AMELIORATIVE EFFECT OF DRIED LEAVES EXTRACT OF CALOTROPIS GIGENEA (L) ON SCOPOLAMINE INDUCED MEMORY IMPAIRMENT IN RATS

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ABSTRACT

C. gigentea is frequently utilised ethnopharmacologically for a variety of disease, including CNS disorders. They can counteract reactive oxygen species (ROS) by acting as reducing agents, hydrogen donors and free radical quenchers. The impact of dried extract leaves of C. gigentea (L) on Scopolamine hydrobromide (ScHBr) induced memory impairment in rats, cholinesterase activity, and oxidative stress is investigated in this study. C. gigantea was chosen for this study because there is insufficient statistical evidence to justify its traditional use in medicine. This plant's extract was tested for various pharmacological activities. Scopolamine induced amnesia in Albino rats of each sex, weighing around 200g to 250g. Nootropic drug used in this trial was Piracetam, which was given at a dosage of 120mg/kg.p.o of body weight. Scopolamine hydrobromide, which tends to enhance AChE activity, is one of the amnesia-causing drugs. The extract dose was given to animals for the Elevated Plus Maze (EPM) and Morris Water Maze (MWM) trials. An effort was made to analyse the plant part, namely the leaves of C. gigentea (L). The presence of bioactive chemical elements such as alkaloids and flavonoids aids in their identification, determination, and demonstration of their ability to treat various ailments. Scopolamine hydrobromide (ScHBr) has been used to induce amnesia in rats. Plant extracts have been shown to lessen TBARS & AChE when compared to Scopolamine-induced rats. C. gigentea leaves extract has been shown to reduce latency times in maze tests, suggesting that it could be employed to treat cognitive impairment disease in the future.

Keywords: Reactive oxygen species (ROS), Scopolamine hydrobromide (ScHBr), Acetyl Cholinesterase (AChE), Thio Barbituric Acid Reactive Substances (TBARS), Transfer latency (TL).

1. INTRODUCTION

Learning is the process of acquiring information whereas the act of encoding, storing and retrieving knowledge is memory. The stored memory is retrieved and accessed (Born and Rasch, 2013)[1]

Classification of Memory
Memory is divided into following types:-

Short-term memory
Memories that last only a few seconds or minutes are included.

Working memory
Working memory is a kind of memory utilised for daily chores or tasks but lost when the activity is done. (Engle et al. 1999).[2]

Long-term Memory
For months, years, or a lifetime, it might be remembered. Furthermore, long-term memory is classified into declarative and non-declarative form.

1.1 Dementia
Dementia is a condition with decreased learning, memory, thought, orientation, numeracy, comprehension, judgment, and capacity to do everyday work. The loss in higher dementia cognitive function varies from normal ageing. Cognitive impairment is

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generally sporadically assessed in dementia and old age. Cognitive dementia impairment generally happens irregularly by degrading the emotional control over social conduct or stimulus.

The reduction in cognitive performance is largely related to a decrease in cholinergic neuron transmission (Gold, 2003). Antimuscarinic medication is a key impairment of both animal and human learning and memory (Boccia et al. 2003) (Atri et al., 2004). Acetylcholine can improve coding by boosting the intensity and modulation of input neurons (Hasselmo, 2006). In addition, the role of acetylcholine controls the care and memory circuits in the neocortex. (Sarter and Hasselmo, 2010).

In the hippocampus, acetylcholine receptors are extensively dispersed. Acetylcholine works in two different receptor classes: Nicotinic ionotropic (nAchR) and Muscarinic Metabotropic (mAchRs) receptors. M2 receptors have an important function in regulating different elements of memory – to improve the action of acetylcholine (Seeger et al., 2004).

_Calotropis gigantea_ (L.) is a shrub of the family of the Apocynaceae. The plant is used in Ayurvedic medicine to treat a range of conditions. A recent study, on the other hand, looked at the criteria utilised to determine the plant's identity, purity, and power. In this study, the shrub was investigated morphologically, microscopically, and phytochemically.

Flavonoids are antioxidants, anti-leukemia radicals and vasodialators. These can contribute to the maintenance of brain and Alzheimer's blood supply. There are anticancer, anti-aging and antimicrobial effects of flavonoids.

The purpose of this research is to examine the neuropharmacological features of _Calotropis gigantea_ (L.). In order to assess the toxicity of mice, a single dosage of 2000mg/kg methanolic plant extract has been given orally. The mice were used with well-established models of methanolic extracts (250 or 500 mg/kg). Preliminary phytochemical exams of the extract of methanol showed that flavonoids are the most abundant phytoconstituents. Because flavonoids are vital for the treatment of brain disease, responsibility for CNS operations for these components has been proposed. Finally, the current results objectively confirmed _C. gigantea_ for its neuropharmacological properties.

(a) (b)

Fig 1. _Calotropis gigantea_ (L.): (a) Flower (b) Leaves Powder

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2. MATERIAL AND METHODOLOGY

2.1. EXPERIMENTAL ANIMALS

The animals were obtained from the main animal shelter in Prayagraj, SIP. Both animals and experiments were authorized and carried out according to the Institutional animal ethics committee (IAEC) standards, Prayagraj (CPCSEA no.........). Rat weighed an average of 150-200 grams. The experimental animal room was maintained at a temperature held at 22ºC (±3ºC). Rh ranged from 50% to 60%. The lighting was artificial, with a 12-hr light-to-12-hr-dark cycle. Standard laboratory meals with ad libitum access to drinking water were employed for eating. The monitoring, normal, and therapy groups were randomly assigned to healthy young adult six rats. Each animal was tagged individually before the test started (by labeling on the base of the tail) and acclimatized in the cages for at least five days.

2.1.1 DRUGS AND CHEMICALS:

Drug: Scopolamine hydrobromide and Piracetam were acquired from Dr. reddy, Sigma Aldrich.

Chemicals: Petroleum ether, ethanol, chloroform, and methanol were all acquired from the same supplier (Sigma Aldrich). Chemicals of analytical reagent grade were employed in the research.

2.2. PLANT MATERIALS

*C. gigantea* (L) leaves were collected in December 2020 in Kaushambi, Uttar Pradesh, India. The leaves are verified in natural environment circumstances by Principal Scientist, Central Regional Center of the Indian Botanical Survey, Prayagraj - 211002.

In the Department of Pharmacy, SIP, Prayagraj and the Indian Botanical Survey (BSI) for future reference the voucher number SIP/2021/047 was retained .The authenticity letter can be obtained here.

2.3. EXTRACTION OF LEAVES:

Extraction of leaves was done by Soxhlet apparatus. The solvents for extraction were selected on the basis of their polarity (Rodgers 1997).[9]

Leaves of *Calotropis gigantea* (L) were dried at room temperature in the shade. The shade dried plant material was pulverised and defatted with petroleum ether in a beaker. The extraction continued till the degradation of the substance. A mark was removed using methanol following the extraction of petroleum ether in a soxhlet process. The extraction has been extended for 72 hours to finish extraction. The extract was condensed and dried with a constant weight rotary evaporator. Take the defatted powdered and then subjected to extract with the help of soxhlet apparatus using mixture of 40% methanol+ 20% ethanol+ 40%water and the resulting obtained solution was placed in water bath (45º C)(Parihar et al., 2011)[10]

2.4 QUANTITATIVE CHEMICAL EVALUATION:

ACUTE ORAL TOXICITY:

The extracts are submitted to an acute toxicity investigation to identify a safe dose using the Organization for Economic Cooperation and Development's acute oral toxic class approach (423). (OECD)The methanol extract of *C. gigantea* leaves did not cause lethality or toxic reactions in mice until the end of the study period. According to the toxicity scale, the methanol extract is "unclassified."
TEST DOSE PREPARATION:

The use of an aqueous solution/suspension of the test dose should first be explored as indicated by OECD recommendations. The methanol extract of *C. gigantea* leaves was largely soluble in 1% tween 80 suspension and 0.5% CMC in normal saline solutions, as indicated before (Dhumal et al, 2013).[11] The test dose was administered in a single dose orally.

2.5 EXPERIMENTAL DESIGN:

Experimental animals were classified as randomly into five groups and total 30 experimental animals were taken in which, each group receive six animals for two successive models.

- **Group- I.** Control group: Animals were given std. research facility diet i.e. vehicle administered in this group is saline solution.
- **Group- II.** Inducing agent: Animals were given amnesia inducing agent i.e Scopolamine hydrobromide about 1mg/kg body weight.
- **Group- III.** Experimental animals of this group received extract of *C. gigantea* about 250mg and scopolamine i.e, (Test compound (250mg)+ scopolamine (1mg).
- **Group- IV.** Test compound(500mg)+ scopolamine(2mg); Experimental animals of this group received extract of about 500mg and scopolamine(1mg) for the test
- **Group- V.** Piracetam(120mg)+ scopolamine (2mg); Experimental animals of this group received standard proprietary drug i.e. Piracetam and scopolamine.

Administration of ScHBr (1mg/kg,.b.w., i.p) was 60 min before the acquisition trials in group 3&4. *C. gigantea* extract at a dose of 250 and 500mg/kg were given for subsequent days respectively and acquisition trial was carried out 60 min after the last dose. Standard Nootropic medication-Piracetam was administered by using normal saline solution at dose of 1mg/kg. Standard drug, Plant extract was administered 30 min before the conducting of the experiments.

2.6 EVALUATION PARAMETERS

USED MODEL

- Morris water maze
- Elevated plus maze

BIOCHEMICAL ESTIMATION:

- Acetylcholinesterase estimation
- GSH estimation
- MDA estimation
- TBARS estimation

MORRIS WATER MAZE:

The rats are divided into eight groups, each with six rats. At the start of the path, animals will be placed in the Morris water maze, where they will be free to swim and sit on a secret platform. If the rat is able to connect to the hidden platform within three minutes, the trial will be considered a success. If the animals are unable to leave the platform within
180 seconds, the researcher will gently place them there for 30 seconds. The time between trials is usually between 5 and 10 minutes. For five days in a row, each rat will be given four escape paths. The amount of time it takes to get to the platform will be recorded.

**ELEVATED PLUS MAZE:**

Using an exteroceptive behavioral paradigm, this model (high plus labyrinth) was utilized to assess learning and memory in rats (in this, the stimulus was external to the body). The device consisted of two open arms (16 cm x 5 cm) and two closed arms (16 cm x 5 cm x 12 cm). The maze was lifted to 25 cm above the floor by extending the arms of the central platform (5 cm x 5 cm). On the first day, all rats were positioned at the far end of an open arm facing the center platform. The time each rat was required to enter the closed four-legged arms was evaluated as the latency of transfer (TL).

**2.7 PREPARATION OF TISSUES:**

On the eight-day, immediately after cervical dislocation, test animals of both groups were sacrificed, and their brains were separated, put on Petridish, and measured. Rinse the whole brain with ice-cold saline. A homogenizer was employed in 0.1 M refrigerated phosphate buffer to homogenate 10% brain homogeneity (pH 7.4). To extract the supernatant, it was centrifuged at a temperature of 10,500 rpm at 4°C for 20 minutes. The biochemical investigation is performed on the supernatant of each animal.

**PROTEIN ESTIMATION:**

GSH, MDA, and AChE function were assessed by measuring protein levels in all brain samples. Lowry's 1951 method was used to determine protein concentrations.

**Chemical Reagents**

1. **Aqueous solution**
   a) 2% (w/v) \( \text{NaCO}_3 \) in 0.1 M \( \text{NaOH} \)
   b) 1% \( \text{CuSO}_4 \), (w/v)
   c) 2% Sodium potassium tartrate,
      \[ 48\text{mL of A + 1mL of B + 1mL of C} \]
1. 1 mg/ml BSA (Bovine Serum Albumin) (Stock standard).
2. The stock was diluted 20 times with working normal BSA (1000g/ml).
3. Folin-Phenol reagent (ice-cold) dissolved in equal parts water prior to use.

**Method of Examination**

The 1mL supernatant was carefully mixed with 0.9 mL DDW and a 5 mL alkaline working reagent before incubation at room temperature for 10 minutes. After 30 minutes, add the Folin-phenol reagent and incubate at room temperature for another 30 minutes. The absorbance was 750 nm and compared to the absorption of a blank reagent. A reference of 50-1000 g was used for bovine serum albumin (BSA) (1 mg/ml) and the sample protein content of mg/ml was measured (Lowry et al., 1951).\cite{12}

**Measurement of lipid peroxidation (MDA):**

A marker of lipid peroxidation has been discovered and quantified by TBARS (Ohkawa et al.). The short-term advantages of lipid peroxidation have been assessed for malondialdehyde (MDA). 10% homogeneous tissue was utilized for 1.2 mL of supernatant extraction. Adding 0.6 ml of 30% TCA (thiobarbituric acid) and adding diluting 95% ethanol of 0.6 ml of 0.8% TBA (thiobarbituric acid). The tubes are covered with aluminum foil and agitated for 30 minutes in a bath of water at 900°C. The tubes

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were then removed and immersed for a further 30 minutes in ice cold water. Then he centrifuged for 15 minutes at 2500 rpm. At 540 nm, the absorption of the supernatant fluid was spectrophotometrically measured against enough void at room temperature. Blanch is composed of 1 mL distilled water, 30% TCA by 0.5 ml, and 0.8% TBA by 0.5 ml. This formulation was used to calculate the TBARS value: n moles MDA/mg protein.

**Measurement of Acetyl cholinesterase (AChE): Acetyl cholinesterase (AChE) assay:**
Acetyl cholinesterase in the brain is an indication of a deterioration of the cholinergic system over time. Concentrations of acetyl cholinesterase in the brain were determined using Ellman method. The test included 0.05 ml of supernatant, 3 ml 0.01 M sodium phosphate (pH8), 0.1 ml of iodine acetylthiocholine and 0.1 ml of DTNB (Ellman reagent). A spectrophotometer was used to determine the change of absorbance at 412 nm instantly. The results are presented in moles of hydrolyzed protein acetylcholine/min/mg and calculated by means of a molar extinction chromophore (1.36x104 M1 cm1) coefficient (Ellman et al., 1961).[13]

\[
R = \frac{\delta OD \times Volume \ of \ Assay}{Ex \ mg \ Protein}
\]

\( R \) denotes the rate of enzyme activity, which is measured in moles of acetylcholine iodide hydrolyzed per minute per milligramme of protein. \( \delta OD \) is the minutely variation in absorption, and \( E \) is the extinction coefficient (1.36 x 104 M – 1 cm- 1)

**STATISTICAL ANALYSIS:**
The values were expressed as mean ± SEM from 6 animals. The results were subjected to statistical analysis by using one-way ANOVA followed by Dennett’s test to calculate the significant difference if any among the groups. P<0.05 was considered as significant.

**3. RESULT**

**Exteroceptive behavioral models**
Assessments of scopolamine-induced amnesia in rats and effect on latent translation of *Calotropis gigentia* linn alcoholic extract (elevated plus maze paradigm).

**Table:1** The effects on transfer latency (elevated pus maze paradigm) of the alcoholic extract from *Calotropis gigentia* linn are shown in Table.1

<table>
<thead>
<tr>
<th>S. no</th>
<th>Treatment groups</th>
<th>TL on acquisition day (sec) 7 day</th>
<th>TL on retention day (sec) 8 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control group</td>
<td>44.50 ± 15</td>
<td>41.25±8.70</td>
</tr>
<tr>
<td>2</td>
<td>Inducing agent( ScHbr) 1mg/kg</td>
<td>87.60± 17.2*</td>
<td>84.17±15.72*</td>
</tr>
<tr>
<td>3</td>
<td>extract(250)+Inducing agent(Schbr) 1mg</td>
<td>51.5±5.5a</td>
<td>48.00±4.2b</td>
</tr>
<tr>
<td>4</td>
<td>extract(500)+Inducing agent(Schbr)1mg</td>
<td>47.5±6.7a</td>
<td>42.02±3b</td>
</tr>
<tr>
<td>5</td>
<td>Piracetam(120)+Inducing agent(Schbr)1</td>
<td>41.2±10.92a</td>
<td>39.27±1.64b</td>
</tr>
</tbody>
</table>
Spatial reference learning and memory
The effects of an alcoholic extract of *Calotropis gigentia* (L) leaves on transfer delay (Morris water maze paradigm) have been evaluated in scopolamine-induced amnesia of rats (Mean ± SD).

**Table: 2** The effects of an alcoholic extract of *Calotropis gigentia* (L) leaves on transfer delay are given in Table 2 in scopolamine-induced amnesia in rats (Mean ± SD).

<table>
<thead>
<tr>
<th>S. no</th>
<th>Treatment groups</th>
<th>TL on acquisition day (sec) 10th day</th>
<th>TL on retention day (sec) 11th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control group</td>
<td>54.2±5.33</td>
<td>50.2±8.18</td>
</tr>
<tr>
<td>2</td>
<td>Inducing agent (ScHbr) 1mg/kg.b.w</td>
<td>108.4±6.52*</td>
<td>103.5±5.78*</td>
</tr>
<tr>
<td>3</td>
<td>extract(250)+Inducing agent(Schbr)1</td>
<td>60.8±4.78a</td>
<td>56±1.93b</td>
</tr>
<tr>
<td>4</td>
<td>extract(500)+Inducing agent(Schbr)1</td>
<td>58.5±5.76a</td>
<td>55±4.34b</td>
</tr>
<tr>
<td>5</td>
<td>Piracetam(120)+Inducing agent(Schbr)1</td>
<td>56.2±4.79a</td>
<td>51±3.24b</td>
</tr>
</tbody>
</table>

Estimation of AChE activity
In amnesia caused by scopolamine in rats, the effects of *Calotropis gigentia* (L) alcoholic extract on AChE activity (mean ±SD) were evaluated.

**Table: 3** Effects of a *Calotropis gigentia* (L) leaf alcoholic extract on AChE activity in rats with scopolamine-induced amnesia (Mean ±SD).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment groups</th>
<th>AChE (g/min/mole of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control group</td>
<td>0.183 ± 0.001</td>
</tr>
<tr>
<td>2</td>
<td>Inducing agent (ScHbr) 1mg/kg.b.w</td>
<td>0.250 ± 0.005*</td>
</tr>
<tr>
<td>3</td>
<td>C.gigentea(250mg/kg)+Inducing agent (Schbr) 1</td>
<td>0.200 ± 0.004*</td>
</tr>
<tr>
<td>4</td>
<td>C.gigentea(500mg/kg)+Inducing agent (Schbr) 1</td>
<td>0.180 ± 0.005*</td>
</tr>
<tr>
<td>5</td>
<td>Piracetam (120mg/kg)+Inducing agent(Schbr)1</td>
<td>0.185 ± 0.006*</td>
</tr>
</tbody>
</table>

Fig 2: Effect of alcoholic extract of plant extract materials on TL and RL on EPM. N=6, and values are expressed as mean± SEM. One way. ANOVA followed by Dunnet’s test, *p < 0.05, as comparing it with control, * a P < 0.05 compared with ScHBr treated group, b P < 0.05 compared with control group.
**Fig 3:** Effect of alcoholic extract of plant part on TL and RL on MWM. N=6, and values are expressed as mean± SEM. One way ANOVA followed by Dunnet’s test, \*p < 0.05, as comparing with control, \( ^a \) P < 0.05 compared with ScHBr treated group, \( ^b \) P < 0.05 compared with control group.

**Fig 4:** Effects of alcoholic extract of *Calotropis gigentea (L)* leaves on AChE level in the brain on scopolamine-induced amnesia in rats (Mean ± SD) N=6. One way ANOVA followed it by Dunnet's test, \*p < 0.05, as comparing with control \( ^a \) P < 0.05 compared with ScHBr treated group.
Homo sapiens often use natural materials generated by plants, animals, and marine sources to preserve their health. There is no definite treatment for cognitive impairment disorders such as Alzheimer's disease. As a consequence, the plant component, especially the Calotropis gigantea leaves, was analyzed (L). To see how well it performs as an Alzheimer's pharmacological therapy. All signs of cognitive shortcomings, which play a significant role in learning and thinking, include neuro-apoptotic, neuro-inflammatory, oxidative stress, dementia, forgetfulness, and reduction in levels of acetylcholine.

Different factors are used to standardize Calotropis gigantea (L) alcoholic extract leaves, such as the presence of undesirable chemical substances and the determination of their various extractive values, physicochemical parameters, and pharmacognostical parameters. Search its multiple indexes, such as index swelling and spraying. The loss of drying reveals the existence of moisture in medicines. The presence in plant extracts of biological, chemical components, such as alkaloids, glycosides, polyphenolic compounds, flavonoids, carbohydrates, steroids, amino acids, and amygdalin, helps them to identify, determine and demonstrate their ability to cure different diseases. C. gigantea contains approximately 53 compounds, of which approximately 38% are cardenolides, 4% are steroids, 4% are flavonoids, 20% are terpenoids, 3% are cardiac glycosides, 4% are resins, 6% are proteinases, and 10% are miscellaneous compounds. Few compounds found in C. gigantea, especially flavonoids and steroids, have good biological evidence.

We also calculated the toxicity of plant extract at different levels (5, 500, 300, 2000 mg/kg) and found that plant leaves extracts materials were safe at any dose. Scopolamine - hydrobromide, a common paradigm for amnesia generation. Experimental rats, utilized. ScHBr has anti-muscarinic effect that inhibits ACh from binding to its receptors and increases cholinesterase activity and, therefore, degradation.

Memory was assessed in rats using two types of behavior: increased paradigms and paradigms of Morris water labyrinth. Doses of Calotropis gigantea (L) extract alcoholic leaves were evaluated for rats inducing scopolamine at 250 mg/kg body weight and 500 mg/kg body weight. ScHBr is given at 1 mg/kg body weight to cause amnesia and dementia. According to our experimental methods, the treatment Group for plant extract was shorter than the ScHBr induction group. This is caused by lower oxidative stress,
greater Ach levels, and reduced cholinesterase enzyme activity in both models, i.e., EPM and MWM.

*C. gigentea* is a rich source of antioxidants as well since it consists of a substantial amount of phenolic compounds, omega three fatty acids, and other antioxidants. These chemicals can quench free radicals, helping to minimize oxidative stress. The TBARS concentration is used to calculate the parameters of oxidative stress. Plant extracts have been demonstrated to lower TBARS levels compared to Scopolamine. The quantity of TBARS in the scopolamine-treated group is considerably greater.

In addition to hematological and biochemical data, the organ weight/body wt ratio results demonstrate that the alcoholic extract of *C. gigentea* leaves extract did not produce organ swelling, atrophy, or hypertrophy. Moreover, the absence of substantial changes in the kidney's organ weight/body weight ratio supports the biochemical findings. Similarly, the liver weight/body weight ratio reveals that the extract groups' livers had no abnormalities.

**CONCLUSION**

The most frequent symptoms of Alzheimer's and associated problems include memory loss, dreamy mood, lack of focus, attention-deficient persons with an increased degree of activity, and other neuro-related diseases. If such a disease is not dealt with, it might lead to death. Unfortunately, no precise treatment has yet been discovered for such a disease. This has led to initiatives to study and cure these diseases. *C. gigentea* contains polyphenolic compounds and other chemical components (L). Many animal models were used to measure such conditions. Seed extracts have been shown to lower latency times in the elevated plus labyrinth and the Morris water labyrinth. Additional biochemical data showed that AChE activity has declined and TBARS levels have declined, indicating that this medication might be used to treat cognitive impairment in the future.

**FUTURE ASPECTS**

Our findings in *Calotropis gigentea* (L) pave the door for additional research on molecular processes and the therapeutic effect of isolated components of plants on neurological conditions such as Huntington's and Parkinson's.

**REFERENCES**


