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PROTECTIVE POTENTIAL OF *Bacopa monniera (Brahmi)* EXTRACT ON ALUMINUM INDUCED CEREBELLAR TOXICITY AND ASSOCIATED NEUROMUSCULAR STATUS IN AGED RATS

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Abstract

The present study attempts to assess the comparative effects of *Bacopa monniera*, (40 mg/kg body weight) and donepezil (2.5 mg/kg b. wt) on aluminum (100 mg / kg b. wt. of $AlCl_3$) mediated oxidative damage in the cerebellum of aged rats (24 months) along with the associated dysfunctioning of neuromuscular coordination and motor activity. A significant decrease in the activities of antioxidant enzymes and increased total reacting oxygen species, lipid and protein peroxidation products observed in aluminum exposed rats. We observed that treatment with *B. monniera* extract restored the altered antioxidant enzyme activities more, when compared with donepezil. However, acetylcholinesterase showed similar effect both in donepezil and *B. monniera* treated groups. The content of aluminum was increased in all experimental groups, however, iron content was found increased in all groups except the *B. monniera* treated groups. Moreover, aluminum treated groups of rats exhibited significant changes in behavioral profiles but these changes were in both *B. monniera* and donepezil treated groups. The light microscopic and ultrastructural studies revealed damaged Purkinje's neurons and altered granular cell layer along with the increased accumulation of lipofuscin granules in aluminum treated animals. These changes were quite less pronounced in *B. monniera* group than that of donepezil and this may be due to the reduction of excess iron content by *B. monniera*. On the basis of our results it may be concluded that Al may be linked with cerebellar degeneration and neuromuscular disorders while *Bacopa monniera* extract helps in reversing these changes.

Key words: Aluminum, Aging, Cerebellum, B. monniera, Donepezil, Motor and learning.

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Abbreviations: AD: Alzheimer's Disease; **Al**: Aluminum; **BM**: Bacopa monniera; **DP**: donepezil; **ROS**: reacting oxygen species; **CD**: conjugated dienes; **CAT**: Catalase; **GSHPx**: glutathione peroxidase; **SOD**: superoxide dismutase; **LF**: lipofuscin; **TP**: Total Proteins; **TL**: total lipid; **PC**: protein carbonyl; **LPO**: lipid peroxidation; **LOOH**: Lipid hydroperoxide; **MDA**: malondialdehyde; **GSH**: glutathione; **GSSG**: oxidized glutathione; **AChE**: acetylcholinesterase; **SMA**: spontaneous motor activity.

INTRODUCTION

Aluminum (Al) is the third most abundant element in the earth crust. Only oxygen (49.5%) and silicon (26%) occur more commonly than aluminum (8%). It can enter into the body via diet, drinking water, vaccines, antacids, inhaled fumes and particles from occupational exposures. Humans consume on an average 7600 µg /day of Al from drinking water and food (55). Al is a potent neurotoxic element, which has been suggested to play an important role in the degeneration of nerve cells of experimental animals as well as human brain. Also, it known to be involved in the etiology of several human pathologies such as dialysis dementia,

amylotropic lateral sclerosis and senile dementia of Alzheimer type (AD) (34,45).

Perturbations of motor and cognitive function occur more commonly in the elderly group of people, and this has been eliciting increasing attention in the past few decades. The cerebellum is known to be critically concerned with muscular coordination and it smoothens motor activity. Nearly 50% of all neurons of the brain are located in this region, which takes up only 10% of the total brain volume and receives nearly 200 million afferent fibers (12). Since, cerebellum is involved in fine coordination and control of voluntary movement it may be vulnerable to injury, particularly toxic insult. Comparatively little efforts have been made to investigate the neuronal alterations of the cerebellum in this wide group of elderly population following exposure to toxic chemicals including those concerning environmental toxins (42).

Evidence from clinical and animal model studies demonstrates that brain aluminum content increases with age (50). It may be either due to increased exposure with age or decreased ability to remove Al from the body (32). Therefore, knowledge of age related effects of Al in tissues and relative risk as well as extent of its retention in tissue is important for the understanding of its effects and associated disorders (14).

Bacopa monniera (BM) is a traditional Indian herb called Brahmi which has been widely recommended as a nerve tonic (2, 48). Its antioxidative property is used for the treatment of free radical mediated oxidative damage to brain (21, 22, 43, 48). It has been reported that bacoside constituents of *B. monniera* repair damaged neurons by enhancing kinase activity. On the other hand, Donepezil hydrochloride (Aricept), a potent and selective acetylcholinesterase inhibitor, has been specially designed for the treatment of AD and other neurodegenerative disorders (46). In this study, donepezil (DP) has chosen for the comparison with B. monniera, due its inhibition of acetylcholinesterase and antioxidant properties. This dual action of donepezil may explain its sustained activity compared to other cholinesterase inhibitors.

In view of the aforementioned considerations, the present study was designed to investigate the comparative effect of BM extract and donepezil on Al induced altered neuromuscular coordination and motor learning capacity along with oxidative damage in cerebellum of aged rats.

MATERIALS AND METHODS

Chemicals

Nitroblue tetrazolium Cat N-5514 (NBT), thiobarbituric acid Cat T-5500 (TBA), phenazinemetho sulphate Cat N-9625 (PMS), nicotinamide adenine dinucleotide Cat N-6754 (NADH), 5.5'-dithio *bis* 2-nitrobenzoic acid Cat D-5420 (DTNB), nicotinamide adenine dinucleotide phosphate Cat N- 7785 (NADPH) tricholoroacetic acid Cat T- 8657 (TCA) and reduced glutathione Cat G-4251 (GSH) were purchased from Sigma Chemical Co., St. Louis, MO, USA, All other reagents used were of high quality and analytical grade.

Animals

Forty-eight aged male (24 months; 462.8 ± 4.3 gram) Rattus norvegicus, Wistar strain rats were purchased from Industrial Toxicology Research Institute, Lucknow, (UP) India. The animals were housed separately in polypropylene cages in a room, which was maintained at a temperature of 22 ±2 ^{0}C, relative humidity of 50 ±10 % and 12h light dark cycles. They were fed a commercial pellet diet (Dayal Industries, Barabanki, UP, India) and allowed access to water ad libitum. The animals were randomly divided in to four subgroups (n=12) for biochemical (N=6) and microscopic study (N=6) in each groups. The Institutional Animal Ethics Committee approved the study prior to the initiation of the experiment and also approved all experimental protocols.

Experimental group

The details of groups prepared are Group -1: $AlCl_3$ (100 mg / kg body wt) treated (Al) (3); Group - 2: *Bacopa monniera* extract (40 mg / kg body weight) (41, 44, 47, 54) with 100 mg $AlCl_3$ / kg body wt treated (BM); Group - 3: Donepezil (2.5 mg/ kg body wt) (15, 25) with 100 mg $AlCl_3$ / kg body wt treated (DP) and Group - 4: equivalent volume of distilled water treated control rats (CT).

Aluminum chloride solution

The 4% Aluminum chloride solution was made in distilled water at natural pH and prepare aqueous suspension mixed with 2.5% of gum acacia as per the previously described method (51).

Bacopa monniera extract

The whole plant of *Bacopa monniera* was dried in shade and then powdered. The powder was extracted with distilled water. The aqueous extract was discarded and the residual plant material was extracted thrice with 90% ethanol. The residue obtained after removing the solvent was dried in vacuo and macerated with acetone to give a free flowing powder. The bacopa extract so prepared contained 40% bacosides estimated as bacoside A by high pressure thin liqid chromatography (6, 11, 23).

Route of Administration

The dose was directly introduced into the rat pharynx via a feeding cannula (The sharp age of the tip of a hypodermic needle no. 16 was blunted by grinding on a stone and thereafter bent to 120° so that the curved needle

could easily be introduced into the rat pharynx via oral cavity without the pointed tip lacerating the passage) (51) to control and experimental groups for 90 days. The drug treatment were given to Al treated rats after 1hour of the $AlCl_3$ treatment.

Behavioral studies

The Motor activity, behavioral changes, muscle coordination, sensory and motor reflex responses were assessed in the control and experimental rats as per our previously described protocol (52).

Spontaneous motor activity

[SMA]: SMA was measured by scoring on a scale of 0 – 9 in which SMA in control group was assigned score 4.

Gait: The rat was allowed to move on the edge of the cage for 1 min and straight movements without fall or staggering was considered as normal. The gait was recorded as escape latency during the period of 1 min.

Catalepsy: A condition in which body or limbs remain passively in any position in which they can be placed. This test is used by placing forepaws on a metallic rod placed at a height of 6 cm, when forepaws were not withdrawn within 10 sec. catalepsy was considered positive.

Muscle coordination test (Rota rod)

The period of stay on rotating rod (speed: 5 rotations / min; Total duration of test 2 min) for each control and treated rat were recorded by Rotamex (Techno Electronics, India). The rats were trained to stay for period of 2 min on rotating rod and only trained rats were included in the study. Motor was measured using Rota Rod at least 5s and it was rotated at speed of 10 rpm for 2 consecutive days on third day the time duration of each rotation speed was also recorded.

Passive avoidance test

Cognitive behavior was assessed by the number of times the animal escapes, in the series of 10 test trials. The apparatus for this test consists of two chambers separated by a partition. One chamber is lit in which the animals are housed. After 10 sec, the buzzer was set on and after 10 sec, an electric shock at 60 V was given. The animal's jumps to the other compartment as soon as the buzzer was set on, it mean the animal has avoided the test. However, on other hand, the animal's jumps to the other compartment after shock or does not jump at all, this is termed as escapism. A total of 10 trials were given to every animal. To qualify, the animal jumps to avoid at least 8 times out of 10.

Biochemical Analysis

Metal Estimations

The cerebellum was collected, cleaned, blotted dry, weighed and then digested in a mixture of HNO_3: H_3PO4 (6:1) till residue remained, the residue was dissolved in an appropriate amount of 0.1N HNO_3 and read for Al and Fe on flame atomic absorption spectrophotometer (Perkin Elmer Analyst- 300) against standard for each metal. The standards were processed identically as test. Table 1 shows validation of the Al and Fe on flame atomic absorption spectrophotometer (Perkin Elmer Analyst- 300).

Tissue Homogenate Preparation

After 90 days of experiment rats were sacrificed. Their brains were removed and weighed individually. Thereafter, cerebellum was dissected out for biochemical analysis. Ten percent (w/v) homogenate of the cerebellum was prepared using York's homogenizer fitted with Teflon plunger in 0.1 M phosphate buffer (pH 7.1). The whole homogenate was first centrifuged at 2500 x g for 10 minutes in a refrigerated centrifuge. The pellet consisting of nuclear fraction and cell debris was discarded. The supernatant was further centrifuged at 11,000 x g for 15 minutes and mitochondrial fraction was separated. The clear supernatant was further centrifuged at 105,000 x g for 90 minutes and the resultant supernatant was used for determining enzyme activities.

Table 1. Results obtained in the validation study.

	Aluminum	**Iron**
Wave length	309.3 nm	248.3 nm
Limit of Detection	0.11 mg /kg	1.1 mg/ kg
Limit of Quantification	0.02 mg /kg	0.05 mg /kg
Accuracy (mean)	102 %	99 %
Standard Deviation	0.004	0.006
Precision (RSD_{n-1})	2.35%	2.09%

Data obtained in the validation study of Iron and Aluminum estimation by Atomic absorption spectrophotometer.

Total Reacting Oxygen Species (ROS) assay

The basal level of ROS was determined by the procedure of Montoliu et al. (35). An appropriate volume of freshly prepared tissue homogenate was diluted in 100 m*M* potassium phosphate buffer (pH 7.4) and incubated with a final concentration of 5 m*M* dichlorofluorescin diacetate in methanol for 15 min at 37°C. The dye-loaded samples were centrifuged at 12,500 *g* for 10 min at 4°C. The pellet was vortex mixed at ice-cold temperatures in 5 ml of 100 m*M* phosphate buffer (pH 7.4) and again incubated for 60 min at 37°C. The fluorescence measurements were performed with a Hitachi 850 spectrofluorometer at 488 nm for excitation and 525 nm for emission wavelengths. The cuvette holder was maintained at 37°C. ROS were quantified from the dichlorofluorescein standard curve in methanol (0–100 n*M*).

Lipid peroxide levels (LPO) and lipofuscin (LIF)

The thiobarbituric acid reacting substances (TBARS) were estimated in the cerebellum (39) and expressed as nmole of MDA /g tissue as. Lipofuscin (LIF) was measured using 2:1 chloroform-methanol extraction mixture (49). The concentration was measured with a fluoro-spectrophotometer at an excitation maximum of 360 nm and emission maximum of 420 nm. The content of the fluorescence was determined using quinine fluorescence as a standard. Data were presented as U/g tissue. One unit (U) of lipofuscin is defined as fluorescence of 0.01 g/ml quinine sulfate.

Protein and protein carbonyl content

The protein content was measured (29) using bovine serum albumin (BSA) as standard. It was represented as mg/g protein. The protein oxidation was measured by estimating the protein carbonyl levels (28) in cerebellum. Protein carbonyl content was determined in the samples by measuring the DNPH adducts at 375 nm. Carbonyl contents were calculated by using a molar extinction coefficient (*e*) of 22,000 M ¯1 cm ¯ 1. Data were expressed as nmoles carbonyl /mg.

Measurement of endogenous enzymes

An aliquot of cerebellum homogenate was used for the assay of enzymatic antioxidants. The superoxide dismutase (SOD EC 1: 15.1.1) activity was determined from its ability to inhibit the reduction of NBT in presence of PMS (33). The reaction was monitored spectrophotometrically at 560nm. The SOD activity was expressed as U/mg protein (1 unit is the amount of enzyme that inhibit the reduction of NBT by one half in above reaction mixture). Catalase (CAT, EC 1.11.1.6) activity was assayed using hydrogen peroxide as substrate; the decomposition of H_2O_2 was followed at 240nm on spectrophotometer. The CAT activity was expressed as U/mg protein (1). The glutathione peroxidase (GSHPx, EC 1.11.1.0) was assayed using GSH, NADPH and H_2O_2 as reactants. The oxidation of GSH into GSSG was measured in terms of oxidation of NADPH to $NADP^+$ and assayed as decrease in the absorbance of reaction mixture at 340 nm on spectrophotometer (40). The activity of GSHPx was expressed as n moles of NADPH oxidized / min / mg protein. Glutathione reductase (EC.1.6.4.2, GR) activity was assayed by the method of (16). Activity of GR was expressed as nmoles of NADPH oxidised/min/mg protein of cell extract.

Measurement of reduced and oxidized glutathione

Reduced glutathione was measured in deproteinized supernatent of the cerebellum. Tissue homogenate was deproteinated with tetrachloroacetic acid, centrifuged and supernatant was used for the estimation of reduced glutathione (GSH) with the help of Ellman reagent (5, 5' dithiobis (2-nitro benzoic acid). The optical density of the pale colour was measured on the spectrophotometer on 412 nm. An appropriate standard (pure GSH) was run simultaneously. The level of GSH was expressed as µg / g tissue (9). The oxidized glutathione (GSSG) was estimated by the decrement of GSSG in the presence of NADPH and glutathione reductase and determined the decrement of NADPH absorbance at 340 nm. The result was expressed as µg /g tissue (13).

Acetylcholinesterase activity

The activity of acetylcholinesterase enzyme was determined using acetylthiocholine iodide as substrate (10). The mercaptan formed because of hydrolysis of ester then reacts with an oxidizing agent, DTNB that splits into two products, one of which is 5 thio –nitro benzoate, which absorbs at 412 nm. The enzyme activity is measured by the increase in absorbance at 412 nm. The results were expressed as nmoles of acetylcholine hydrolyzed/min /mg protein

Microscopic studies

The rats were anesthesized with nembutol (sodium pentobarbitol) solution, 50mg/ kg b.w. perfused through heart with Karnovsky's fixative (0.1 M paraformaldehyde and glutaraldehyde solution in cocodylate buffer, pH 7.3). The brains were quickly removed from the cranium, placed on ice and the cerebellum was dissected out.

Light microscopy

For the evaluation of histopathological changes in cerebellum, small section of the tissue was immediately fixed with formalin. Thereafter, the specimens were embedded in paraffin, sectioned at 5 µm and stained with hematoxyline and eosin.

Electron microscopy

Ultrastructural changes in cerebellum of the central nervous system were assessed using standard electron microscope techniques. Small pieces of cerebellum 2-3 mm size, were immersed in the same fixative for four hours, and thereafter washed with 0.1M-cacodylate buffer (pH 7.3). The samples were post fixed for three hours at 4^0C in 1% osmium tertraoxide prepared in 0.1 M cacodylate buffer. The specimens were washed with distilled water and left in 1% aqueous uranyl acetate overnight. Subsequently, dehydration was carried out in ascending grades of alcohol, acetone and in pure acetone. Following dehydration, the specimens were embedded in Epon 812 at room temperature. Sections were cut on an LKB-Ultramicrotome with a glass knife. Thereafter, sections were mounted on 300 mesh copper grids, stained with 1% uranyl acetate and lead citrate and examined with a Phillips (FEI Tecnai 12 twin) Transmission Electron Microscope.

Statistical Analysis

Experimental data were summarized as Mean ± SEM. Groups were compared together by one way analysis of variance followed by Student Newman-Keuls post hoc test. The acceptance level of significance was $p< 0.05$. InStat

(version 3) was used for analysis of data. Significant comparison between groups described by mean percentage change of the control vs Al treated and Al treated vs Brahmi and Donepezil.

RESULTS

Behavioral profiles

The motor and learning responses namely spontaneous motor activity (SMA), catalepsy, righting reflex, gait, rota rod and passive avoidance test are presented in table 2. SMA was found to be not significant between groups. The catalepsy and righting reflex were significant changed in Al treated and DP treated rats while BM was insignificant changed. Gait, rota rod and passive avoidance test were found to be significantly altered in Al treated groups when compared with the controls while BM and DP treated rats reveres significantly as compared to the Al treated rats.

Body, brain and cerebellum weight

Reduction in terminal body weight (TBW) was observed in Al treated rats and DP treated rats when compared with the age matched control rats. Increased TBW was observed in BM treated rats. The terminal brain and cerebellum were also found to be reduced in Al treated and DP treated rats as compared with the controls and similarly, these changes were reversed in the BM treated rats (Table 3).

Table 2. Effect of *Bacopa monniera* and Donepezil on aluminum induced neurobehavioral changes

Parameters	Groups			
	CT	**AL**	**BM**	**DP**
SMA	4.2 ± 0.2	3.5 ± 0.2	3.8 ± 0.3	3.8 ± 0.2
Catalepsy	10.7 ± 0.7	8.0 ± 0.5[a]	9.5 ± 0.8	7.8 ± 0.5[a]
Righting Reflex	3.5 ± 0.3	5.0 ± 0.3[a]	3.8 ± 0.5	4.7 ± 0.3[a]
Gait	72.3 ± 5.7	43.8 ± 4.1[a]	65.8 ± 4.1[b]	67.3 ± 3.3[b]
Rota Rod	52.5 ± 2.6	33.3 ± 2.7[a]	50.2 ± 3.3[b]	43.5 ± 3.6[b]
Passive Avoidance	3.2 ± 0.4	5.1 ± 0.3[a]	4.0 ± 0.3[b]	3.3 ± 0.2[b]

Values are expressed as mean ± SEM for six animals in each group. The superscripts relate significant ($p<0.05$; one way ANOVA) comparison between control and Al treated (a), Al treated and BM treated or DP treated (b) and between BM treated and DP treated (c).

Table-3. Effect of *Bacopa monniera* and donepezil on Aluminum induced changes in the terminal body weight along with brain and cerebellum weight. of rats.

Groups	**Initial Body wt (g)**	**Terminal Body wt (g)**	**Terminal Brain wt (g)**	**Terminal Cerebellum wt (g)**
CT	462.5 ± 2.1	481.7 ± 4.5	2.17 ± 0.1	0.25 ± 0.02
AL	460.2 ± 2.6	415.0 ± 6.1[a]	1.63 ± 0.12[a]	0.17 ± 0.01[a]
BM	458.3 ± 2.1	452.5 ± 6.4[b]	2.09 ± 0.11[b]	0.22 ± 0.01[b]
DP	459.2 ± 3.0	423.3 ± 7.1[ac]	1.99 ± 0.15	0.19 ± 0.02[a]

Values are expressed as mean ± SEM for six animals in each group. The superscripts relate significant ($p<0.05$; one way ANOWA) comparison between control and Al treated (a), Al treated and BM treated or DP treated (b) and between BM treated and DP treated (c).

Aluminum and Iron concentration

The table 4 shows Al and Fe concentration in the cerebellum of different groups of rats. We observed significantly high concentration of Al in the cerebellum of Al treated, BM and DP treated rat when compared with the age matched control rats. However, the concentration of Fe was found to be increased in Al treated and DP treated rats as compared to the controls while, Fe content was decreased in the BM treated rats as compared with the Al treated and DP treated rats.

Table-4. Effect of *Bacopa monniera* and donepezil on the concentration (µg/g tissue) of aluminum and Iron concentration in rat cerebellum.

	Aluminum content (µg / g tissue)	Iron content (µg / g tissue)
Control	2.1 ± 0.07	19.2 ± 2.2
Aluminum (AL)	14.8 ± 0.9[a]	34.5 ± 3.1[a]
AL + BM	12.2 ± 0.8[a]	22.6 ± 1.8[b]
AL + DP	13.4 ± 1.0[a]	28.3 ± 1.9[a]

Values are expressed as mean ± SEM for six animals in each group. The superscripts relate significant ($p<0.05$; one way ANOWA) comparison between control and Al treated (a), Al treated and BM treated or DP treated (b) and between BM treated and DP treated (c).

Biochemical profiles

Figure - 1 embodies ROS, protein and lipid peroxidation products. The level of ROS was found to be significantly increased by 52% and 35% in Al treated and DP treated rats respectively as comparison to the controls. However, BM treated rats showed significantly decreased (21%) level of ROS when compared with the Al treated rats. The total protein in the cerebellum was significantly reduced by 33% in Al treated rats in comparison with the controls which, however, recovered by 35% and 39% in BM treated and DP treated rats. Protein oxidation products in term of protein carbonyl content in cerebellum were increased by 75% in Al treated rats and it got reversed by 34% and 32% in BM and DP trated groups respectively. The lipid peroxide levels were found to be increased by 53% in Al treated rats when compared with the controls, which got reduced by 23% and 17% in BM treated and DP treated rats respectively. Lipofuscin content of the cerebellum was found to be elevated by 158% in Al treated rats in comparison with controls which remarkably recovered by 27% in BM treated rats than that of Al treated rats.

Figure-2 shows enzymatic antioxidant status of control and experimental rats. The activity of SOD was found to be decreased by 31% when compared with the control while it reversed by 33% and 18% in BM and DP treated rats. Similarly, the catalase activity was also reduced by 42.5% in Al treated rats in comparison with the control but it was found to be increased by 72% in BM treated rats as compare with the Al treated rats. The activity of Se dependent antioxidant enzymes i.e, GSHPx and GR was markedly reduced by 32.5% and 39% in Al treated rats respectively. The GSHPx was significantly elevated by 45% and 32% in BM and DP treated rats respectively when compared with Al treated rats. On the other hand, the activity of GR was increased only by 45.6% in BM treated rats when compared with the Al treated groups. Figure 4 also shows the concentration of acetylcholinesterase (AChE) in the cerebellum of control and experimental groups. The activity of AChE was found to be significantly increased by 27% in the Al treated rats as compared to the controls. However, it was reduced by 26% and 18% in DP and BM treated rats respectively when compared with Al treated rats.

Figure-3 shows the concentration of GSH and GSSG and their redox ratio. The GSH was reduced by 59% in Al treated rats when compared with the controls while there was insignificant recovery in BM and DP treated rats when compared with the Al treated rats. The increased GSSG concentration (68%) was found in Al treated cerebellum as compared with controls and it was revered in BM treated groups (31%) when compared with Al treated rats. The redox ratio of GSH / GSSG was significantly reduced by 59% in Al treated rats when compared with the controls, while it was increased by 88% in BM treated groups, in comparison with the Al treated rats.

Microscopic Observation

Light Microscopy

Figure 5 depicts photomicrographs of the H &E stained section of the cerebellum of control and experimental rats. The control section of the cerebellum (fig, 2-A) shows normal histological features with well-organized three cortical layers.

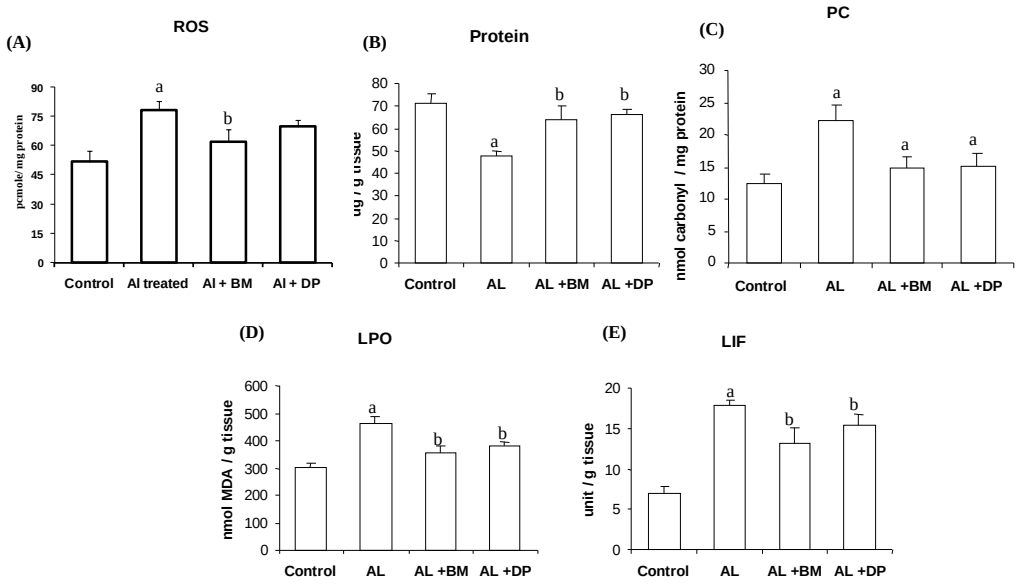

Figure 1. Levels of reacting oxygen species (ROS), Protein, Protein carbonyl content (PC), lipid peroxide levels (LPO) and lipofuscin in control and aluminum treated group. The results are expressed as Mean ± SEM in six rat of each group. Superscripts relate significant ($p < 0.05$) comparison with Control (a), Al treated (b) and BM treated (c).

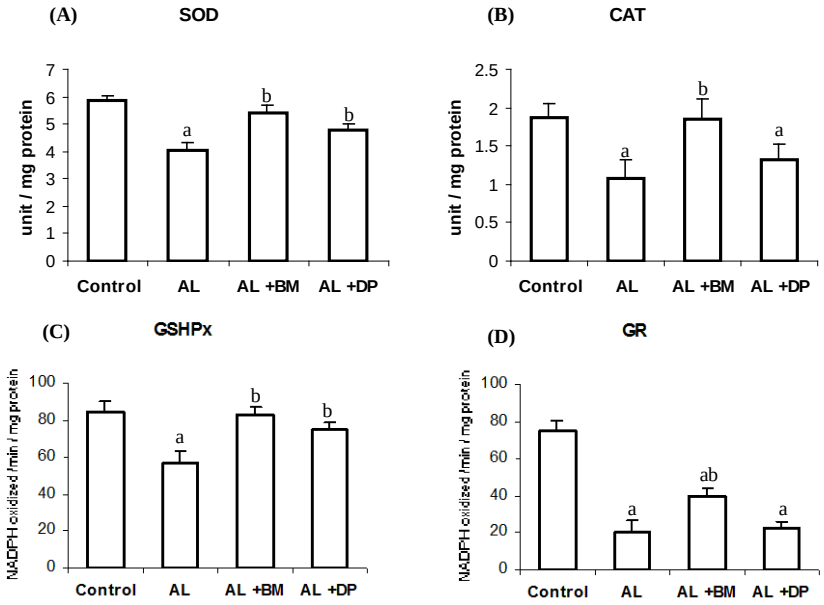

Figure 2. Activity of superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSHPx) and Glutathione reductase in control and aluminum treated group. The results are expressed as Mean ± SEM in six rat of each group. Superscripts relate significant ($p < 0.05$) comparison with Control (a), Al treated (b) and BM treated (c).

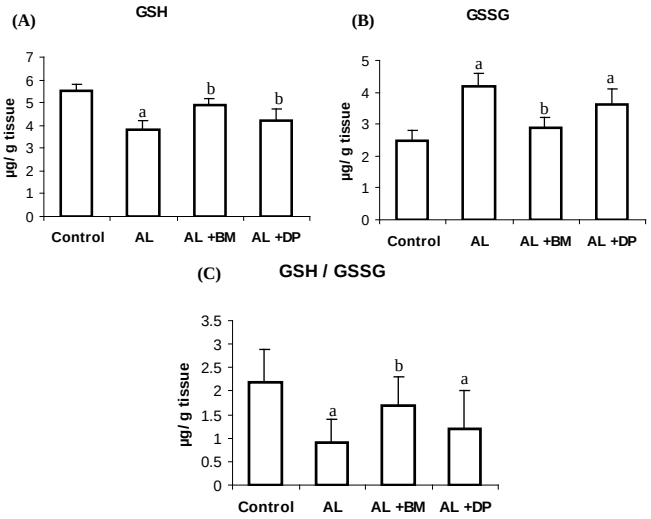

Figure 3. Levels of reduced glutathione (GSH), oxidized glutathione (GSSG) and their ratio (GSH/GSSG) in control and aluminum treated group. The results are expressed as Mean ± SEM in six rat of each group. Superscripts relate significant ($p< 0.05$) comparison with Control (a), Al treated (b) and BM treated (c).

The superficial molecular layer (M) is occupied mostly by axons and dendrites, middle monolayer of Purkinje cells (P), the dense layer of granular cells (G) and the white matter in the center of folium are clearly visualized (40X). The Al treated section of the cerebellar cortex (Fig, 2-B) show dystrophy of the Purkinje and granular cells and associated with karyolysis of Purkinje neurons (X 400). The section obtained from Bacopa monniera treated rats (fig, 2-C) exhibited well maintained architecture resembling control rats. There were no apparent degenerative changes in any of the three layers (X 100). The section obtained from the cerebellum of donepezil treated rats (fig 2-D) shows sparsely populated granular cell layer with occasional necrotic changes but the Purkinje neurons were nearly normal (X 100).

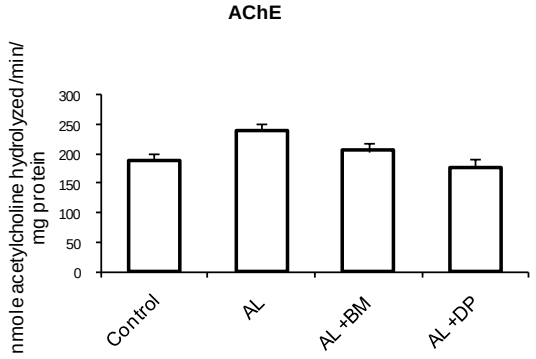

Figure-4. Activity of acetylcholinesterase (AChE) in the cerebellum of control and aluminum treated group. The results are expressed as Mean ± SEM in six rat of each group. Superscripts relate significant ($p< 0.05$) comparison with Control (a), Al treated (b) and BM treated (c).

Electron Microscopy

Figure 6 shows the electron micrographs of the cerebellar cortex of control and experimental rats. Electron micrograph (A) depicts well preserved nucleus of the Purkinje neuron of control rats. The perikaryon shows parallel cisternae of rough endoplasmic reticulum, many mitochondrial profile and coated vesicles. Electron micrographs B shows prominent granular cell layer of the control rats. Figure C, D and E are from the cerebella of Al treated rats. Clustered pleomorphic lipofuscin are seen (fig C) and remarkable accumulated lipofuscin also in Purkinje neurons of the cerebellum (fig D). Electron micrograph of granule cell layer of cerebellum shows four nuclei (nu) of granule cells exhibiting peripheral clumping of chromatin granules (E). Electron micrograph F and G showing prominent nucleus along with minor granules of lipofuscin (fig F) and granule cell layer of the cerebellum from characteristic peripheral condensation of chromatin and well preserved perikarya containing mitochondria (M), ribosome (R) and a few electron dense bodies. The Donepezil treated (fig H) exhibited neurolipofuscin and maintained structure of the nucleus. Moreover, we observed damaged mitochondria in the frontal cortex and hippocampus region of Al treated rats. However, these changes were not seen following treatment with Brahmi.

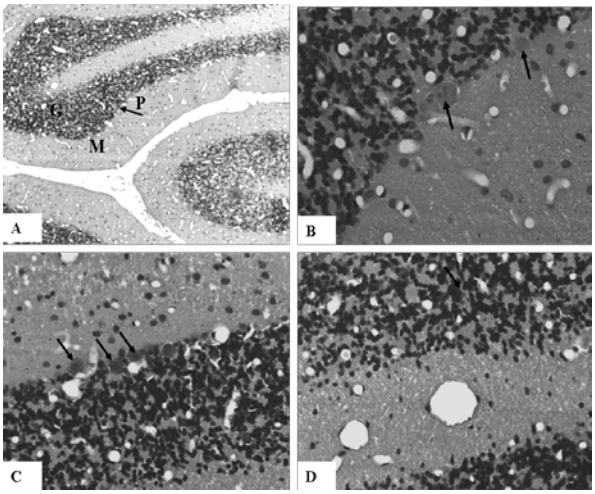

Figure 5. Light photomicrograph of H&E stained section of rat cerebellum. (A) Representative section from the control group shows three cortical layers namely molecular (M), purkinje cell layer (P) and granular cell layer (G). Photograph (B) shows damaged purkinje cells of Al treated rats. Section of cerebellum (C) shows maintained architecture of three layers of the bacoside treated rats. Dispersed granular cell layer in the section of donepezil treated rats (D).

DISCUSSION

In the present study we evaluated Al induced morphological and neurochemical alterations in the cerebellum and correlated them with the motor and learning functioning of aged rats. Moreover, the protective effect of *Bacopa monniera* extract and donepezil was also tested in Al induced cerebellar toxicity in rats. Primarily, we observed reduced motor activity, depleted neuromuscular coordination, gait, catalepsy and altered learning and memory response in aged rats following 90 days of Al administration. These findings indicated that Al induced neuronal changes and they are in consonance with our earlier observations (30, 52). Several neurological manifestations have already been attributed to Al intoxication in humans, including memory loss, tremors, jerky movements, loss of curiosity, ataxia, myoclonic jerk and convulsions (56). Furthermore, we observed that co-administration of Al alongwith *B. monniera* and Al with donepezil to rats significantly reversed their behavioral changes. Our results clearly demonstrated that B monniera inhibits anticholinergic enzyme AChE, almost to the same extent as donepezil, and this may be responsible for protection of synaptic morphology leading to enhancement in the behavioral performance (18, 48). An elevated AChE activity in cerebellum was observed following Al administration and this may be directly correlated with cholinergic sign and symptoms (23). It may be pointed out that loss of cholinergic neuronal activity is one of the cardinal features of dementia (11). Both the Al induced neurotoxicity and impairment of behavioral performance was dependent on each other. Results from the present study also showed that Al insult retard somatic developments as indicated by lower body weight and subsequent brain and cerebellum weight loss.

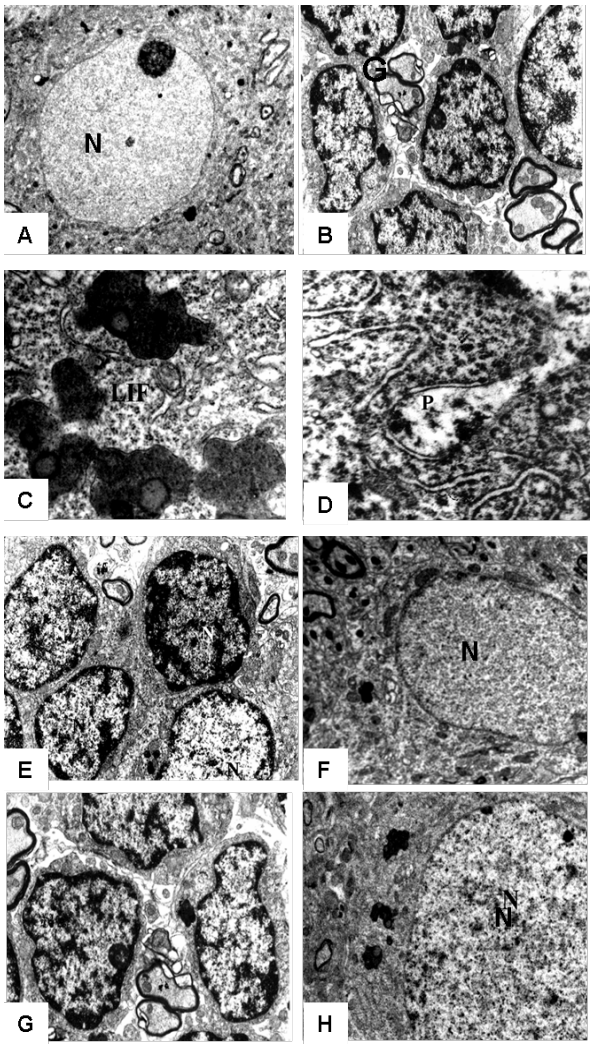

Figure 6. Electron micrograph of the cerebellar cortex of control rat exhibiting prominent nucleus (A) and well preserved granular cells (B). The micrograph of Al treated rat exhibited accumulation of large size of lipofuscin granules (C), purkinje neurons (D) containing electron dense bodies and diffused perikarya of the granular cell (E). The micrograph of the *B. monnieri* treated rats shows promimant nucleus (F) and well defined granular cell (G). The donepezil treated rats showed irregular cytoplasmic dispersion in the nucleus and three clumped lipofuscin grnules in the vicinity of the nucleus (H).

Our neurochemical findings from Al treated rats provide unequivocal evidence of damage in cerebellum with increased rate of oxidative metabolism of lipids and proteins (38). We found elevated lipid peroxide levels in Al treated rats. Earlier it has been reported that the increased lipid peroxidation and protein oxidation in Al neurotoxicity may be due to the accumulation of excess iron and it may further lead to an increase in Fe catalyzed Fenton reaction resulting in generation of more reactive oxygen species (ROS) (52).

It has been reported that Fe and Al bind to transferrin receptor before crossing the blood brain barrier via transferrin-mediated endocytosis and thus enter into the brain, where they are retained for a prolonged period (36). This has been explained to be due to the increased permeability of blood brain barrier or due to decreased ability of the body to remove Al with advancing age. In this study we detected Al content in the cerebellum along with significantly increased levels of iron in the Al exposed rats. We reported that B. monniera reduces the excess Fe accumulation in cerebellum. It may be due to regulation of Fe homeostasis and chelating capacity of *Bacopa monniera* to Fe.

Moreover, previous studies also documented that iron accumulates in the human CNS with normal aging (57). This accumulation of iron is reported in astrocytes and microglia of the cerebral cortex, cerebellum, hippocampus, basal ganglia, and amygdala in people between 60 and 90 years of age (5). This age-related iron accumulation may be caused by increased leakiness of the blood– brain barrier and increased oxidative insult. Moreover, as the brain contains large amounts of lipids that are rich in polyunsaturated fatty acids, therefore they can readily react with free radicals and undergo lipid peroxidation (31). Furthermore, we observed high lipofuscin levels in Al treated rats. Increased lipifuscinogenesis is one of the well known marker of neuronal aging (20, 37). Similarly, earlier Kumar et al. (26) had also reported that increased Al and lipofuscin concentration could deleteriously affect the neurons, leading to depletion of antioxidants. However, we observed that in *B. monniera* treated rats cerebellum there was significantly reduced level of lipid and protein peroxidation products and reduced lipofuscin content.

We also found reduced levels of the enzymes involved in antioxidant defense, viz. SOD, CAT, GPx and GR in Al treated rats. These findings are in concordance with the earlier reports (21, 22, 24) 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 co-administration of *B. monniera*. These biochemical modifications indicate that B. monnieri possess strong antioxidative property and it also acts as an anti-aging agent (8). In the present study, we also found reduced a GSH/GSSG ratio in Al treated rats. GSH/GSSG ratio determines the relative amount of reduced or working glutathione (GSH) compared to the oxidized 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 GSSG reductase (GR). We found that administration of B. monnieri extract minimizes these changes by increasing of GSH/GSSG ratio and this may be due to the antioxidative properties of this herb.

Results of our histopathological studies (H&E) revealed that Al distort the architectural integrity of the cerebellar cell layers in aged rats. The alteration of Purkinje's and granular cell layers of the cerebellum as reported here may be responsible for the associated functional changes (53). The human cerebellum contains on the order of 60 to 80 billion granular cells. The Golgi bodies provide inhibitory feedback to granular cells forming a synapse with them and projecting an axon in to the molecular layer. The results of our studies suggest that the alterations in Purkinje's and granular cells of the cerebellum could have directly contributed to gait, catalepsy, neuromuscular disorders and learning and memory impairment. Moreover, the electron microscopic observations revealed accumulation of clustered lipofuscin in Al treated rats, alongwith axonal swelling, demyelination of nerve fibers and increased neuronal degeneration. It may be due to the Al induced oxidative damage of cerebellum which may be responsible for such changes. However, these changes were not discernible in *B. monnieri* treated rats. We also observed that occurrence of lipofuscin was more significantly reduced with the co-administration of *B. monniera* extract than that of donepezil. These finding further support our biochemical studies.

On the basis of our results, it may be concluded that *B. monnieai* may prove

efficacious in ameliorating the aluminum-induced alterations in neuromuscular and cognitive behavior and neurochemical changes of aged rat cerebellum. Moreover, our results also showed that the protective effect of *B. monniera* was similar to that of donepezil. However, *B. monniera* additionally, also significantly recovered endogenous antioxidants (i. e., CAT, SOD, GPx, GR and GSH) which protect neurons against ROS along with improving AChE. The active components of *B. monniera* i.e., bacoside A and B are the triterpenoid saponins and they may be responsible for the protective effect of this herb (4, 17, 21, 22, 27). Moreover, it seems that *B. monniera* extract possess certain additional antioxidative and neuroprotective properties due to which it may be used as herbal supplement for the treatment of neurodegenerative disorders and neuron degenerations like donepezil. However, there is a need for further in depth studies to confirm these findings.

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Other articles in this theme issue include references (58-73).

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