

## **Original Research Article**

### **The learning and memory enhancing properties of *Bacopa monnieri* plant leaves protein: A systematic study in Wister Albino rats model system**

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**Abstract:** Man and other animals have shown characteristic features towards learning and memory. Disorders of the central nervous system will have a significant bearing on the healthcare concerns of the human population worldwide associated with loss of memory, cognitive deficits, impaired mental function, back in learning etc. Traditional knowledge says that *Bacopa monnieri* has memory enhancing property. The current systematic study aimed to examine the scientific evidence as to whether *Bacopa monnieri* plant leaves proteins can enhance learning memory in Wistar albino rats. Rats were given 200mg/kg dose of *Bacopa monnieri* plant leaves proteins orally along with scopolamine (i.p) for 14 days. Rats were subjected to elevated plus maze and passive avoidance testing on 14<sup>th</sup> day to evaluate learning. Same models were repeated on 15<sup>th</sup> day to evaluate retention (memory). The results were compared with the results of negative control group treated only with scopolamine and positive control group treated with scopolamine and piracetam for 14 days. Significant learning and memory enhancement was seen with *Bacopa monnieri* plant leaves proteins as compared to negative and positive control group with p value < 0.05. This result that treatment with *Bacopa monnieri* plant leaves proteins is beneficial in Alzheimer's disease and has potential disease modifying effect.

**Keywords:** *Bacopa monnieri* plant leaves proteins, learning, memory, Alzheimer's disease, elevated plus maze, passive avoidance tests.

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#### **INTRODUCTION**

Factors like psychological stress, pollutants, genetic predilection increases or encourages the risk of neurocognitive or neurodegenerative disorders like Alzheimer's disease (AD), depression, multi dementia, senile dementia which leads to impairment of learning and memory [1, 2]. Though AD is common in aged individuals but the symptom of Alzheimer's disease is progressive memory loss followed by a general cognitive decline due to cholinergic deficiency in cortical regions.

In India, it is time immemorial practice of learning and memory enhancing by certain exercises like Yoga and using few dietary components, medicinal plants and herbs. It is believed for several centuries that, memory enhancement has been a theme for science fiction, but now we understand the concept of the neurobiological basis of memory [3]. Based on the medical knowledge of memory function, many researchers are in the direction of developing a drug, or finding a source of natural compounds that might improve our capacity to remember [4, 5].

Brahmi (*Bacopa monniera* Linn.) is an herb which is available naturally in India and has a long history of usage in the Ayurveda, Unani, and in Chinese medicine tradition in the treatment of anxiety, intellect and poor memory [6]. In India, it is used to increase the cognitive and memory enhancing power in children and age old people. It is reported that, *Bacopa monniera* contains active constituents like proteins, phenols, alkaloids and saponins, Bacosides A and B [7, 8, 9, 10]. However there is lack of evidence about role of proteins isolated from *Bacopa monniera* plant leaves. Keeping this information in view the learning and memory enhancing property of proteins of *Bacopa monniera* plant leaves has been evaluated following administration in mice. Based on the behavioral studies with rats, described above, it is expected that it will improve performance on tasks tapping long-term memory.

#### **MATERIALS AND METHODS**

Brahmi (*Bacopa monnieri*) plant leaves were collected from authentic source, piracetam, scopolamine, 5% dextrose (for suspending test compound) procured from Adichunchanagiri Hospital

and Research Centre drug house. Healthy male Wistar albino rats strain, 150 – 200g were used. The rats were maintained under a reverse photo cycle of 12 h day and 12 h night in temperature and humidity controlled environment with free access to food and water. All experiments were conducted between 9:00 am and 12:00 pm in a noise free environment. The study was approved by Institutional Animal Ethics Committee.

#### Methods of protein extract preparation

10g of shade dried Bramhi (*Bacopa monnieri*) plant leaves, finely powdered (100 mesh British Pharmacopea) was mixed with 200 ml of double distilled water. It was vortexed for 6 hours at 20°C. The mixture was centrifuged at 8000 rpm for 20 minutes and the supernatant was separated. Further, the supernatant was subjected to 65% ammonium sulphate precipitation and vortexed over night. The mixture was centrifuged at 10000 rpm. The precipitated Ammonium sulphate protein precipitate was collected and subjected to dialysis using 2.5kDa molecular cutoff biomembrane against double distilled water for 72 hours with an interval of 6 hours. The dialyzed crude protein was separated and stored at -10°C for further studies.

#### Isolation of human peripheral lymphocyte

Human peripheral lymphocytes were isolated from 10ml of venous blood drawn from young, healthy donors. Blood was collected in ACD (85mM citric acid-71mM trisodium citrate-165mM D-glucose) in the ratio of 5:1. 4 volumes of solution A (hemolyzing buffer-150mM NH<sub>4</sub>Cl in 10mM Tris buffer, pH 7.4) was added, mixed well, incubated at 4°C for 30 min. Centrifuged at 1200 rpm for 12 min, the supernatant (hemolysate) was discarded, pellet was washed again with 5ml of hemolyzing buffer and the pellet containing cells were washed thrice with 10 ml of solution B (250mM m-inositol in 10mM phosphate buffer pH 7.4) and suspended in same solution. The cell viability was determined by Trypan dye blue exclusion method [11]. To 10µl of lymphocyte sample added 10µl of Trypan blue (0.02%) and the cells were charged to Neuberg's chamber and the cell number was counted. The dead cells being permeable to Trypan blue appear blue against white color of the viable cells. The survival rate of lymphocytes was determined at time intervals 20th, 40th and 60th minutes of incubation. Viability was tested by Trypan blue exclusion and exceeded 96% in each isolation. Percentage viability was calculated by the formula.

$$\% \text{ viability} = \frac{\text{Total no. of viable cells}}{\text{Total no. of viable cells} + \text{dead cells}} \times 100$$

**Time course study of the effect of H<sub>2</sub>O<sub>2</sub> and protection antioxidants like *Bacopa monnieri* plant leaves proteins, *Curcuma longa* proteins and *Curcuma longa* peel proteins on the viability of lymphocytes.**

Lymphocyte cells (1x10<sup>6</sup>) were treated H<sub>2</sub>O<sub>2</sub> in the presence or absence of antioxidants in 1ml of HBSS pH 7.4 at 37°C. After the incubation time up to 30 minutes, the viability of the cells was determined by Trypan blue exclusion analysis [11] and the percentage of viable cells was calculated.

#### *In vivo* experimental Protocol

Learning and memory was assessed with elevated plus maze and passive avoidance tests. 3 study groups with 6 animals in each were used for the studies. Memory impairing dose of scopolamine 1mg/kg i.p was administered for 14 days in group 1 rats. Group 2 received scopolamine 1mg/kg i.p for 14 days and piracetam 200 mg/kg i.p from 8<sup>th</sup> day to 14<sup>th</sup> day. Group 3 received scopolamine 1mg/kg i.p for 14 days and proteins of *Bacopa monnieri* plant leaves 200mg/kg P.O from 8<sup>th</sup> day to 14<sup>th</sup> day.

On the 14<sup>th</sup> day, 90 min after the administration of the last dose of drugs in the respective groups, rats were exposed to elevated plus maze and passive avoidance task for acquisition [learning]. Retention [memory] was recorded 24hrs later on the 15<sup>th</sup> day. Groups were as follows;

Proteins of Bramhi (*Bacopa monnieri*) leaves were suspended in 5% dextrose.

Group 1- Treated with scopolamine 1 mg/kg alone i.p. [negative control group]

Group 2- Treated with scopolamine 1 mg/kg+ piracetam 200mg/kg i.p. [standard group/ positive control]

Group 3- Treated with scopolamine 1 mg/kg + Proteins of Bramhi (*Bacopa monnieri*) leaves 200mg/kg P.O

#### Elevated plus maze

The elevated plus maze test was conducted according to [12] Vijayalakshmi *et al.*, 2012. This consists of a central platform of 10x10 cms connected to two open arms of 50x10cms and two closed arms of 50x40x10 cms in dimension and elevated 50 cms above the floor. Wistar albino rats weighing 150 to 250 g was used.

The experiment was performed in 2 stages. On day 14, the day of acquisition testing, each rat was placed at the end of an open arm facing away from the center. The time taken to enter any one of the closed arms was recorded as transfer latency [TL]. All four legs inside the closed arm were counted as an entry. Cut off time allotted for each rat was 180 s. Those animals which did not enter the closed arms within the cut off time were excluded from the study. Retention testing was conducted 24 hrs after the first trial and transfer latency was recorded in a similar manner as mentioned before. Shortened transfer latency was considered as an index of improvement of memory.

### Passive avoidance test

The passive avoidance test was conducted according to [13] Hock et al, 2008. This is one trial which is fear motivated avoidance task in which rats learn to refrain from stepping through a door, to an apparently safer, but previously punished compartment. The latency to refrain from crossing into the punished compartment serves as an index of the ability to avoid and allows memory to be assessed

The apparatus consisted of a square box with a grid floor [50x50 cms] and wooden walls of 35 cms height. This box was illuminated with a 7W/12V bulb placed 150 cms above the centre of the compartment. In the centre of one of the walls, there was an opening [6x6 cms] which can be opened or closed using a transparent plexy glass sliding door which leads to a small dark compartment [15x15 cms]. This compartment was provided with an electrifiable grid floor which can be connected to a shock source and a removable ceiling.

The experiment was conducted in 2 stages. Test animals were given an acquisition trial on 14<sup>th</sup> day followed by a retention trial 24 hrs later, on 15<sup>th</sup> day. In acquisition trial, the animal was placed in the

illuminated compartment at maximal distance from guillotine door and the latency to enter the dark compartment was measured as step through latency [STL]. Rats that did not step through the door within a cut off time 180 s was not used. Sliding door between the two compartments was closed as the rat entered dark chamber and unavoidable foot shock [1.5mA, 50HZ, 2 s] was delivered. The ceiling was opened and the rat was returned to home cage. Retention was tested after 24 hrs on 15<sup>th</sup> day and STL was recorded. Cut off time allotted for retention was 600 s. Increase in STL was considered as an index of improvement of memory.

### STATISTICAL ANALYSIS

Data entry was done on MS EXCEL and 'SPSS version 17' software was used for data analysis. One way ANOVA test is used to compare the effect of the drugs on different groups. Turkey's HSD test was used for post-hoc analysis of significant overall differences.

For all the tests a 'P' value of 0.05 or less was considered for statistical significance.

### RESULTS & DISCUSSION

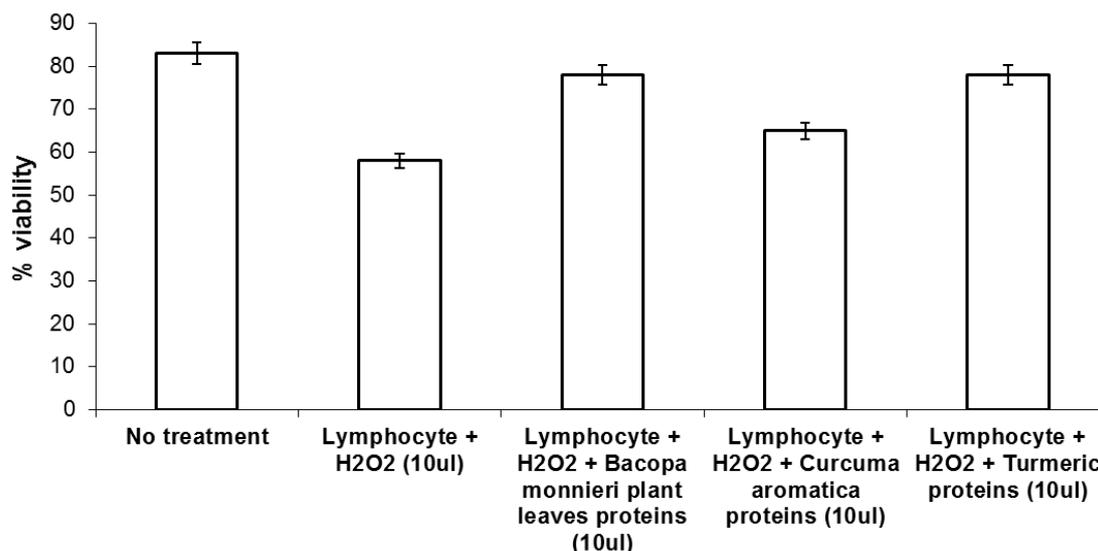


Fig. 1: Study of cell toxicity of Bramhi leaves proteins (*Bacopa monnieri*) and other antioxidants

Lymphocytes (10<sup>6</sup>cells) pretreated with or without antioxidant proteins at indicated concentrations in 0.5ml HBSS pH 7.4, incubated at 37°C for 20min., then H<sub>2</sub>O<sub>2</sub> (144µM) was added, incubated at 37°C for 60 minutes in final volume of 1ml HBSS, pH 7.4.

After the desired incubation time (60 minutes), viability of the cells was determined by Trypan blue exclusion and the percentage of viable cells was calculated as mentioned in methods.

**Table-1: Effect on transfer latency using elevated plus maze**

Group	Transfer latency in seconds	
	Learning day (day 14)	Retention day (day 15)
1.(scopolamine only)	77.83 ± 30.04	102.55 ± 37.1
2.(scopolamine + piracetam)	50.66 ± 4.67	53.98 ± 6.42
3. (scopolamine + T1)	25 ± 12.1	16.6±13.7

P < 0.001 [on day 14], P < 0.05 [on day 15], [Learning day: group-1 versus group-3: p< 0.01, Retention day: group-1 versus group-2: P< 0.01, Group-1 v/s Group-3: p<0.01, Group-2 v/s Group - 3: P< 0.05]

The rats treated with Proteins of Bramhi (*Bacopa monnieri*) leaves 200mg/kg showed statistically significant improvement in mean transfer latencies on both learning (P<0.01) and retention day (P<0.01) as compared to scopolamine only treated

group indicating learning and memory enhancing effect. There was a significant enhancement in learning and memory in Proteins of Bramhi (*Bacopa monnieri*) leaves treated group as compared to piracetam treated on both the days.

**Table 2: Effect on Step through latency using light and dark apparatus**

Group	Step through latency in seconds	
	Learning day (day 14)	Retention day (day 15)
1 (scopolamine only)	49.20 ± 35.7	18.78 ± 8.6
2 (scopolamine + piracetam)	13.71 ± 5.65	29.23 ± 11.3
3 (scopolamine + T1)	65.5 ± 6.5	75.3 ± 7.9

Learning day: p< 0.01, Group-1 v/s Group - 2: p< 0.05, Group-1 v/s Group- 2: p< 0.05, Retention day p <0.01, Group-2 v/s Group -3 p< 0.05

The rats treated with protein extracts of *Withania somnifera* 200mg/kg showed statistically significant improvement in mean transfer latencies on retention day (P<0.01) as compared to scopolamine only treated group indicating memory enhancing effect.

age related dementia. It has been reported that, scopolamine induced amnesia mice model resembles the oxidative stress associated neurodegenerative disorder in human [19]. Hence, the scopolamine induced amnesic mouse is widely used as an animal model for AD [20].

Alzheimer's disease is an age-associated, irreversible, progressive neurodegenerative disease, characterized by severe memory loss, behavioral changes, and a notable decline in cognitive function [14, 15]. Management of the neurodegenerative disorder like AD and increasing memory enhancement power is considered as one of the greatest challenges. It is well known that, a natural or herbal medicine offers options to modify the progression and symptoms of AD. It is reported that, oxidative stress, free radicals, beta amyloid, cerebral deregulation caused by bio-metal toxicity and abnormal inflammatory reactions contribute to the key event in Alzheimer's disease pathology and Curcumin- the so called active principle of Turmeric prevents the same. Herbs and medicinal plants are used as medicine to prevent memory loss in elderly age people and improve their cognitive capacity [16, 17, 18]. Scopolamine, a muscarinic antagonist that induces central cholinergic blockade, produces a reversible impairment in both maintaining attention and processing of information with the acquisition of new knowledge in rodents and human. This impairment similar to the memory disturbances found in AD and

The present study is undertaken to evaluate the non-toxic nature and effect of protein of *Bacopa monnieri* plant leaves on learning and memory performance in rats using two models namely, two compartments passive avoidance test and elevated plus maze test. As explained in the materials and methods, the freshly isolated lymphocytes were treated with proteins *Bacopa monnieri* and Turmeric proteins along with cytotoxic hydrogen peroxide. Figure -1 result shows that, protective effect of *Bacopa monnieri* plant leaves proteins against hydrogen peroxide induced cell death and proved that, it is non-toxic to human peripheral lymphocytes. The latency to enter the dark compartment (STL) and closed arm (TL) was taken as a parameter for assessment in experimental models. The rationale being, if the drug has positive effect on learning, it would be reflected as decrease in latency to enter dark compartment/closed arm. However, interpretation of TL/STL in retention trial is different in both the models, in that, decreased TL in EPM model is inferred as improvement in memory, whereas increased STL after shocking the animal in PA model is

interpreted as memory enhancement. Response to *Bacopa monnieri* plant leaves proteins (200mg/kg) was compared against negative and positive control in both the models.

In elevated plus maze, on learning day, noteworthy improvement in learning was noticed in *Bacopa monnieri* plant leaves proteins treated group as compared to scopolamine only treated group and piracetam treated group. In the same way, significant enhancement in memory was seen in *Bacopa monnieri* plant leaves proteins treated group as compared to scopolamine only treated group and piracetam treated group.

In passive avoidance task, on learning day, improvement was evident in group treated with *Bacopa monnieri* plant leaves proteins when compared with scopolamine only treated group, which suggests an enhancement of learning though it was not statistically significant. On retention day, significant enhancement in memory was seen in *Bacopa monnieri* plant leaves proteins treated group and piracetam treated group as compared to scopolamine only treated group. The memory enhancement in *Bacopa monnieri* plant leaves proteins treated group appeared to be more than the piracetam treated group, it was statistically insignificant.

Results of the study indicate that *Bacopa monnieri* plant leaves proteins has learning and memory enhancing effect as noted by changes in the TL/STL in learning and retention trials. Since the amnesic effect of scopolamine which is a muscarinic receptor antagonist has been reversed by *Bacopa monnieri* plant leaves proteins successfully, it indicates that proteins of *Bacopa monnieri* plant leaves acts on Ach receptors. Beneficial effect on learning and memory enhancement was superior in *Withania somnifera* treated group as compared to piracetam treated.

## CONCLUSION

This is the first study of its kind where proteins from *Bacopa monnieri* plant leaves have been evaluated for learning and memory enhancing potential in rats. It is evident from the above findings that the beneficial effects of *Bacopa monnieri* plant leaves proteins on memory can be attributed to facilitation of cholinergic transmission. Hence *Bacopa monnieri* plant leaves proteins would be beneficial and it can also be added as an adjuvant to existing therapies for the treatment of dementia. It is also necessary to find further which particular protein of the plant leaves is showing more / effective activity.

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