Cognitive effects of ethanolic extract of *Boerhaavia diffusa* and its silver nanoparticles in ethanolic dementia model

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**ABSTRACT**

Dementia is a condition of progressive deterioration of intellectual or cognitive function. Several factors cause dementia such as excessive alcohol consumption, elevated cholesterol, obesity, hypertension, diabetes (treat to avoid frequent hypoglycaemia) etc. Present study investigated the effects of ethanolic extract of *Boerhaavia diffusa* (EEBD) and its silver nanoparticles (AgNPsBD) in Ethanol induced interoceptive dementia rat model. Silver nanoparticles of *Boerhaavia diffusa* (AgNPsBD) were biosynthesized and characterised analytically by UV, SEM, and DLS. Male Wistar albino rats received Ethanol (2.5 mg/kg, i.p.) for 15 days. Animals were divided into five groups. Group I contained normal rats, Group II contained Control rats that received Ethanol 2.5 mg/kg i.p. and Group III, Group IV and Group V were received Piracetam, EEBD (300 mg/kg) and AgNPsBD (30 mg/kg) p.o. respectively along with Ethanol. Elevated plus maze and Morris water maze were used as exteroceptive models for analysing cognitive performances. The animals were sacrificed and estimated lipid peroxidase and reduced glutathione in rat brain. Dementia induced animals showed reduced cognitive performance and increased oxidative stress when compared to other groups. It is concluded that the EEBD causes a reduction in both transfer latency and retention latencies and the biochemical parameters also indicate the cognitive enhancing effect of EEBD compared to its silver nanoparticles.

**Keywords:** Dementia, Silver Nanoparticles, *Boerhaavia diffusa*, Scanning Electron Microscopy, Dynamic Light Scattering.

**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 2014 found that by 2015 there will be 850,000 people with dementia in the UK and that dementia costs the UK £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 are used as herbal medicines for dementia.

*B. diffusa* is a traditionally used plant for the treatment of various ailments in traditional health care system and its nootropic activity has not yet scientifically evaluated. In the present study the nootropic activity of ethanolic extract of *B. diffusa* was investigated. Study followed evaluation of the biosynthesised silver nanoparticle form of *B. diffusa* was also investigated for its nootropic activity.

Nowadays green chemistry is been more promoted as it is ecofriendly and to avoid toxins. The field of nanotechnology recently used plants as bio-source for the reduction and stabilizing the metal nanoparticles. The size property of nanoparticles thus helps in the penetration into the brain tissues. As the biosynthesised silver nanoparticle containing the biological molecules it may get easily delivered and the therapeutic effect can be assessed. As the plants contains biological compounds that assists the reaction to be more compactable and it overcomes the tedious process of maintaining cell cultures.

**MATERIAL AND METHODS**

**Extraction and fractionation**

Healthy male Wistar albino rats (150-200gm) were obtained from the animal house of Dept. of Pharmaceutical Sciences, Mahatma Gandhi University, Cheruvandoor campus, Kottayam, Kerala, India. They were housed in well ventilated, large spacious hygienic cages under standard animal husbandry conditions (22-28°C) with relative humidity of (55 ±5)% and alternate 12hour 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), Dept. of Pharmaceutical Sciences, Mahatma Gandhi University, Cheruvandoor, Kottayam, Kerala, India and the number was assigned as (IAECNO:016/MPH/UCP/CVR/14).

Drugs and chemicals

Piracetam was purchased from Sigma Aldrich, Bangalore, India. Silver nitrate (3Mm) and ethanol (60%) and all other reagents were obtained from the chemical store of Dept. 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 and authenticated by Rojimon Thomas, Asst. Professor Department of Botany, CMS College, Kottayam, Kerala, India. The specimen Voucher No: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 by soxhlation process using Ethanol as the solvent and dried. The obtained extract was subjected to preliminary phyto chemical analysis.

Biosynthesis of Silver nanoparticles of Boerhaavia diffusa

Ethanolic extract of B. diffusa was taken in proportion of 2g extract in 20 ml distilled water added to 80 ml of 3M\(\text{AgNO}_3\) solution and incubated for 24 h. The solution turned to brown colour indicating the formation of AgNPsBD. After that, the solution was 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. 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 as in Annex 2d, test procedure 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 behavioral and then every 30 minutes for next 3h and finally for mortality after 24h till 14 days [5].

Treatment of animals

Animals were divided into 5 groups and were treated accordingly for duration of 15 days.

- **Group I** - vehicle treated (normal), 2ml 5%CMC, p.o.
- **Group II** - Control (inducing agent only- Ethanol 2.5 mg/kg i.p.)
- **Group III** - Standard- received Piracetam 50mg/kg,p.o.
- **Group IV** - EEBD (300mg/kg), p.o.
- **Group V** - AgNPsBD (30 mg/kg) p.o. treated [6]

Evaluation of nootropic activity of ethanol induced model

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 sec. After the measurement of TL, rats were returned to their home cages after the first trial [7]. On 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]. All rats were trained in a standard Morris water maze task. Maze consisted of large circular pool (75cm &30cm) 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

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 1000g 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 analyzed by using one way ANOVA followed by Dunnet’s post hoc test. The p<0.05 was considered to be statistically significant.
RESULT

Table 1: List of preliminary phytochemical tests

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Test performed/ reagents used</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Mayer test, Hager test, Dragendorff's test</td>
<td>-</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>Shinoda test</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>Salkoswki test</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Borntrager’s test</td>
<td>+</td>
</tr>
<tr>
<td>Saponines</td>
<td>Foam test</td>
<td>+</td>
</tr>
<tr>
<td>Protein and amino acid</td>
<td>Millions test, Ninhydrin</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td>Biuret test, Benedicts test, Fehlings tests</td>
<td>+</td>
</tr>
<tr>
<td>Phenol</td>
<td>Ferric chloride and lead acetate</td>
<td>-</td>
</tr>
<tr>
<td>Terpinoids</td>
<td>Chloroform and sulphuric acid</td>
<td>+</td>
</tr>
</tbody>
</table>

+ 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 $\lambda_{\text{max}}$ 435 on the visible region of UV-Visible spectra (Fig: 1) was arised by the excitation of surface plasmon vibration caused the appearance of brown colour \[12\]. 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 heterogeneous and possesses good stability (Fig: 3).

![Fig 1: UV spectrum of AgNPsBD showing $\lambda_{\text{max}}$ at 435nm](image1)

![Fig 2: SEM image of AgNPsBD revealed the existence of very small and uniformly spherical nanoparticles](image2)

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.

Nootropic activity of ethanol induced model:

![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<0.01 and *denotes P<0.05 are considered as significant by One-way ANOVA followed by Dunnet’s multiple comparison. Significance is between inducing agent (ethanol) group and groups receiving EEBD and AgNPsBD, which showed a significant effect of EEBD which was showed effect similar to that of Piracetam. AgNPsBD also showed a significant effect, but was comparatively lesser than that of EEBD and standard](image4)

![Fig 5: Experiment results of Morris water maze (Retention latency in sec) test. 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. Significance is between inducing agent (ethanol) group and groups receiving EEBD and AgNPsBD, which showed a significant effect of EEBD which was showed effect similar to that of standard drug. AgNPsBD also showed a significant effect, but was comparatively lesser than that of EEBD and standard](image5)
Estimation of Reduced Glutathione Level (GSH)

**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, **denotes P<0.01, *denotes P<0.05 are considered significant by One-way ANOVA followed by Dunnet’s multiple comparison. Significance is between inducing agent Ethanol group and groups receiving EEBD and AgNPsBD and standard (Piracetam), which showed a significant effect of EEBD similar to that of Piracetam. AgNPsBD also showed significant effect, less than other EEBD and standard.

Estimation of Lipid peroxidises:

**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, *denotes P<0.05 are considered significant by One-way ANOVA followed by Dunnet’s multiple comparison. Significance is between inducing agent Ethanol group and groups receiving EEBD and AgNPsBD and standard (Piracetam), which showed a significant effect of EEBD similar to that of Piracetam. AgNPsBD also showed significant effect, less than other EEBD and standard.

**DISCUSSION**

Dementia is the deterioration of intellectual or cognitive function with little or no disturbance of consciousness and perception [13].

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 ethanol in relation to cognition was assessed by using Wistar albino rat ethanol 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 [14].

Various studies conducted on *B. diffusa* are on either root part only [15] or leaf part only [16]. Similarly, the extraction processes generally conducted were aqueous [17] hydroalcoholic [18] or methanol [19] and ethanolic soxhlet extractions [20]. In this study the ethanolic extraction by soxhlation process was carried out. The preliminary phytochemical analysis revealed the presence of components such as glycosides, terpenoids, flavanoids, 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 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 were as 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 formations 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 that is 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 [21] whereas zinc sulphide (ZnS) nanoparticles are around 315 nm [22]. 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.

According to findings of this study intraperitoneal injection of ethanol 2.5mg/kg body weight, causes 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 rats receiving Ethanol 2.5mg/kg was observed compared to the treated groups. The reason to choose ethanol 2.5mg/kg was that chronic alcohol intake in rodents causes impairment of hippocampal-dependent learning and memory [23,24], which occurs as a consequence of the vulnerability of the hippocampus to ethanol-induced neurodegeneration [25,26]. Ethanol intake also causes frontal cortex and corpus callosum shrinkage and hippocampal Ach hypofunction. The negative influence of ethanol on NMDA mediated synaptic transmission of hippocampus may contribute to deleterious effects on learning and retention (Walker and Hunter; 1978). The ethanolic extract of *B. diffusa* which already proven to have antioxidant effect and the presence of flavonoids especially the 3, 7', 5-trihydroxy-7'-methoxy flavones, 4',7-dihydroxy -3'methyl flavones and the urosilic acid may have contributed to nootropie activity. Reactive oxygen species affects the antioxidant defence mechanism by reducing the intracellular concentration of GSH as well as increasing lipid peroxidase. Significantly increased level of GSH and decreased lipid peroxidase levels were seen in the rats with EEBD and Piracetam.
treated as compared to Ethanol group. These facts indicate that the antioxidant mechanism required for the neuron cell survival, its nourishment and above all the neurogenesis are distorted or impaired. Neurogenesis generally involves generation, maturation, migration and functional integration of new neurons into the hippocampal dentate gyrus and has been implicated in hippocampal-mediated learning [27,28]. Alteration in these functions by the ethanol intake may lead to reduced neurogenesis which causes dementia. Even the binge ethanol exposure reduces both proliferation and survival of pluripotent cells [29]. Thus the reduction in neurogenesis is associated with decreased hippocampal learning and memory [30,31].

CONCLUSION

The present study showed that ethanolic extract of B. diffusa and its silver nanoparticle possess significant nootropic activity. The study shows that the contradicted existed facts about silver nanoparticles are correct, yet when the silver nanoparticle contains biosynthesised particles it doesn’t harm much as expected but gave an effect even though its effect was comparatively lesser than the extract. From the study it is concluded that ethanolic extract of B. diffusa has nootropic activity and such bio molecules in nano form if they are site targeted form they can give better results.

Conflicts of Interests

No conflicts of interest.

REFERENCES


HOW TO CITE THIS ARTICLE