Defence Life Science Journal, Vol. 2, No. 2, April 2017, pp. 152-162, DOI : 10.14429/dlsj.2.11365

RESEARCH PAPER

Brahmi Herbal Drink Mitigates Aluminium Chloride Induced Cognitive Impairments

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ABSTRACT

Aluminium chloride ($AlCl_3$) is neurotoxic and has been proposed to be one of the environmental factors responsible for neurodegenerative disease like Alzheimer's and Parkinson's disorders. It also causes learning and memory deficit. *Bacopa monniera* is well known for its memory enhancing property in traditional Indian system of medicine. Cognitive-enhancing and neuromodulatory property of brahmi herbal drink (BHD), a nutraceutical product from *Bacopa monniera* extract. BHD was evaluated for physicochemical, sensory attributes and stability studies. Overall acceptability of BHD was good according to hedonic scale/ratings. Stability of the drink is for 6 months without losing its activity. Further, cognitive enhancing and neuromodulatory propensity of BHD was evaluated against $AlCl_3$ treatment in rats. Administration of AlCl3 (100 mg/kg) daily for 23 days significantly increased cognitive impairment as evaluated in elevated plus maze (EPM) and Morris water maze (MWM) tests. BHD supplementation improved cognitive ability by decreasing the transfer and escape latency of EPM and MWM tests respectively. Results further elucidate that BHD supplementation decreased acetylcholine esterase activity and nitric oxide levels by down-regulating AChE and iNOS expression respectively. BHD supplementation showed it neuroprotective efficacy by up-regulating BDNF expression. AlCl3 induced lipid peroxidation and reactive oxygen species generation was significantly alleviated by BHD and restored antioxidant status levels. All these results demonstrated the cognitive-enhancing and neuromodulatory potential of BHD in counteracting the damage inflicted by $AlCl_3$ on rat brain.

Keywords: Brahmi herbal drink; Antioxidants; Aluminium chloride; Acetylcholine esterase; Inducible nitric oxide synthase

1. INTRODUCTION

Aluminium chloride ($AlCl_3$) is neurotoxic and has been proposed to be one of the environmental factors responsible for neurodegenerative disease like Alzheimer's and Parkinson's disorders[1]. Aluminium can easily cross blood brain barrier via. specific high affinity receptors and upon entering leads to cognitive dysfunction by impairing cholinergic function and inducing oxidative stress[2]. Studies have also shown that long-term exposure affects axonal transport, Aluminium acts synergistically causes. Structural abnormalities in synaptic cleft results in memory impairment[3]. In addition, aluminium induces inflammatory responses[4], protein like neurofilaments, microtubules associated protein and Aβ of highly phosphorylated cytoskeletal implicated in Alzheimer's disease[3,5]. Aluminium chloride has also shown have negative effects on behavioural aspects like anxiety-related behaviour in wistar rats[6]. Histology studies have also reveals the neurodegenerative effects of aluminum in the cerebral cortex and hippocampus of rats especially at higher dose. The mechanism of $AlCl_3$-inducted neurotoxicity and identification of effective treatment is an important public and occupational health priority for industrial and developing nations.

Phytomedicine obtained from herbal sources are in great demand as they are able to alleviate various infectious diseases, physical and cognitive disorders and there by provide outstanding contribution to modern therapeutics. *Bacopa monniera* is a member of Scrophulariaceae, hold great promise for the improvement of cognitive and endurance enhancing function. It is a perennial prostrate or creeping, juicy, succulent, glabrous annual herb, found throughout India in wet, damp and marshy areas[7]. It is commonly known as Brahmi. In ayurveda it is an important constituent of medicine classified into medhyarasayana, a medicine known for intellect and improve memory[7]. The cognition facilitating effect of *B. monniera* was extensively investigated and was due to the active saponins, bacoside A content[8]. These active principles, apart from facilitating learning and memory in normal rats, it has several other health benefits such as anti-stress[9], vasodilator[10], anti-inflammatory[11], anxiolytic[12] and anti-fatigue properties[13].

Received : 28 March 2017, Revised : 15 April 2017
Accepted : 21 April 2017, Online published : 12 May 2017

Neuroprotective property of *Bacopa monniera* against various neurotoxicants such as hydrogen peroxide, scopolamine, sodium nitroprusside and crackers smoke were already demonstrated[14-16]. Since, the bacoside rich extract is bitter in taste. It was blended with date syrup, honey, ginger extract, lemon as base to improve the sensory attributes, nutritive and nutraceutical value of the drink. The present study was further undertaken to investigate the effect of developed BHD against $AlCl_3$ induced neuronal impairments in rats. Despite strong experimental and clinical evidence for aluminium effects on the nervous system, the mechanism is still not completely clear. To gain further insight into potential mechanisms of toxicity and to explore the possible treatment effects of BHD we studied the effects of exposure to $AlCl_3$ and BHD on behavioural and neurochemical endpoints in rats.

2. MATERIALS AND METHODS

2.1 Chemicals

Aluminium chloride, thiobarbituric acid, trichloro acetic acid, ferric chloride, dinitro salicylic acid, p-hydroxydiphenyl, copper suphate, were purchased from Himedia, India and glycine, hydrochloric acid, potassium hydroxide, sodium hydroxide, calcium hydroxide, sodium dodecyl sulphate, sodium sulphate, disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate were purchased from Merck, Germany. RNeasy mini kit was purchased from Qiagen (Valencia, CA, USA). Transcriptor first strand synthesis kit was purchased from Roche (Mannheim, Germany). All the dehydrated media and chemicals were purchased from Himedia, Mumbai, India. Lion Date syrup (Lion Dates Impex Pvt. Ltd, Chennai), honey, ginger extract, lemon were purchased from the local market, Mysuru, India.

2.2 Isolation, Purification and Quantification of Bacoside A from *Bacopa Monniera* Extract (BME)

Bacopa monniera plant material was collected from the foot hills of Tirumala, Tirupati, Andhrapradesh. Identification and authentication was done by Dr. Nagaraju, Taxonomist from the Dept. of Botany, Sri Venkateswara Degree college, Tirupati, A.P and the herbarium collection No: DR 470 (1991) and the sample was preserved at the herbarium collection. The hydroethanolic (90 per cent ethanol) bacoside rich extract was isolated and quantified for bacoside A by RP-HPLC and LC-MS-MS analysis and was found to be 16 per cent as reported in our previous studies[14-16].

2.3 Preparation of Brahmi Herbal Drink

Based on the amount and activity of bacosides, drink was prepared with a combination of BME (0.050 per cent), date syrup (4 per cent), honey (10 per cent), Ginger extract (1.5 per cent) with lemon juice (2 per cent) and sodium benzoate (0.015 per cent) as preservative and packed in 4 layer co-extruded film pouches (12 micron/PET/9 micron aluminum foil/15 micron nylon/7 micron CAST polypropylene and pasteurised at 85 °C for 15 min). Herbal drink without brahmi extract was also prepared and studied as negative control.

2.4 Physico-Chemical Analysis of BHD

The developed BHD was analysed for moisture, pH, acidity, according to the standard methods (AOAC, 2000). Total soluble solids (° Brix) were estimated by means of Abbe refractometer (Milton Roy, USA).

2.5 Sensory Analysis

Thirty trained panelists comprised of scientists of the Defence Food Research Laboratory, Mysuru, carried out sensory evaluation of the BHD to access the acceptability of the product. Sensory parameters evaluated were, colour, taste, appearance, flavour and over-all acceptability (OAA) using a score scale of 1 to 9 where 1 indicates extreme dislike and 9 indicates extreme like[17].

2.6 Stability Studies and Microbial Analysis

BHD was stored at 4 °C and room temperature for 12 months. Every 3months duration, drink was tested for microbial load as well as for its performance enhancement by conducting forced swim test as the benchmark for its function (Data not shown).

The microbiological analysis of the product was carried out to ensure its quality and stability at different storage temperatures at regular intervals. The parameters like aerobic mesophilic counts, coliform, spores, yeast and mould counts of the processed food sample was assessed. The sample was further evaluated for the presence of food pathogens such as Bacillus cereus, Salmonella, Shigella, Staphylococcus aureus and Escherichia coli. All the experiments were performed following APHA guidelines (1992)[18]. The protocol for the microbiological analysis of the food sample was performed as described by Harrigan (1998)[19]. Briefly,10 ml of the food sample was aseptically added to 90 ml of 0.1 per cent sterile peptone water and homogenised. The homogenate was serially diluted decimally. For the enumeration, one ml of the inoculum from each dilution was plated using respective media. The enumeration was performed in duplicates. For determining the spore count the initial inoculum was heated in water bath at 80 °C for 15 min. Dextrose Tryptone Agar (DTA) was used for the enumeration of spores. Red Bile Agar (VRBA) was used for the selective growth of coliforms and plate count agar (PCA) was used for the growth of aerobic mesophilic bacteria. Violet. Yeast and Mould were grown on acidified potato dextrose agar (PDA). VRBA plates were incubatedat 37 °C for 18 h. PCA and DTA plates were incubatedat 37 °C for 48 h. PDA plates were incubatedat 30 °C for 3-5 days.

The presence of *Bacillus cereus* and *Staphylococcus aureus* was checked using mannitol yolk polymyxin agar and Baird Parker agar respectively. For the detection of *Escherichia coli,* the homogenised sample was inoculated in MacConkey broth and incubated at 37 °C for 24 h. The sample was tested for the absence of *Escherichia coli* by culturing the sample on eosine methylene blue agar. For incidence of *Salmonella* and *Shigella*, 25 ml of sample was incubated with 225 ml of buffered peptone water at 37 °C for 24 h followed by selective plating on Salmonella Shigella Agar.

2.7 Animals and Feeding

Animal experiments were conducted according to the Institute Animal Ethical Committee regulations approved by the Committee for the Purpose of the Control and Supervision of Experiments on Animals (CPCSEA). For experiments from the stock colony thirty Wistar strain male albino rats weighing 130 g - 150 g were selected from the stock colony, Defence Food Research Laboratory, Mysuru, India, housed individually in stainless steel cages under maintained 12 h light/dark cycle with controlled room temperature (25 ± 2 °C). The rats were randomly divided into the following five groups:

(a) Group I- control

(b) Group II- BHD group (supplemented with BHD),

(c) Group III- $AlCl_3$ group ($AlCl_3$ administered at dose of 100 mg/kg body wt.)

(d) Group IV- BHD+$AlCl_3$ groups (BHD Supplementation against $AlCl_3$ treatment at a dose of 100 mg/kg body wt.) and

(e) Group V- HD+$AlCl_3$ groups (HD is herbal drink consisting all the ingredients present in BHD except bacopa extract), HD administration against $AlCl_3$ treatment at a dose of 100 mg/kg body wt.).

All the rats were fed with standard commercial available pellet diet supplied by Sri Venkateswara Enterprises, Bengaluru, India. Fluid intake and body weight were monitored daily. BHD treated groups (group II and group IV) rats were supplemented with BHD whereas group V was supplemented with HD from day-1 to day-21. Cognitive impairment was induced by administration of $AlCl_3$ (100 mg/kg, orally by gavage) from day-3 onwards. For elevated plus maze acquisition and retrieval trial experiments were performed on day-17 and 18 with a gap of 24 h for each trial. On day-19 rats were subjected for morries water maze test.

2.8 Behavioural Tests

2.8.1 Elevated Plus-Maze Test

In animal models like rats and mice evaluate learning and memory elevated plus-maze served as the exteroceptive model. It consisted of two open (50 × 10 cm^2) and two closed (50 × 10 × 40 cm^3) arms, connected by a central platform of 10 × 10 cm^2 elevated to a height of 80 cm above the floor, In the middle of the floor fine line was drawn to each closed arm. Each animal was individually placed at the one end of an open arm facing away from the central platform of the plus-maze. All the animals were given a single trial on the plus-maze. Transfer latency (TL) was taken as the time taken by the rat to move from an open arm to any one of the closed arms crossing the middle linewith all its four legs. In case, the animal fail to enter the closed arm within 90 s, it was gently moved into the closed arm and the transfer latency of 90 s was assigned to it. Later the animal was allowed to explore the plus maze for 5 min and send back to cage. On 17th and 18th day TL was measured on and serves as a parameter for acquisition (learning) while TL on day 18th indicates retention (memory).

2.8.2 Morris Water Maze Test

Spatial memory in animals was assessed by Morris water-maze (MWM) test[20, 21]. It is an experimental model based on swimming where the animal is taught to escape into a hidden platform. It consisted of huge circular pool (60 cm in height, 120 cm in diameter, water maintained at 28 ± 1°C temperature was filled to a depth of 30 cm). The pool was equally divided into four quadrants with the help of two threads, which were at right angle to each other. At the center of the four quadrants white platform was placed 1 cm below the surface of water so as to make it invisible at the level of water. The continuous swimming trail of each rat was followed from the start point to the platform. In case of failure of the rat in finding the platform within 120 s, it was guided to reach the platform and allowed to stay there for 20 s and 120 s were recorded as trail time. Four trails for each rat per day were performed with an interval of 2 min each. On daily basis escape latency per rat was calculated as the average of 4 trials on 23rd day the probe trail was performed. After the last trail, rats were returned to water and were tested with a single 120 s probe trail during which the target platform was removed from the pool. The time spent by the rats in the target quadrant was measured. Immediately after the probe trial of Morris water maze test animals were sacrificed by cervical dislocation, Brain tissue was dissected out under standard conditions and stored at -80 °C until further analysis. The hippocampus was dissected out from the brain and homogenised in physiological saline, using hand homogeniser fallowed by centrifugation at 5,000 × g for 15 min at 4 °C. The supernatants were preserved at −80 °C for further experimental use. For gene expression studies hippocampus tissue was rinsed with DEPC-treated water and stored in RNA later solution for total RNA isolation.

2.9 Estimation of Acetylcholine Esterase Activity

Tissue samples were homogenised in ice-cold Tris-HCl buffer (100 mM; pH 8.0). The content was centrifuged at 13,000 × g for 10 min at 4 °C. The supernatants was collected and analysed for AChE activity by the method described by Ellman[22], *et al.* with few modifications. 10 µl of cell homogenate was taken and 2870 µl of assay buffer, 100 µl of 10 mM DTNB and 20 µl of 100 mM acetylthiocholine iodide were added, mixed well and absorbance wasmeasured at 412 nm for 3 min at an interval of 1 min interval.

2.10 Total RNA Isolation, c-DNA Synthesis and Semi-Quantitative PCR

Total RNA of tissue isolated was extracted using RNeasy spin columns (Qiagen, USA) as per manufactures instructions. c-DNA synthesis was performed following the manufactures instructions (Roche Diagnostics, Germany) and preserved at -20 °C until further use. Transcript abundances for the AChE, BDNF and iNOS genes were tested by semi-quantitative PCR. Primers were designed using GeneTool 1.0 software and primer synthesis was done at imperial life sciences (P) Ltd, India (Table 1). β-actin was used as standard housekeeping gene. The PCR reaction mixture (1 µl of c-DNA, 2x ready mix PCR reagent comprising dNTP`s, 10 µl Taq-Polymerase) was used and added 10 pmol of forward and reverse primers with a final volume of 20 µl with PCR grade water. The PCR reaction was performed with DNA Thermal Cycler (Veriti, ABS, USA). The cycling programs were set as follows: initial denaturation at 94 °C for 5 min, proceeded by denaturation

Table 1. Primers sequences used for semi-quantitative PCR

Gene	Accession No.	Primers		Product Size (bp)
β-actin	NM_007393	Forward: 5' GGCCCAGAGCAAGAGAGGTATCC 3'	251-273	378
		Reverse: 5' CGGCCAGCCAGGTCCAGAC 3'	610-628	
AChE	NM-172009	Forward-5' TCGCAGCCTTTGGGGGAGAC 3'	653-672	227
		Reverse-5' AGCGCCACCTGGGGGACA 3'	862-879	
BDNF	NM-001270630	Forward-5' CGGCCCAACGAAGAAAACCATAA 3'	607-629	154
		Reverse-5' GGCGCCGAACCCTCATAGACAT 3'	739-760	
iNOS	NM_012611	Forward -5' GCCCCACGGAGAACAGCAGAG 3'	143-163	284
		Reverse-5' GGGCGGGTCGATGGAGTCAC 3'	407-426	

for 30 s at 94 °C, followed by annealing for 30 s at 55 °C, extension for 50 s at 72 °C and final extension for 5 min at 72 °C. After completion of PCR (25 cycles for β-actin; 35 cycles for AChE, BDNF and iNOS), the thermal cycler was stopped. The amplified products obtained were loaded onto 1 per cent agarose containing 0.1 mg/ml ethidium bromide in the gel and run at 100 V for 45 min. The band intensity of control and treated sample in agarose gel was quantified using NIH Image analysis software.

2.11 Measurement of Lipid Peroxidation

Lipid peroxidation as malondialdehyde was analysed by Buege and Aust method[23] with minor modifications. 100 mg Brain tissue was homogenised in 2 ml of phosphate buffer (pH 7.0). To 0.5 ml of tissue homogenate 0.5 ml of 10 per cent TCA and 2 ml of TBA mixture were added. The TBA mixture contained 0.35 per cent TBA, 0.2 per cent SDS, 0.05 mM $FeCl_3$ and BHT in 100 mM glycine - HCl buffer (pH 3.6). The reaction mixture was palced in boiling water bath for 30 min and allowed to cool. The above mixture was centrifuged at 8,000 rpm for 10 min and the absorbance was quantified at 532 nm. The MDA content was calculated using molar extinction co-efficient $1.56x10^5$/mol/cm[24].

2.12 Estimation of Nitrite Levels

The supernatant was analysed for nitrite content using Griess reagent by colorimetrically. Griess reagent comprised of 1 per cent sulphanilamide in 2 per cent H_3PO_4 and 0.1 per cent N-(1-naphthyl) ethylenediamine dihydrochloride. Equal volumes of the Griess reagent and supernatant were mixed, incubated for 10 min at room temperature in the dark. The absorbance was then quantified at 540 nm using a spectrophotometer. The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve.

2.13 Estimation of Reactive Oxygen Species (ROS) Levels

The ROS level was determined by the procedure of Montoliu[25], *et al.* with slight modification. Tissues at a concentration of 10 per cent (w/v) homogenised in 50 mM phosphate buffer (pH 7.4). To the tissue homogenate 50 µl of 10 µM dichlorofluorescin diacetate solution in DMSO was added and incubated at 37°C for 30 min. The fluorescence developed will be directly proportional to ROS levels. The fluorescence intensity was measured by using spectrofluorometer with the excitation wavelength at 485 nm and emission wavelength at 535 nm respectively. The data was expressed in terms of percentage inhibition.

2.14 Estimation of Antioxidant Enzymes Activity

Tissues concentration of 10 per cent (w/v) was homogenised in 50 mM phosphate buffer (pH 7.4). Glutathione peroxidase (GPx), Superoxide dismutase (SOD), and glutathione reductase (GR) activities were measured with a commercially available kits (Randox, Cat no. SD. 125, RS 504, GR 2368, Canada). Catalase (CAT) activity was determined by measuring the decay of 6 mM H_2O_2 solution at 240 nm by spectrophotometric degradation method[13]. To determine the enzyme activity an extinction coefficient of 43.6 M^{-1} cm^{-1} was used and values were expressed as mmol H_2O_2 degraded/min/mg of protein. Glutathione S-transferase (GST) activity was assayed by the method of Habig and Jakoby[26]. Briefly, 2.7 ml of phosphate buffer, 0.1 ml of reduced glutathione, 0.1 ml of 1-chloro-2,4-dinitrobenzene as substrate and 0.1 ml of supernatant were mixed. The increase in the absorbance was recorded for 5 min at 1 min intervals at 340 nm. The results were represented as nanomoles of CDNB conjugated/min/mg of protein.

2.15 Estimation of Reduced and Oxidised Glutathione Levels

Deproteination of tissue homogenate was done by using tetrachloroacetic acid, centrifuged and supernatant was collected for the estimation of reduced glutathione (GSH) with the help of Ellman reagent (5, 5' dithiobis (2-nitro benzoic acid). The absorbance of the pale yellow colour was quantified on the spectrophotometer at 412 nm. A pure GSH standard was run simultaneously. The GSH level was expressed as nmol/mg protein. The oxidised glutathione (GSSG) was determined by the decrement of GSSG in the presence of NADPH and glutathione reductase and estimated the decrement in the absorbance of NADPH at 340 nm. The result was represented as nmol/mg protein[27].

2.16 Western Blot Analysis

Total cellular protein was run to separate on SDS-PAGE and transferred onto a nitrocellulose membrane using an electro blotting apparatus (Cleaver Scientific Ltd, UK) as per earlier method[13]. After transfer, the membranes were probed with GAPDH (sc-25778) and anti HSP-70 C92F3A (sc-66048)

(Santa Cruz Biotechnology, CA, USA) at 1: 1,000 dilutions and incubated at 37 °C for 3 h. The membranes were washed with TBST for four times at an intravel of 15 min followed by incubation at room temperature for 2 h in horseradish peroxidase conjugated rabbit anti- mouse secondary antibodies (DAKO, Denmark) at 1:10,000 dilutions. The membranes were washed again with TBST and developed using an enhanced chemiluminescence detection system (ProteoQwest®, Sigma). The membranes were exposed to x-ray film and the developed band intensity was captured. The band intensity was measured using NIH image J software.

2.17 Statistical Analysis

All The data were expressed as mean ± standard deviation (SD). The $AlCl_3$ group was compared with CON group and BHD+$AlCl_3$ group were compared with $AlCl_3$treatment group. Data were analysed by One-way ANOVA followed by Tukey's post hoc test using SPSS 15.0 software for Windows. Differences at $p < 0.05$ were considered to be significant.

3. RESULTS

3.1 Physicochemical Analysis of Bacoside Rich Juice

The moisture content of BHD was 88.0 per cent soluble. The total soluble solid (ºBrix) of the sample was 11. Total titratable acidity of sample was found to be 0.321 per cent as citric acid equivalents of juice with pH 4.5. Based on sensory evaluation colour of the juice scored 7.4± 0.25, flavour scored 7.4± 0.28, taste scored 7.3± 0.27 and the overall acceptability (OAA) of the product was good (7.3± 0.32). In the present study, Date syrup, lemon juice, honey and ginger were used to mask the bitterness and color given by the bacoside rich extract and thereby enhancing the sensory attributes as well as nutraceutical value of the drink.

3.2 Stability Studies and Microbial Analysis

The periodical microbiological analysis showed absence of major food borne pathogens including coliforms. The presence of TPC was within the permissible limit throughout the period of storage.

3.3 Effect of BHD on Behavioural Tests

3.3.1 Elevated Plus-Maze Test

EPM test was used to evaluate memory and expressed as transfer latency. Administration of $AlCl_3$ significantly increased transfer latency in animals treated only with $AlCl_3$ compared to normal controls ($p < 0.05$), which indicates that the memory deficits were due to $AlCl_3$ administration. In contrast to animals treated with $AlCl_3$ along with BHD supplementation significantly alleviated the $AlCl_3$ induced learning and memory deficits in Wistar rats ($p < 0.05$) as shown in Fig. 1.

3.3.2 Morris Water Maze Test

Control group, BHD supplemented group rats showed a marked reduction in escape latencies from day-1 to 4thday. Administration of $AlCl_3$ results in the deterioration of learning and memory deficits in the Morris water maze task compared to normal control group ($p < 0.05$). However, BHD and

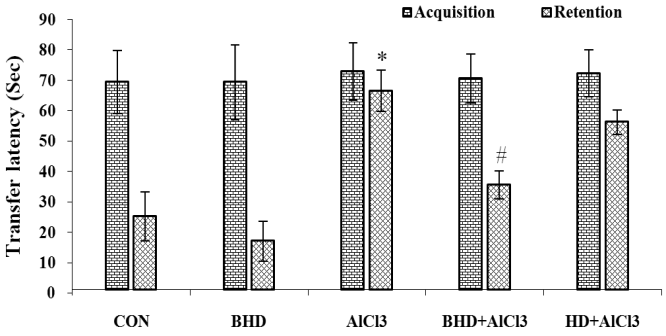

Figure 1. Effect of BHD on transfer latency by using elevated plus-maze test.

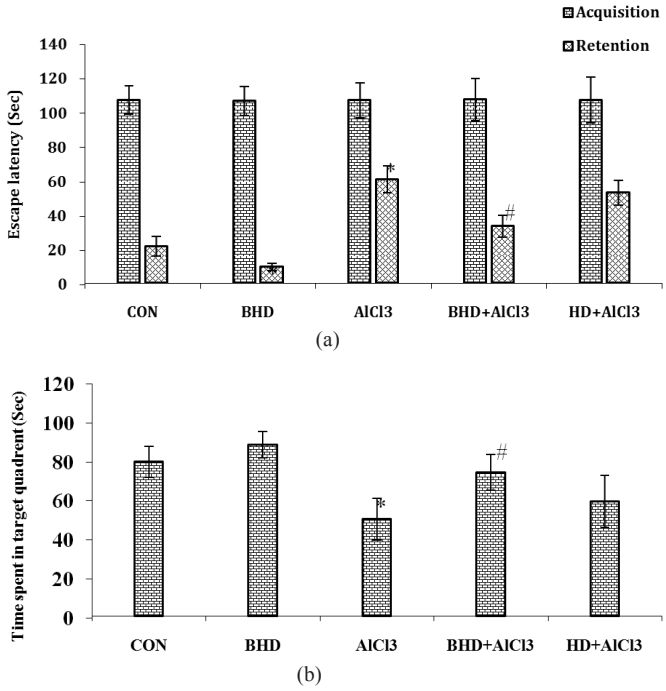

Figure 2. (a) Effect of BHD on escape latencies in the hidden platform of Morris water maze. (b) The escape latencies in the hidden platform MWM.

BHD+$AlCl_3$ groups showed significant protection against $AlCl_3$ induced learning and memory deficits. BHD alone administered group was found to be therapeutically more potent than other treatment groups as shown in Fig. 2.

3.4 Effect of BHD on AChE, BDNF and iNOS Expression

AChE, BDNF and iNOS are markers of cognitive function and neurodegenerative disorder. In the current study, we have estimated the expression level of these genes in rat hippocampus region in brain. The semi-quantitative RT-PCR results showed $AlCl_3$-induced down-regulation of BDNF expression by 78.90 per cent fold, in hippocampus region in comparison to control groups (100 per cent) whereas the AChE and iNOS gene expression was up-regulated by 159.20 per cent and 158.10 per cent within hippocampus region comparison to control group. Upon BHD supplementation expression levels of the three genes were brought to normal as shown in Fig. 3.

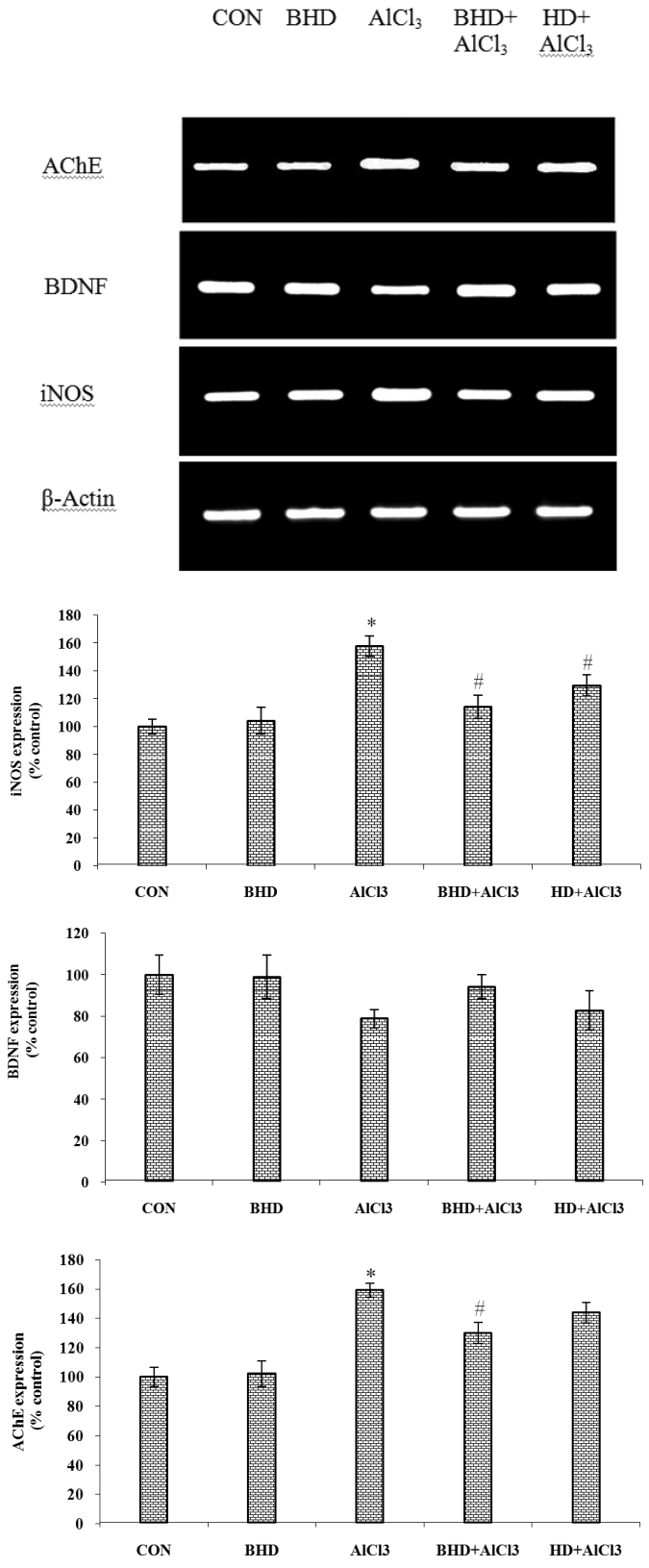

Figure 3. Effect of BHD on BDNF, iNOS and AchE expression.

3.5 Effect of BHD on AChE Activity

AChE activity was increased by 170.19 per cent and 161.12 per cent in cortex and hippocampus of $AlCl_3$ administered group respectively in comparison to control group. Supplementation with BHD had significantly inhibited AChE activity in both cortex (123.02 per cent) and hippocampus (124.15 per cent) whereas the HD supplementation did not show significant results ($p < 0.05$) as shown in Table 2.

Table 2. Effect of BHD on AChE activity in cortex and hippocampus of rats

	AChE activity (U/mg protein)	
	Cortex	**Hippocampus**
CON	12.24 ± 2.89	8.84 ± 1.04
BHD	11.71 ± 1.44	8.39 ± 0.74
$AlCl_3$	20.83 ± 2.68*	14.24 ± 1.00*
BHD+$AlCl_3$	15.06 ± 1.84$^{\#}$	10.97 ± 0.86$^{\#}$
HD+$AlCl_3$	19.35 ± 1.74	13.68 ± 0.97

3.6 Effect of BHD on Lipid Peroxidation, Nitrite and ROS Levels

$AlCl_3$ exposure leads to lipid peroxidation, which is measured as MDA levels in rat cortex and hippocampus. $AlCl_3$ administration has significantly increased the MDA levels by 170.19 per cent in cortex and 161.12 per cent in hippocampus in comparison to control group. Supplementation with BHD had significantly decreased the rate of lipid peroxidation in both cortex and hippocampus rat brain ($p < 0.05$) as shown in Table 3. An increased level of lipid peroxidation is due to elevated levels of ROS and nitrate in cortex (165.53 per cent , 168.73 per cent) and hippocampus (169.23 per cent , 177.22 per cent). BHD supplementation had significantly neutralised ROS and nitrite levels in both cortex and hippocampus rat brain ($p < 0.05$) (Table 3).

3.7 Effect of BHD on Antioxidant Status

The cellular antioxidant enzymes GPx, SOD, CAT, GR and GST levels was markedly declined in $AlCl_3$treated group ($p < 0.05$) compared to that of normal control group. Significant protection was achieved by BHD supplementation ($p < 0.05$). The cellular reduced GSH levels and GSH:GSSG ratio was significantly reduced by $AlCl_3$ treatment ($p < 0.05$). The glutathione status was improved by BHD supplementation as shown in Table 4.

3.8 Effect of BHD on HSP-70 Expression

Immunoblotting study of HSP-70 protein expression is up-regulated by 136 per cent in cortex of $AlCl_3$ group compared to control group (100 per cent). Supplementation of BHD has significantly brought down their levels to normal as shown in Fig. 4.

4. DISCUSSION

Brahmi herbal drink is a nutraceutical product developed to relieve neuronal stress, improved cognitive functions against aluminium induced toxicity animals. Aluminium and its salts are known to induce neurodegenerative dysfunction[1]. Hippocampusreagion and cerebral cortex is the region of memory and learning ability which is affected by $AlCl_3$ treated neurodegenerative disease[27]. But the mechanism behind amelioration still remains unclear. Hence, in the present study

Table 3. Effect of BHD on lipid peroxidation, ROS and NO levels in cortex and hippocampus of rats

	MDA (µM/ cm/ g of tissue)		ROS (per cent fluorescence)		NO (µg/ g of tissue)	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
CON	63.11 ± 6.54	79.91 ± 3.06	100.00 ± 12.67	100.00 ± 10.32	12.76 ± 1.33	8.09 ± 0.84
BHD	61.91 ± 6.88	77.31 ± 5.78	100.38 ± 13.70	100.03 ± 9.21	12.32 ± 0.94	7.98 ± 0.73
$AlCl_3$	90.74 ± 6.84*	98.02 ± 2.71*	165.53 ± 10.00*	169.23 ± 11.21*	21.53 ± 1.06*	14.34 ± 0.64*
BHD+$AlCl_3$	74.05 ± 4.24#	82.34 ± 2.53#	119.62 ± 10.10#	126.32 ± 7.24#	16.21 ± 1.11#	10.23 ± 0.85#
HD+$AlCl_3$	87.86 ± 4.38	91.39 ± 2.36#	132.39 ± 8.76#	138.43 ± 8.72#	20.60 ± 0.98#	12.42 ± 0.94#

Table 4. Effect of BHD on antioxidant status in cortex and hippocampus of rats

	SOD (U/mg protein)		CAT (mM H_2O_2 degraded/min/mg protein)	
	Cortex	Hippocampus	Cortex	Hippocampus
CON	2.95 ± 0.13	3.03 ± 0.14	14.08 ± 1.03	9.42 ± 0.54
BHD	3.01 ± 0.21	3.10 ± 0.17	14.72 ± 1.10	9.46 ± 0.68
$AlCl_3$	1.67 ± 0.20*	1.58 ± 0.16*	9.48 ± 1.00*	4.93 ± 0.50*
BHD+$AlCl_3$	2.53 ± 0.14#	2.65 ± 0.12#	12.34 ± 0.68#	7.42 ± 0.42#
HD+$AlCl_3$	2.19 ± 0.17#	2.25 ± 0.14#	10.04 ± 0.67	6.98 ± 0.69#

	GPx(U/mg protein)		GR (U/mg protein)		GST (U/mg protein)	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
CON	12.06 ± 0.92	9.67 ± 0.23	23.76 ± 1.69	17.96 ± 1.35	13.87 ± 0.81	11.84 ± 0.75
BHD	12.63 ± 0.87	9.63 ± 0.15	23.98 ± 1.07	18.58 ± 1.64	14.53 ± 1.02	11.93 ± 0.73
$AlCl_3$	8.63 ± 0.64*	6.13 ± 0.56*	13.75 ± 2.07*	11.13 ± 0.93*	10.03 ± 0.86*	8.24 ± 0.47*
BHD+$AlCl_3$	11.38 ± 1.04#	7.78 ± 0.34#	19.67 ± 1.33	15.36 ± 0.90#	12.94 ± 0.78#	10.73 ± 0.83#
HD+$AlCl_3$	10.66 ± 0.90#	7.10 ± 0.49	18.58 ± 1.42	13.86 ± 1.36#	11.02 ± 0.81	9.89 ± 0.54#

	GSH (nmol/mg protein)		GSSG (nmol/mg protein)		GSH/GSSG ratio	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
CON	32.17 ± 2.13	12.65 ± 1.10	10.09 ± 0.74	2.99 ± 0.06	3.19 ± 0.47	4.24 ± 0.77
BHD	33.17 ± 1.70	12.99 ± 0.89	10.01 ± 0.81	2.87 ± 0.05	3.32 ± 0.84	4.53 ± 0.66
$AlCl_3$	19.17 ± 2.00*	7.64 ± 0.63*	18.90 ± 0.79*	3.97 ± 0.03*	1.01 ± 0.15*	1.92 ± 0.32*
BHD+$AlCl_3$	24.67 ± 3.14#	9.67 ± 0.96#	12.69 ± 0.77#	3.07 ± 0.01#	1.94 ± 0.16#	3.15 ± 0.48#
HD+$AlCl_3$	18.17 ± 2.65#	7.66 ± 0.95	13.85 ± 0.83#	3.43 ± 0.07#	1.31 ± 0.13#	2.23 ± 0.54

was aimed to understand the effect of $AlCl_3$ on cognitive function. Elevated plus maze served as the exteroceptive behavioural model and Morris water-maze test was employed to assess spatial memory in mice and rats[20,21]. $AlCl_3$interferes with memory and cognitive function and subsequently causes cognitive impairments. In this study, rats were administered with $AlCl_3$ at a dose of 100 mg/kg body wt. to induce cognitive impairment[27]. Cognitive-enhancing activities of BHD was evaluated by elevated plus maze and Morris water maze tests. In the present investigation, elevated plus maze results showed that administration of $AlCl_3$ exhibited increased transfer latency (TL) as compared to the normal control group. Thus, $AlCl_3$ induces memory impairments in rats. The rats supplemented with BHD significantly reversed memory deficits against $AlCl_3$-induced impairments ($p < 0.05$) as shown in Fig. 1. Morris water maze test is well established behavioural test for detecting changes in the central cholinergic system and especially influences on spatial memory[28]. In Morris water maze test, BHD supplementation showed significant shorter escape latencies than the $AlCl_3$treated group during the training period as shown in Fig. 2, which indicates that the BHD improves the impairment in memory induced by $AlCl_3$. These results were confirmed by earlier reports where the *B. monniera* significantly reversed aluminium-induced memory and learning deficits as monitored by Morris water maze test[30].

Previous studies have demonstrated that learning and memory can be modified in animals and humans by aluminium chloride affecting the central cholinergic system[31]. Studies have shown that acetylcholine esterase has been a potential target for treatment of Alzheimer's disease and for prevention strategies[28]. BHD significantly inhibited acetylcholine esterase expression in hippocampus by 143.95 per cent compared to $AlCl_3$ group (159.19 per cent). AChE activity was also significantly increased in the cortex and hippocampus by 170.19 per cent and 161.12 per cent , respectively, as compared to control rats whereas BHD supplementation significantly

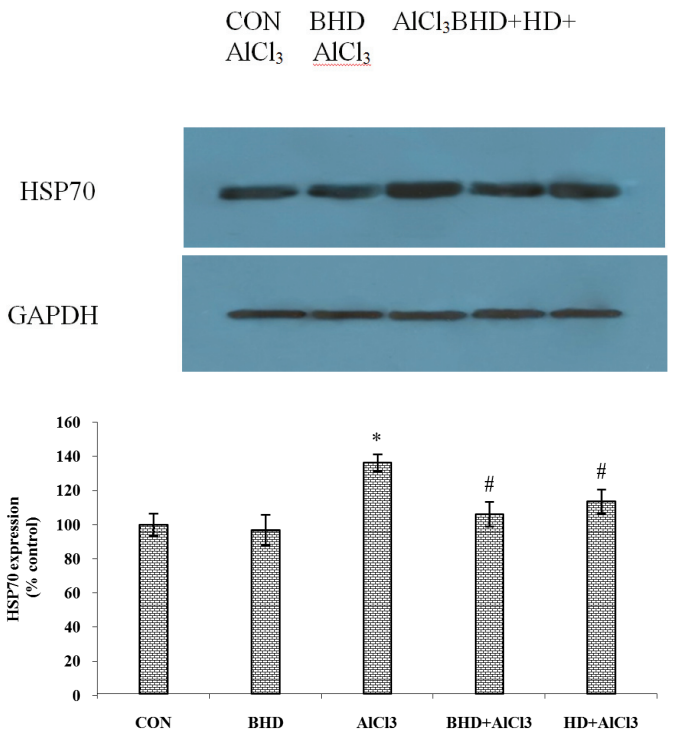

Figure 4. Effect of BHD on HSP70 expression.

brought down there levels to normal. In this regard, many investigations have shown that the compounds which increase synaptic acetylcholine via. inhibition of the acetylcholine esterase were considered to be neuroprotective molecules.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, which helps in neuronal cell survival, growth-promoting and differentiation in the developing nervous system. BDNF is extensively implicated in synaptic and structural plasticity[33,34], neuroprotective effects[35,36], anxiety[37,38]and learning and memory[39, 40]. Many studies have documented evidence of decreased expression of BDNF in neurological disease like Alzheimer's disease, Parkinson's disease, Huntington's disease and schizophrenia patients because of oxidative and apoptotic stress insults[41, 43]. Hence, in the present study, we have examined the effect of BHD on BDNF expression in $AlCl_3$ treated rats. BDNF expression was significantly decreased by 78.90 per cent in $AlCl_3$ treated rats compared to control group whereas BHD supplementation has normalised the levels to control ($p < 0.05$).

Nitric oxide (NO) is a highly reactive molecule and gets quickly converted to more stable nitrates and nitrites, which are the markers of NO levels. Exposure to $AlCl_3$ will elevate the levels of NO in brain by the expression of nitric oxide synthase[44]. The present study data is also in agreement with the increased levels of NO ($p < 0.05$) due to up-regulation of iNOS expression induced by $AlCl_3$ treatment. BHD supplementation has significantly brought down the NO levels to normal ($p < 0.05$) by down regulating iNOS expression (Fig. 3). Studies have shown that inhibition of nitric oxide synthase protect cholinergic neurons against $AlCl_3$ excitotoxicity in the rat brain[45]. Studies have also shown the activation of stress response results primarily in the increased expression of heat shock proteins like HSP-70. HSP-70 is a 70 kDa protein protect the tissues from several injuries caused by the oxidants and has been studied extensively for its potential. HSP-70 is up-regulated in response to different stress conditions, such as exposure to toxic compounds, metal ions, cigarette smoke etc., which results in the induction of HSP-70 expression, mitochondrial depolarisation and even cell death[13,46]. In the present study, exposing the rats to $AlCl_3$ has attributed to over-expression of HSP-70 compared to control group. Supplementation of BHD has significantly prevented the over-expression of heat shock proteins as a cellular defense mechanism as shown in Fig. 4.

As evidenced by many investigators oxidative stress plays role in the pathogenesis of Alzheimer's disease [47-49]. Oxidative stress is mainly due to elevated rate of lipid peroxidation and decreased antioxidant status. Studies have reported that $AlCl_3$-induced memory impairment in mice and rats model is associated with elevated oxidative stress within the brain[50]. This is because of continuous generation of ROS in brain during neuronal activitynormal and metabolism. The nervous system is specifically vulnerable to the deleterious effects of ROS. Because the brain has a high oxygen consumption rate, large amount of polyunsaturated fatty acids and low levels of antioxidants defense compared to other organs[51]. Moreover, many studies have shown that increased in the oxidative status of amnesic rats resembled the clinical status of oxidative stress and membrane lipid peroxidation in demented patients[47,49]. In the present study, $AlCl_3$ treatment has significantly increased the generation reactive oxygen species, which reacts the membrane lipids, as a result formation of lipid peroxidation products will be increased. In the current investigation we also found reduced levels of the enzymes involved in antioxidant defense, viz. SOD, CAT, GPx, GR and GST in cortex and hippocampus of $AlCl_3$ treated rats. These findings are in concordance with the earlier reports[43, 52-53] which also documented a significant decrease in the activities of SOD and CAT in brain after Al insult. Furthermore, we observed a significant reversal in above stated changes by the BHD supplementation. These biochemical modifications indicate that BHD possess strong antioxidative property. In the present study, we also found reduced GSH/GSSG ratio in $AlCl_3$ treated rats. GSH/GSSG ratio determines the relative amount of reduced glutathione (GSH) when compared to the oxidised glutathione (GSSG). A larger ratio reflects a more efficient glutathione redox system, because GSH is normally maintained in a highly reduced state via NADPH dependent enzymes, specifically GR. We found that BHD supplementation minimizes these changes by increasing of GSH/GSSG ratio and this may be due to the antioxidative properties of this drink.

5. CONCLUSION

On the basis of results, it may be concluded that the nutraceutical product developed (i.e., BHD) is efficacious in ameliorating the aluminium chloride induced alterations in cognitive behaviour and neurochemical changes of rat cortex and hippocampus. Moreover, our results also showed that the protective effect of BHD is because of bacosides present in it.

Herbal drink consisting of all the ingredients as that of BHD except bacosides failed to show significant in most of the $AlCl_3$ induced impairments. This is mainly due to its neuromodulatory propensity by ameliorating AChE, BDNF and iNOS expression. In addition, BHD also significantly recovered endogenous antioxidants (i.e., SOD, CAT, GPx, GR, GST and GSH) which protect brain against reactive oxygen species.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

ACKNOWLEDGEMENT

The authors are thankful to Director, Defence Food Research Laboratory, Mysuru, India for his constant encouragement and support to carry out the research work.

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