Nootropic Potential of Silver Nanoparticles of Boerhaavia diffusa and its Ethanolic Extract in High Fat Diet Model of Dementia in Rats

Sibi P Ittiyavirah[1*], Reshma Ghosh[2]

1) Assistant Professor and Head of Department, Division of Pharmacology, Dept of Pharmaceutical Sciences, Mahatma Gandhi University, Cheruvandoor, Ettumanoor, Kottayam, Kerala, India.

2) Research Scholar, Division of Pharmacology, Dept of Pharmaceutical Sciences, Mahatma Gandhi University, Cheruvandoor, Ettumanoor, Kottayam, Kerala, India.

INTRODUCTION

Dementia is the condition of progressive deterioration of intellectual and cognitive function resulting in loss of social independence, usually seen to progress as aging. In dementia, at least one among the functions such as language, decision making, calculation, judgment, spatial orientation and abstract reasoning of cortical area may be impaired along with the memory loss. The symptoms progress over months to years, and alertness is preserved until the very late stages of disease. Alzheimer society's Dementia UK in 2014 predicted that by 2015 there will be 850,000 people with dementia in the UK and that will cost them £26 billion a year. Prevalence of overall dementia and its subtypes are increasing in India. Several plants were proved for nootropic activity such as *Bacopa monniera* [1], *Celastrus paniculatus* [2], *Centella asiatica* [3] and those were used as herbal medicines for dementia.

Boerhaavia diffusa is a traditionally used plant for the treatment of various ailments in traditional health care system even though its nootropic activity has not yet been scientifically evaluated. In the present study the nootropic activity of ethanolic extract of *B.diffusa* was investigated. In this study the nootropic activity of the biosynthesised silver nanoparticle form of *B.diffusa* was investigated.

Green chemistry is being promoted to a large extent these days as it is eco-friendly as well as nontoxic. The field of nanotechnology recently used plants as bio-source for the reduction and stabilization of the metal nanoparticles. The size property of nanoparticles thus helps in the penetration into the brain tissues. Since the biological molecules were present as biosynthesized silver nanoparticle, it may get easily delivered and the therapeutic effect can be assessed. Plants containing biological compounds may assist the reaction to be more compatible and overcomes the tedious process of maintaining cell cultures.

* For Correspondence:
E-mail:sibitho@gmail.com

MATERIALS AND METHODS

Experimental animals

Healthy male Wistar albino rats (150-200gm) were obtained from the animal house of Dept. of Pharmaceutical Sciences, Mahatma Gandhi University, Cheruvandoor, Kottayam, Kerala, India. They were housed in well ventilated, large spacious hygienic cages under standard animal husbandry conditions (22-28^{0}C) with relative humidity of 75±5% and alternate12hour light-dark cycle. The animals were fed with standard food and water *ad libitum*. All animals were acclimatized to the experimental environment prior to study. The study protocol was approved by Institutional Animal Ethical Committee (IAEC), Department of Pharmaceutical Sciences, Mahatma Gandhi University, Cheruvandoor, Kottayam, Kerala, India and the number was assigned as (IAEC:016/MPH/UCP/CVR/14).

Drugs and chemicals

Piracetam was purchased from Sigma Aldrich, Bangalore, India. Silver nitrate (3mM) and Ethanol from Spectrum chemicals, Kerala, India and all other reagents were obtained from the chemical store of Department of Pharmaceutical Sciences, Mahatma Gandhi University, Cheruvandoor, Kottayam, Kerala, India.

Plant material

The whole plant of *B.diffusa* was collected from nearby places of Changanacherry during the month of June 2014 and authenticated by Rojimon Thomas, Assistant Professor Department of Botany, The CMS College, Kottayam, Kerala, India. The specimen Voucher No is 758.

Plant extraction

Plant of *B.diffusa* was collected locally and dried under the shade. The dried plant was crushed and ground to fine powder. The plant powder was extracted using Soxhlet apparatus using ethanol as the solvent and dried. The obtained extract was subjected to preliminary phytochemical analysis.

Biosynthesis of Silver nanoparticles of *B.diffusa*

Ethanolic extract of *B. diffusa* was taken in proportion of 2g extract in 20 ml distilled water added to 80 ml of 3mM $AgNO_3$ solution and incubated for 24 h. The solution turned to brown colour indicating the formation of silver nanoparticles of *B.diffusa* (AgNPsBD). The solution was then centrifuged at 7000 rpm for 10 minutes and the mixture was collected after discarding the supernatant. The collected AgNPsBD were allowed to dry in a watch glass.[4]

Characterisation of biosynthesized Silver nanoparticles

The optical properties of silver nanoparticles were studied using UV–VIS (UV–VIS 1800, Shimadzu Japan) spectral analysis respectively. The morphological, structural and chemical composition of biosynthesized silver nanoparticles was analyzed by employing SEM-EDX (Jeol JSM-3600) equipment. Dynamic light scattering (DLS) analysis was done by using Nano- ZS, Malveren Instrument, Navi Mumbai, India.

Acute oral toxicity study

The acute toxicity study was carried out in adult female albino rats according to OECD guideline No.420. The fixed dose method with a starting dose of 2000 mg/kg body weight and lower indexed doses was adopted. Then the animals were observed continuously for 3h for general behaviour and then every 30 min for next 3h and finally for mortality after 24h till 14 days.[5]

Treatment of animals

Animals were divided into 5 groups. Group I received normal pellets for a period of 28 days, Group II served as the Control group that received only high fat diet (HFD) for 28 days. Group III received HFD and were treated with Piracetam 50mg/kg p.o for 28 days. Group IV and Group V received HFD for 28 days and treated with ethanolic extract of *B.diffusa* (EEBD) (300mg/kg), p.o. and AgNPsBD (30 mg/kg) p.o. respectively.[6]

Table1: Composition of High Fat Diet

Contents	Carbohydrate	Protein	Fat	Fibers	Salt	Mineral	Vitamins
mg/100g	26	38	21	9.5	4	0.5	1

Evaluation of nootropic activity

Elevated plus maze

Elevated plus maze consisted of wood with two open arms (35 × 6 cm) and two enclosed arms (35 × 6 × 15 cm) were used. The maze was elevated to the height of 40 cm. The rats were placed individually at the end of open arm facing away from central platform and the time taken by the rat to move from open arm to either of the closed arms with all its four legs (Transfer latency, TL) was recorded. On the first day, the rats were allowed to explore the plus maze for 20 s. After the measurement of TL, rats were returned to their home cages after the first trial[7]; in second trial the transfer latencies were measured on the elevated plus–maze as before and TL was recorded again. The transfer latency was represented as inflexion ratio. Inflexion ratio is the time taken for the animal to enter either of the closed arms from its initial position. Inflexion ratio (IR) is calculated as (L1/L0)/L0, where L1 and L0 represents first and final trial respectively.

Morris water maze

Morris water maze technique was used to measure learning degree and spatial memory.[8] Rats were trained in a standard Morris water maze task. Maze consisted of large circular pool (75cmx30cm) filled with water at a depth of 20cm. The pool was divided into four quadrants. A circular platform was placed at the centre of one quadrant. The rats performed four trials per day for four consecutive days. In the swimming trials, each individual rat was released gently into the water at a randomly chosen quadrant. The rat swam and learned how to find the hidden platform within 60 s. After reaching the platform rat was allowed to stay on the platform for 15 s and was then taken back into the cage. The rats were placed on the platform by hand for 15 s, if they could not escape to the platform within 60 s by themselves, and their escape latency was accepted as 60 s. During the inter-trial intervals, animals were kept in a dry home cage for 60 s. The time to reach the platform (latency) was recorded. 24h after the last day of training, subjects were tested on a probe trial, during which the escape platform was removed and the time spent in the correct quadrant was measured for a 60 s trial.[9]

Estimation of biochemical parameters

The rats were decapitated, brains were removed quickly and placed on ice-cold saline. Frontal cortex, hippocampus and septum are quickly dissected out on a Petri dish chilled on crushed ice. The tissues were weighed and homogenized and used for the following estimations.

Reduced glutathione

Tissue was homogenized in 5ml precipitating solution (1.6g of Glacial meta phosphoric acid, 0.2g of Disodium or Dipotassium ethylene diamine tetra acetic acid (EDTA) and 30g of Sodium chloride (NaCl) were placed in a 100 ml flask and brought to volume with distilled water).The tubes were incubated for 5 min at room temperature and then filtered through course grade filter paper. To 0.2ml filtrate, 3ml of 0.3M phosphate solution and 1ml of 0.04% DTNB was added. The tubes were capped, mixed by inversion and contents were read at 412nm within 4 min.[10]

Lipid peroxidation

The tissue homogenate was prepared in 0.1N Tris- HCl buffer. Homogenate (1ml) was mixed with 2ml of TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated in a boiling water bath for 15 min. After cooling the flocculent precipitate was removed by centrifugation at 1000xg for 10 min. The absorbance of the sample was read at 535 nm.[11]

Statistical Analysis

All results were expressed as mean ± SEM. Data was analysed by Graph Pad Prism (version6.0) by using one way ANOVA followed by Dunnet's post hoc test. The $p<0.05$ was considered to be statistically significant.

RESULTS

Table 2: List of preliminary phytochemical tests

Phytoconstituents	Test performed/reagents used	Result
Alkaloids	Mayer test, Hager test, Dragendroff's test	-
Flavonoids	Shinoda test	+
Steroids	Salkoswki test	+
Glycosides	Borntrager's test	+
Saponins	Foam test	+
Protein and amino acid	Millons test, Ninhydrin	+
Reducing sugar	Biuret test, Benedict's test, Fehling's tests	+
Phenol	Ferric chloride and lead acetate	-
Terpenoids	Chloroform and sulphuric acid	+

+ indicates presence and – indicates absence

Silver nanoparticles:

The formed silver nanoparticle turned its colour to deep brown which indicated the formation of silver nanoparticle. The AgNPsBD λ_{max} 435 on the visible region of UV-Visible spectra (Fig: 1) was raised by the excitation of surface plasmon vibration caused the appearance of brown colour. The SEM image of AgNPsBD confirms the presence of very small and spherical nanoparticles (Fig: 2). The SEM analysis revealed the existence of very small and uniformly spherical nanoparticles. DLS revealed 129.9nm as the average particle size, the solution is found to be heterogeneous and possesses good stability (Fig: 3).

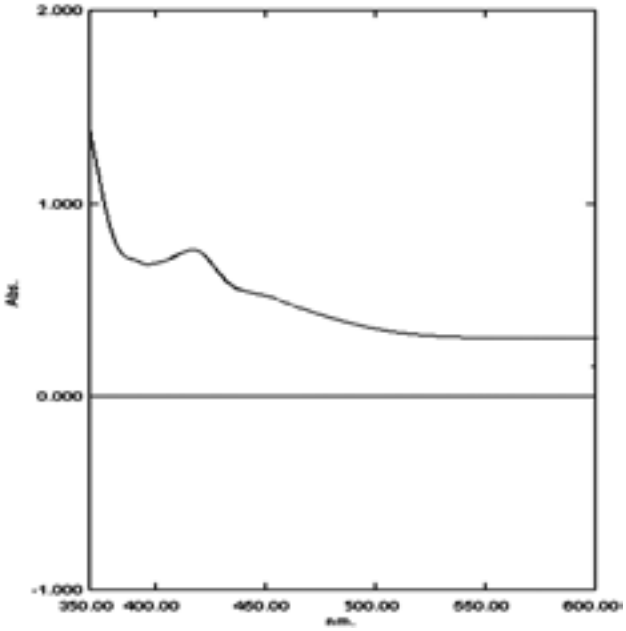

Fig 1: UV spectrum of AgNPsBD showing λ_{max} at 435nm

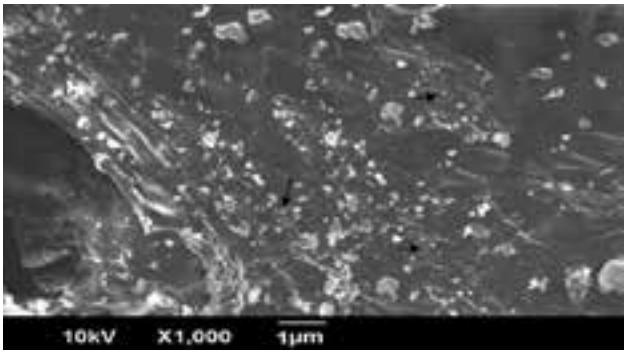

Fig: 2 SEM image of AgNPsBD revealed the existence of very small and uniformly spherical nanoparticles.

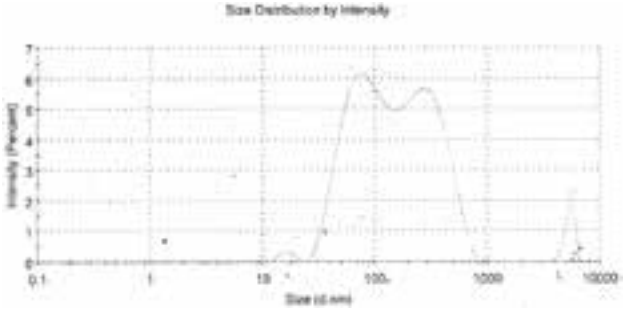

Fig 3: DLS showing 129nm as the average particle size, the sample is heterogeneous and possesses good stability of AgNPsBD. X-axis represents the diameter size of particles in nm and y-axis represents mean percentage intensity of particles present.

Acute oral toxicity study

Signs of toxicity were reported for 2000mg/kg p.o, therefore the lower indexed dose of 300mg/kg p.o was tested and no signs of toxicity found throughout the period of observation.

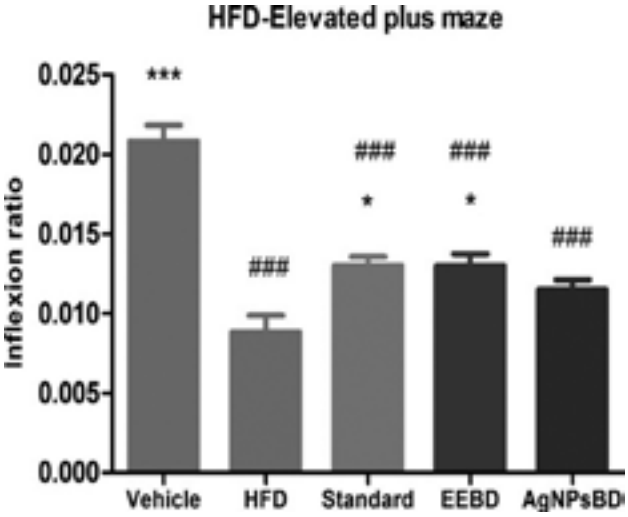

Fig 4: Experiment results of Elevated plus maze (IR). Each group consists of 6 animal each (n=6). Values are expressed Mean±SEM, ### denotes p<0.001, *** denotes P<0.001 and * denotes p<0.05 are considered as significant by One-way ANOVA followed by Dunnet's multiple comparison. Significance is between inducing agent (HFD) group vs other groups. A significant effect of EEBD treated was shown similar to that of standard drug (Piracetam). AgNPsBD also showed a non-significant effect increase in inflexion ratio.

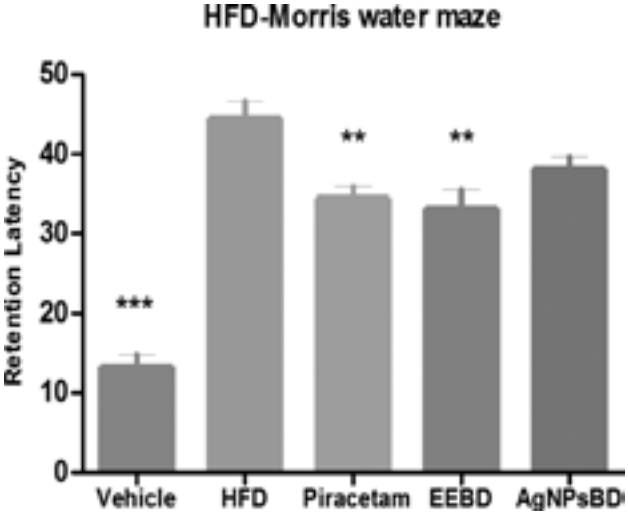

Fig5: Experiment results of Morris water maze (RL). Each group consists of 6 animal each (n=6). Values are expressed Mean±SEM, *** denotes P<0.001 and ** denotes P<0.01,* denotes p<0.05 are considered as significant by One-way ANOVA followed by Dunnet's multiple comparison. Significance is between inducing agent (HFD) group vs other groups. A significant effect of EEBD treated was shown similar to that of standard drug (Piracetam). AgNPsBD also showed a non-significant effect.

Biochemical parameters

Estimation of Reduced Glutathione

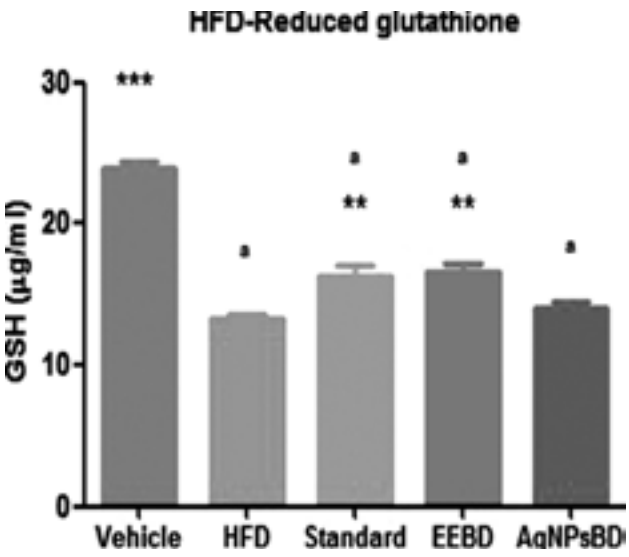

Fig 6**:** GSH level in rat brain. Each group consists of 6 animal each (n=6). Values are expressed Mean±SEM, *** denotes P<0.001 and ** denotes P<0.01,* denotes p<0.05 are considered as significant by One-way ANOVA followed by Dunnet's multiple comparison. Significance is between inducing agent (HFD) group vs other groups. EEBD treatment showed effect similar to that of standard drug (Piracetam). AgNPsBD also showed a non-significant effect.

Estimation of Lipid peroxidase

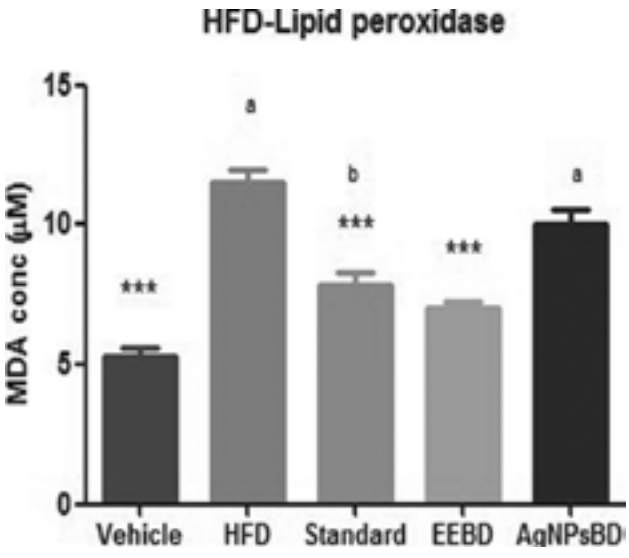

Fig 7: Lipid peroxidase level in rat brain. Each group consists of 6 animal each (n=6). Values are expressed Mean ±SEM, *** denotes P<0.001, ** denotes P<0.01 and * denotes p<0.05 are considered as significant by One-way ANOVA followed by Dunnet's multiple comparison. a denotes p<0.001 and b denotes p<0.01. Significance is between inducing agent (HFD) group Vs other groups. A significant effect of EEBD treated was shown similar to that of standard drug (Piracetam). AgNPsBD also showed a non-significant effect.

DISCUSSION

Dementia is the deterioration of intellectual or cognitive function with little or no disturbance of consciousness and perception.[12]

Non-Alzheimer's dementias are disorders characterized by problems with memory and cognitive function plus other unique clinical features like smoking, excessive alcohol consumption, obesity, diabetes (treat to avoid frequent hypoglycaemia), hypertension, and elevated cholesterol. In the present study the role of HFD varying corticosterone level in relation to cognition was assessed by using Wistar albino rat HFD induced dementia model. The cognitive defectiveness and the effectiveness in cognition of EEBD and AgNPsBD were assessed.

The plant *B.diffusa* has been used in various ailments in traditional health care system and Ayurvedic formulations containing *B.diffusa* as one among ingredients in polyherbal formulations.[13]

Various studies conducted on *B.diffusa* are on either root part only or leaf part only.[14]Similarly, the extraction processes generally conducted were aqueous [15], hydroalcoholic [16] or methanol[17] and ethanolic soxhlet extractions.[18] In this study the ethanolic extraction by soxhlet process was carried out. The preliminary phytochemical analysis revealed the presence of components such as glycosides, terpenoids, flavonoids, proteins, carbohydrates and steroids.

In the biosynthesis of silver nanoparticles different studies showed usage of different concentration of silver nitrate solutions. Trial and error method was employed with various molar concentrations of silver nitrate solutions during the biosynthesis of AgNPsBD starting from 1M silver nitrate solution. However the toxicity of silver nitrate in animals and formation of AgNPs has to be considered. The study was aimed to find the least molar effective silver nitrate solution required for the formation of AgNPsBD, and thus in this study 3mM of silver nitrate solution were used.

Certain studies showed the formation of AgNPs immediately with the addition of silver nitrate solution whereas some others required heating. As heat may eliminate the bio molecules present in the extract in this study no heat was applied but it was kept for 24h duration, for the formation of AgNPsBD.

The formation of biosynthesised nanoparticles usually exhibit the visual property of colour change from green to brown or yellow to brown. In the present study AgNPsBD exhibited the similar colour change property i.e., from green to deep red brown.

Most studies revealed that AgNPs give typical spectrum having maximum absorption in range of 420-450 nm. This absorption is unique property of metal nanoparticles known as surface plasmon resonance (SPR). Generally SPR arises due to conduction of electrons on surface of AgNPs. The SPR of different biosynthesised metal nanoparticles are different, and mainly depend upon the nature of metal used. Previously reported studies showed that for gold nanoparticles it is around 540 nm[19] whereas zinc sulphide (ZnS) nanoparticles are around 315 nm.[20] In this study AgNPsBD showed maximum absorption range at 435nm, which is within the range of general SPR range (420-450) of silver nanoparticles.

DLS generally shows the mean percentage size and determines the stability of the particles. In this study a mean percentage of 129.9 nm is obtained for the prepared silver nanoparticles and also shows a good stability. In the SEM images larger particles are also seen. The variation in the formed nanoparticles may be due to evaporation of solvent during the preparation.

This study revealed that HFD fed rats showed a significant decrease in memory or inflexion ratio of elevated plus maze, learning procedure and spatial memory acquisition of Morris water maze. Consequently an increase in time for the entry to the closed arm in elevated plus maze and covered distance to find platform in Morris water maze of HFD fed rats were observed when compared to that of treated groups and normal group.

In HFD only fed group exhibited a reduction in hippocampal neurogenesis and impeding hippocampal neurogenesis blocks hippocampal dependent learning.[21] HFD causes increase in the corticosterone levels in the male rats, which inhibit the neurogenesis. Generally studies suggest that newborn hippocampal neurons have role in memory and learning. HFD in rodents reported to possess learning impairment.[22] The corticosterone maintaining effect of *B. diffusa* and the flavonoids contained in the extract may have role in controlling serum corticosterone level in male rats. These facts may have decreased the effect of high fat diet induced dementia.

If oxidative stress increased in high fat diet intake, then lipid peroxidase acts as a marker for oxidative injury. In the

present study the elevated lipid peroxidise value in HFD rat brain indicated the oxidative injury or oxidative stress and reversal of the effects were obtained in the treated groups especially that of EEBD treated. The enzymatic antioxidant like that of GSH scavenges the reactive oxygen species and free radicals to stop their formation.[23] The present study showed that there was depletion of GSH content in the rats fed with HFD only and were restored by EEBD treatment. These facts showed its antioxidant effect in protecting the neurogenesis or its nourishment, which can protect the brain from dementia.

CONCLUSION

The present study revealed that ethanolic extract of *B. diffusa* possesses significant nootropic activity, as they have increased the inflexion ratio and decreased the retention latencies in elevated plus and Morris water mazes respectively. The antioxidant properties showed in the estimation of biochemical parameters also further confirms the nootropic potential of EEBD. The study also shows that, the existed facts about silver nanoparticles are correct, since the silver nanoparticles were biosynthesised it doesn't harm the tissues when compared to chemically synthesized, instead gave an effect. Even though the effect of AgNPsBD was comparatively lesser than that of the EEBD. It is concluded that ethanolic extract of *B.diffusa* has nootropic activity and if the nano particles were site targeted, there might be better activity.

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