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

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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 *Cajan 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 (flavonol, flavonones, isoflavones and chalcone) and stilbenes [7–10]. It has shown protective effect against hypoxic-ischemic brain damage in rats [11–13]. It also possesses antimicrobial, hepato-protective and centrally acting analgesic activities [14,15]. It has been used as hepatoprotective, antidiabetic, antiulcer, antiinflammatory, antimicrobial, CNS depressant, anticancer, analgesic.
and anthelmintic 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 (Chemikbiochemika 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].
2.8.3. Estimation of lipid peroxide activity

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

\[
\text{Activity} = \frac{4 \times \text{Absorbance} \times \text{Volume of assay}}{0.156 \times \text{Volume of sample}}
\]

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 formula

\[
\text{Activity} = \frac{(A - B) \times 100}{500}
\]

2.8.2. Evaluation of antioxidant activity

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

3. Results

3.1. Evaluation of extractive value

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 comparisons 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.

3.2. 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 non-significantly (p > 0.05) increased.

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

The activities of drug treated-2 group were almost significantly equivalent to that of standard treated group (Table 1, Fig. 1).
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 [23]. Results of the study have clearly indicated that the lipid peroxide activity was 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 disorders that cause irreparable damage [21,31,32].

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 H2O2 consumed/min/mg protein) | SOD (µg/mg protein) | Lipid peroxide (nmol MDA/mg protein) |
|---------------------------------------------|------------------------------|-------------------|-----------------------------|-------------------------------------------------|---------------------------------------|-------------------|-----------------------------------|
| Sham control                               | 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                      |
| Stress control                             | 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*                     |
| Drug treated-1                              | 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***                    |
| Drug treated-2                              | 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†                     |
| Standard treated                            | 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 intertrabecular 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 trabecular 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).
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.
Effects of hydroalcoholic extract of *Cajanus cajan* Linn leaves against memory impairment indicating its therapeutic efficacy.

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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