s and a TLT of 90s was assigned to it. The rat was allowed to explore the maze for an additional 10s after the measurement of TLT. It was repeated on day 2nd and 3rd also with an aim to achieve a low level of TLT. Then after the trial, each rat of all the groups was given their respective treatment according to experimental protocol and they were put on the elevated plus maze and TLT was measured. TLT measured on plus maze on third training trial served as index of learning or acquisition [23].

2.6.2. Evaluation of effect of hydroalcoholic extract of Cajanus cajan Linn leaves on locomotor activity in SD rats

The locomotor or horizontal activity was measured by using an Actophotometer. Each rat of all the groups was given their respective treatments according to the experimental protocol and the rats after 60 min of last treatment were placed in the Actophotometer for recording the activity score. Each rat was placed individually in the Actophotometer for 5 min and basal activity was obtained [24].

2.7. Evaluation of effect of hydroalcoholic extract of Cajanus cajan Linn leaves on brain AChE activity in SD rats

Isolated brain was used to measure AChE activity. A known weight of the brain tissue was homogenized in 0.32 M sucrose solution to get a 10% homogenate that was centrifuged at 3000 rpm for 15 min followed by centrifugation at 10000 rpm for 10 min at a constant temperature of 4 °C. Following centrifugation, 1 mL of the supernatant was mixed with 9 mL of sucrose solution to get a 1% post mitochondrial supernatant (PMS). Reaction mixture containing 2.7 mL of phosphate buffer, 0.1 mL of DTNB and 0.1 mL of 1% PMS was taken in a cuvette and pre-incubated at 37 °C for 5 min. Reaction was initiated by addition of 0.1 mL acetylthiocholine iodide substrate. Absorbance of the yellow colored compound formed during reaction was measured after every 1 min interval for the period of 3 min at 412 nm [24,25]. A blank was determined without 1% PMS. AChE activity was calculated using the formula [$R = (\Delta A/\text{min} \times \text{volume of assay})/(\text{extinction co-efficient} \times \text{mg of protein})$], where; R = rate of enzyme activity (nmol acetylthiocholine iodide hydrolyzed per min per mg protein); extinction co-efficient = 13600/M/cm; and volume of assay = 3 mL.

2.8. Evaluation of effect of hydroalcoholic extract of Cajanus cajan Linn leaves on antioxidant activity in brain tissues isolated from SD rats

2.8.1. Estimation of catalase activity

Isolated brain tissue was homogenized in a 50 mM, pH 7.4 potassium phosphate buffer solution in the ratio of 1:10 (w/v). The homogenate obtained was centrifuged in a cooling centrifuge at 4 °C and 10000 rpm for 20 min. The 50 μL supernatant was added to a cuvette containing 2.95 mL of hydrogen peroxide (19 mM/L) prepared in the phosphate buffer. CAT activity was evaluated on the basis of principle that the CAT enzyme decomposes hydrogen peroxide leading to a decrease in absorbance. Absorbance was recorded at 240 nm wavelength for 3 min at the interval of 1 min each. CAT activity was calculated by the formulae [CAT activity = (ΔA/min × volume of assay)/(0.081 × volume of homogenate × mg of protein)] [26].

2.8.2. Estimation of superoxide dismutase activity

Hundred microliter of obtained cytosolic supernatant was added to Tris HCl buffer (pH 8.5) and volume was made 3 mL with the buffer. 25 μL of 24 mM pyrogallol solution was added to it and absorbance was recorded at 420 nm for 3 min at the interval of 1 min each. The enzyme SOD was evaluated on the basis of principle that the SOD has the ability to inhibit the auto-oxidation of pyrogallol. 1 unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 3 mL of assay mixture and is given by the formula [Unit of SOD per ml of sample = (ΔA − ΔB) × 100/(ΔA × 50)], where; ΔA is the absorbance difference in 1 min in control; ΔB is the absorbance difference in 1 min in test sample; Data was expressed as SOD units per mg protein [26,27].

2.8.3. Estimation of lipid peroxide activity

A 0.5 ml each of 30% trichloroacetic acid (TCA) and 0.8% thiobarbituric acid (TBA) reagents were added to the tubes containing 1 mL of the suspension medium taken from the 10% tissue homogenate. Tubes were covered with aluminium foil, kept in shaking water bath at 80 °C for 30 min, then, kept in ice-cold water for 30 min, and lastly centrifuged at 3000 rpm for 15 min. Absorbance of the supernatant obtained in centrifuge was read at a wavelength of 540 nm at room temperature against appropriate blank using UV-spectrophotometer. Amount of malondialdehyde (MDA), product of lipid peroxidation damage which forms a chromogenic adduct with two molecules of TBA, was read from standard curve prepared by using different volumes (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ml) of standard reagent 1,1,3,3-tetraethoxy propane (4 μg/mL) and calculated according to the equation [nmol of MDA/mg protein = (absorbance × final volume of test solution)/0.156] [28].

2.9. Histopathological examination

Brain tissues were collected from the sacrificed rats. Fragments from brain tissues were fixed in 10% neutral formalin solution, embedded in paraffin, cut into sections of 6 μm size, stained with hemotoxylin-eosin stain onto the slides, observed under light microscope for any pathological changes and captured for photomicrographs [29].

2.10. Statistical analysis

All the experiments were performed thrice and the obtained values were expressed as mean ± standard error of mean (mean ± SEM). Data were analyzed by one way analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests using the GraphPad Prism V 5.0 (GraphPad Software, Inc., San Diego, California, USA). The p values < 0.05 were considered as statistically significant.

3. Results

3.1. Evaluation of extractive value

The extractive value of hydroalcoholic extract of *C. cajan* leaves (HECC) was found to be 14% w/w.

3.2. Evaluation of effect of hydroalcoholic extract of Cajanus cajan Linn leaves on behavioral activity, locomotor activity, brain AChE activity and antioxidant activity such as CAT, SOD and lipid peroxide activities in SD rats

The percent number of entries, number of entries in open arm, AChE activity and lipid peroxide of stress-induced group were significantly ($p < 0.001$) increased as compared to sham control group, while, the activity score, CAT and SOD activities of stress-induced group were significantly ($p < 0.001$) decreased.

The percent number of entries, number of entries in open arm, AChE activity, lipid peroxide of drug treated-1 group were significantly ($p < 0.01$) decreased as compared to stress-induced control group, while, the CAT and SOD activities of drug treated-1 group were significantly ($p < 0.01$) and the activity score non-significantly ($p > 0.05$) increased.

The percent number of entries, number of entries in open arm, AChE activity, lipid peroxide of drug treated-2 group were significantly ($p < 0.01$) decreased more in quantity as compared to stress-induced control group, while, the CAT and SOD activities of drug treated-2 group were significantly ($p < 0.01$) and the activity score significantly ($p < 0.05$) increased.

The percent number of entries, number of entries in open arm, AChE activity, lipid peroxide of standard drug treated group were significantly ($p < 0.01$) decreased as compared to stress-induced control group, while, the CAT activity, SOD activity and the activity score of standard drug treated group were significantly ($p < 0.01$) increased. The activities of drug treated-2 group were almost significantly equivalent to that of standard treated group (Table 1, Fig. 1).

Table 1
Effects of hydroalcoholic extract of *Cajanus cajan* Linn leaves on different brain specific variables in control and experimental groups of animals.

Brain specific variables & Treatment groups	Number of entries in open arm	% Number of entries	Activity score (counts/5 min)	AChE activity (mole of ACh hydrolyzed/min/mg protein)	CAT (nmol H_2O_2 consumed/min/mg protein)	SOD (μg/mg protein)	Lipid peroxide (nmol MDA/mg protein)
I **Sham control** 1 ml/kg b. wt. *po* of 1% CMC once a day for 14 days	5 ± 0.21	35.83 ± 1.12	125.24 ± 1.43	0.032 ± 0.001	73.48 ± 1.03	60.5 ± 1.32	26.94 ± 0.55
II **Stress control** 1 ml/kg b. wt. *po* of 1% CMC once a day for 14 days + sleep deprivation from 15th to 19th day	7 ± 0.24$^{\#}$	60.25 ± 1.71$^{\#}$	76.26 ± 1.03$^{\#}$	0.132 ± 0.014$^{\#}$	53.79 ± 5.01$^{\#}$	42.5 ± 3.21$^{\#}$	47.21 ± 2.12$^{\#}$
III **Drug treated-1** 200 mg/kg b. wt. *po* of HECC once a day for 14 days + sleep deprivation from 15th to 19th day	5 ± 0.19***	40.75 ± 0.92***	96.66 ± 1.2*	0.064 ± 0.008***	61.59 ± 1.09***	50.73 ± 1.04***	38.52 ± 0.3***
IV **Drug treated-2** 400 mg/kg b. wt. *po* of HECC once a day for 14 days + sleep deprivation from 15th to 19th day	3 ± 0.14***	48.66 ± 0.81***	157.35 ± 1.2**	0.054 ± 0.005***	65.1 ± 1.4***	55.93 ± 1.77***	32.21 ± 0.21***
V **Standard treated** 200 mg/kg b. wt. *po* of piracetam once a day for 14 days + sleep deprivation from 15th to 19th day	4 ± 0.18***	35.75 ± 1.1***	142 ± 1.24***	0.057 ± 0.009***	68.33 ± 2.5***	57.01 ± 1.35***	30.73 ± 0.284***

$^{\#}$ Indicates $p < 0.001$ as compared to sham control group and $^{*}p > 0.05$, $^{**}p < 0.05$ and $^{***}p < 0.01$ as compared to stress control group.

3.3. Histopathological examination

[A]. Sham control group: Trabeculae of connective tissue are seen in all area of section in which neurological cells having round or oval vesicular nuclei and indistinct cytoplasm is seen. Intertrabecular space is filled with eosinophilic amorphous material.

[B]. Stress control group: There is reduction in number of neurological cells and increased connective tissue cells are seen. Number of trabecular is increased with reduction in intertrabeculae space and eosinophilic material.

[C]. Drug treated-1 group: Increase in connective tissue cells and fiber is seen. Neurological cells are reduced to minimum with increase in thickness of trabeculae. Inter trabeculae space does not show eosinophilic material.

[D]. Drug treated-2 group: Trabeculae of connective tissue are seen in all area of section in which neurological cells having round or oval vesicular nuclei and indistinct cytoplasm is seen. Inter trabecular space is filled with eosinophilic amorphous material. [E]. Standard treated group: Further increase in connective tissue cells and fiber is seen. Neurological cells having round or oval vesicular nuclei and indistinct cytoplasm is seen. Inter trabecular space is filled with eosinophilic amorphous material (Fig. 2).

4. Discussion

Formation of memory is a very complex process involving multiple neuronal pathways and neurotransmitters. ACh is the neurotransmitter present in cholinergic neuronal system playing an important role for memory in humans and animals [30].

Loss of memory is main symptom of death of brain central cholinergic neurons and for a variety of disorders including AD [2]. A decreased level of ACh or increased AChE activity is thought to be one of the factors for loss of memory as in AD [31]. Elevated plus maze performance is an appetitive motivation task that is useful to assess the spatial reference as well as spatial working memory performance [23]. Results of the study have clearly indicated that the percent number of entries, number of entries in open arm, AChE activity of stress-induced group were significantly ($p < 0.001$) increased while, the activity score significantly ($p < 0.001$) decreased as compared to sham control group indicating loss of memory in stress-induced group where sleep deprivation leads to disorders that cause irreparable damage [21,31,32].

Normal brain functioning including memory is impaired when connections in the neurons are lost. Oxidative stress is one of the factors causing neuronal injury leading to memory impairment [33,34]. Results of the study have clearly indicated that the lipid peroxide activity was significantly ($p < 0.001$) increased while, the CAT and SOD activities were significantly ($p < 0.001$) decreased in stress-induced group as compared to sham control group indicating oxidative stress in stress-induced group due to sleep deprivation causing neuronal injury leading to memory impairment [21].

Piracetam belongs to a class of drugs called pseudo-irreversible AChE inhibitors which increase the concentration of ACh in the brain by blocking AChE and this increase is believed to be responsible for the improvement in memory with reversal of memory impairment [35]. Results of the study have clearly indicated that the percent number of entries, number of entries in open arm, AChE activity, lipid peroxide activity of standard drug treated group were significantly ($p < 0.01$) decreased while, the CAT activity, SOD activity and the activity score of standard drug treated group were significantly ($p < 0.01$) increased as compared to stress-induced

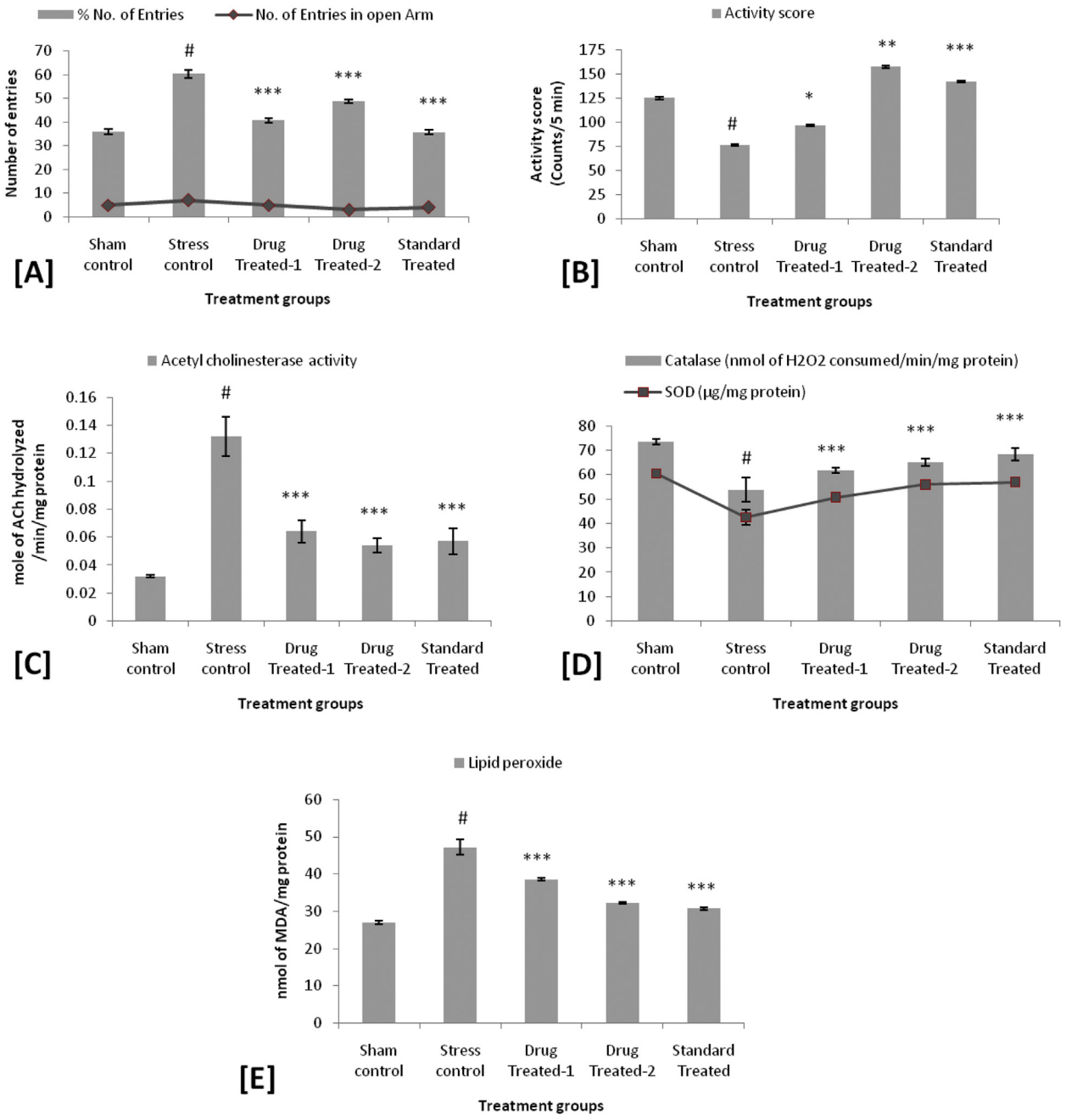

Fig. 1. Effects of hydroalcoholic extract of *Cajanus cajan* Linn leaves: [A] Memory elevated plus maze model, [B] Locomoter activity through actophotometer model, [C] Brain acetylcholinesterase activity, [D] Catalase and superoxide dismutase activity, [E] Lipid peroxide activity, where; # indicates $p < 0.001$ as compared to sham control group and *$p > 0.05$, **$p < 0.05$ and ***$p < 0.01$ as compared to stress control group.

control group indicating improvement in memory with 'reversal of memory impairment' by standard drug piracetam.

Results of the study have clearly indicated that the percent number of entries, number of entries in open arm, AChE activity, lipid peroxide of drug treated groups were significantly ($p < 0.01$) decreased while, the CAT and SOD activities of drug treated groups were significantly ($p < 0.01$) increased in dose dependent manner as compared to stress-induced group. The activity score non-significantly and significantly ($p > 0.05$; $p < 0.05$) increased in drug treated-1 and drug treated-2 groups respectively. It suggested that the activities of drug treated-2 group were almost significantly equivalent to that of standard treated group. Thus, *C. cajan* extract prevented the higher reference memory and working memory errors suggesting that it prevented the memory impairment. It significantly inhibited whole brain AChE activity increased by sleep deprivation in dose dependent manner and thereby could increase the availability of ACh in brain and in cholinergic synapse which might be one of the possible mechanisms to encounter with memory impairment. It also prevented the rise in MDA levels and loss of antioxidant enzymes CAT and SOD showing an antioxidant potential. The protective effect of HECC was well supported with the brain histopathological study.

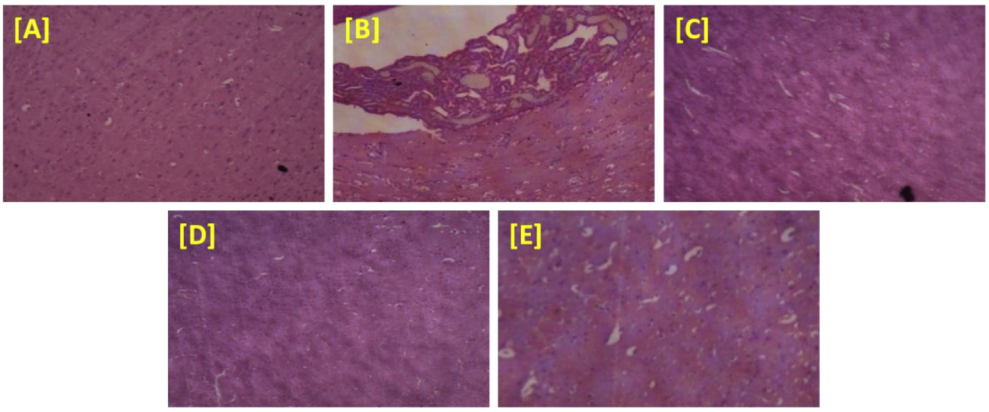

Fig. 2. Effects of hydroalcoholic extract of *Cajanus cajan* Linn leaves on histopathological changes: [A] Sham control group, [B] Toxic control group, [C] Drug treated-1 group, [D] Drug treated-2 group, [E] Standard treated group.

5. Conclusion

From the results of study, it can be concluded that hydroalcoholic extract of *C. cajan* Linn leaves possesses a protective effect against memory impairment indicating its therapeutic efficacy against memory loss as in Alzheimer's disease. Probable underlying mechanisms may be brain's AChE inhibition and increased antioxidant potential by hydroalcoholic extract of *C. cajan* Linn leaves. Further, it can be investigated for the isolated bioactive compounds like quercetin to confirm the responsible phytoconstituents for the nootropic potential of *C. cajan* leaves extract and its application in the treatment of Alzheimer's disease and other cognitive disorders.

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Conflict of interest

None

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