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# Neuroprotective Effects of *Bacopa monniera* Whole-Plant Extract against Aluminum-Induced Hippocampus Damage in Rats: Evidence from Electron Microscopic Images

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## Abstract

**Impaired antioxidant system and structural changes in hippocampus are considered as key instigators of neurodegenerative diseases. The present study aimed to investigate the antioxidant and tissue protective properties of *Bacopa monniera* whole-plant extract (BME) against aluminum (Al)-induced oxidative stress and hippocampus damage in rats. Male Wistar rats were evenly divided into four groups, nine in each and labeled as control, Al treated (10 mg/kg), BME administered (40 mg/kg) and combination of both Al plus BME (Al+BME) treated groups. After one month of treatment by oral administration, antioxidant status was determined, and structural changes in the hippocampus were evaluated by electron microscopy. Al-induced increased oxidative damage in the hippocampus was revealed by elevated thiobarbituric acid reactive substances (TBARS). This increased lipid peroxidation was associated with significantly decreased antioxidant enzyme activities, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). However, Al intoxicated rats treated with BME for 30 days showed significantly restored antioxidant enzyme activities along with decreased TBARS ($P < 0.01$). Further evidences from electron micrographs clearly indicated that Al-induced vacuolation, lipofuscin deposition and pyramidal cell degeneration in the hippocampus was attenuated with co-administration of the whole-plant extract. Our results demonstrate that structural derangement in hippocampus by Al is directly proportionate with increased lipid peroxidation. Nevertheless, *B. monniera* treatment potentiates the antioxidant status and suppressed the tissue damage induced by Al-intoxication. These findings suggest that *B. monniera* whole-plant extracts can be considered as a possible remedy to counteract Al-associated neurological disorders.**

**Key Words: antioxidant enzymes, brain damage, lipid peroxidation, neuroprotective herb, oxidative stress**

## Introduction

*Bacopa monniera (B. monniera),* a small creeping herb locally known as 'Brahmi' in India, belongs to the family of Scrophulariaceae, and is mostly found throughout India, Nepal, Sri Lanka, China and Taiwan.

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Extracts of *B. monniera* have been recommended by ancient Ayurvedic treatises for improvement of memory and for treatment of mental disorders (32). Regular use of *B. monniera* leaf as a natural health supplement is beneficial in the treatment of neurological disorders associated with free radical-induced damages (5, 6, 35). *Bacopa* extracts are able to cure anxiety, epilepsy, bronchitis and asthma, irritable bowl's syndrome and gastric ulcers (31). Furthermore, *B. monniera* extracts have been shown to increase endogenous antioxidant enzyme activities in brain tissues of rats (5).

Aluminum (Al) is a common nonessential metal presents in processed foods, cosmetics, toothpastes, antiperspirant and adjuvants in various parenteral preparations (4); however, it has no identified biological function in the body. Several studies have demonstrated that Al is a possible causative factor in Alzheimer's disease (14, 18). Population residing nearby cement factories are more susceptible to Al-induced neurodegenerative diseases, since these residents contain high amounts of Al deposits in their bodies (13). Al is biologically considered as a pro-oxidant, and chronic exposure to this metal results in the production of excessive free radicals/reactive oxygen species (ROS), which are responsible for neurotoxicity (16, 19). Al-induced ROS can diminish the activities of primary antioxidant enzymes, and trigger membrane peroxidation (37). The brain is the most sensitive organ to oxidative damage due to high levels of ROS and lipofuscin pegmentation (17, 30, 34). Complexing with Al, therefore, enhances the ability of β-amyloid protein to penetrate the blood brain barrier (3). Al-induced oxidative damages in brain-cell components lead to neurotoxicity (29, 33), and continuous exposure to Al may produce structural derangements and impairment of redox balance in the hippocampus.

We hypothesized that occurrence of oxidative damage and impaired antioxidant status by Al exposure may be proportionate with structural derangements in the hippocampus of the rat brain. On the other hand, improved antioxidant status in the brain may attenuate the hippocampus structural damage. In this context, treatment with whole-plant extract of *B. monniera* that possess antioxidant properties may halt further damages against continuous exposure to Al. Therefore, we proposed in this study to evaluate the neuroprotective properties of *B. monniera* whole-plant extract on ruined antioxidant status in Al-intoxicated rats. The lipid peroxidation marker and associated architectural damage, and subsequent rescue by the treatment were evaluated through electron microscopic images of the hippocampus.

## Materials and Methods

*Animals*

In this study, male albino rats of 3 months of age, with bodyweights 200-250 g, were used. The rats were procured from an authorized vendor (Sri Venkateswara Enterprises, Bangalore, India). After one week acclimatization to laboratory conditions, the rats were randomized into groups (n = 9 per group) and maintained in polypropylene cages (47 × 34 × 20 cm) containing sterile paddy husk as bedding, and maintained at 22 - 25°C under a bell-regulated light and dark cycle (12 h:12 h) at the animal facility of the Sri Padmavati Mahila University. The rats were fed on standard rodent diet (Sai Durga Feeds and Foods, Bangalore, India) and water provided *ad libitum*. The study design and protocols were approved by the Institutional Animal Ethics Committee of Sri Venkateswara University, India (No. 03/2012-13/(i)a/CPCSEA/IAES/SVU/PJD-RPC/dated 1/2/2012).

*Preparation of* B. monniera *Whole-Plant Extract (BME) and Dose*

Fresh *B. monniera* plant was obtained from the Tirumala hills, Andhra Pradesh, India, and the whole plant was dried under shade dust-free conditions, and was ground into fine powder. The powder was extracted with double distilled water. The aqueous extract was discarded and the residual plant materials were extracted thrice with 90% ethanol. The residue obtained after removing the solvent was dried in vacuum, and macerated with acetone to give the free-flowing powder. Without further purification, the whole-plant extract was used in this study. The whole-plant extract of *B. monniera* has previously been claimed to be composed of a variety of chemical ingredients, including alkaloids (brahmine, herpestine) and saponins (bacosides) (32). The dose equivalent to 40 mg/kg body weight was chosen based on the previous studies performed on rats (20).

*Chemicals and Drugs*

Al maltolate was purchased from the Sigma Chemical Co., (St. Louis, MO, USA), $H_2O_2$, BSA and other chemicals were obtained from Merck (Mumbai, India), Ranbaxy (New Delhi, India), Qualigens (Mumbai, India).

*Experimental Design*

Thirty six rats were equally randomized into four groups, 9 animals in each, and were treated as follows;

*Group I: Control (Con):* Rats in this group were

administered with 0.9% saline for 30 days.

*Group II:* *Al treated*: Al maltolate, dissolved in 0.9% saline solution, was administered orally at the dose of 10 mg/kg body weight (b.w.) for one month.

*Group III:* *B. monniera treated*: All rats in this group were treated with BME at the dose of 40 mg/kg b.w. *via* an orogastric tube for one month.

*Group IV:* *Al maltolate plus B. monniera treated (Al+BME)*: Rats in this group were administered with Al maltolate and simultaneously received *B. monniera* extracts as described in Groups II and III for one month.

After the last treatment, six rats from each group were sacrificed for biochemical analyses, and three rats were used for transmission electron microscopy (TEM) studies. Hippocampus was separated from the brain region under cold conditions, and homogenates were prepared in 10 mM sodium phosphate buffer using an electric motor with teflon glass and pestle.

*Determination of Lipid Peroxidation Index*

Thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation, was estimated in the hippocampus as described by Ohkawa *et al*. (28). The amount of TBARS was determined spectrophotometrically by UV-Vis spectrophotometer UV-2450 (Shimadzu Corporation, Kyoto, Japan) at 532 nm, and the values were expressed as milligram of TBARS per mg protein.

*Measurement of Antioxidant Enzyme Activities*

Superoxide dismutase (SOD) activity was estimated in tissue homogenates as described by Misra and Fridovich (25). SOD activity was measured as the inhibition of photo-reduction of nitro blue tetrazolium (NBT) by the enzyme. Enzyme activity was expressed as unit of SOD per min per mg protein. Hippocampus catalase (CAT) activity was assayed spectrophotometrically by the method of Aebi *et al*. (1). The decrease in absorbance was read for 60 s with 15 s interval at 240 nm. CAT activity was expressed as µmol $H_2O_2$ decomposed per min per mg protein. Activity of glutathione peroxidase (GPx), the major antioxidant enzyme in the glutathione cycle, was measured as described by Flohe and Gunzler (15). GPx activity was expressed as µmol per min per mg protein. Protein concentration in tissue homogenates were determined by the standard protocol of Lowry *et al*. (23). Bovine serum albumin (BSA) was used as standard, and the color developed was read at 660 nm.

*Transmission Electron Microscopy (TEM)*

The hippocampus tissues of different groups were fixed in 2.5% - 3% glutaraldehyde prepared in 0.1 M phosphate buffer, pH 7.2, for 24 h at 4°C, and post fixed in 2% aqueous osmium tetroxide in the same buffer for 2 h. The slides were then dehydrated in a series of graded alcohols, infiltrated and embedded in Araldite® 6005 Resin or Spurr Resin. Ultrathin (50-70 nm) sections were made with a glass knife on an ultramicrotome (Leica ultra cut UCT-GA-D/E-1/00). Then slides were mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead citrate was done by the method of Bozzola and Russel (8). These sections were carefully viewed under the transmission electron microscope (Hitachi H-7500, Hitachi Ltd., Tokyo, Japan), and images were captured to demonstrate structural changes in the hippocampus.

*Statistical Analysis*

The obtained results are expressed as mean ± SD (standard deviation). All the data were analyzed using one way analysis of variance (ANOVA) and statistical significance was done by Scheffe's *post hoc* test. The level of significant was set at $P < 0.05$.

## Results

B. monniera *Attenuates Al-Induced Lipid Peroxidation*

Al-induced chemical damage to polyunsaturated fatty acids of cell membranes was determined by measuring the TBARS, the products of lipid peroxidation process. We found that Al administration significantly ($P < 0.01$) elevated TBARS in the hippocampus compared to the control. However, *B. monniera* treatment to the Al group resulted in significantly ($P < 0.01$) decreased TBARS compared to the Al alone administered group (Fig. 1). Regard-less of mild elevation in TBARS with *B. monniera* alone, Al-induced further exacerbation was prominently suppressed with a combination of *B. monniera* and Al administration. The decreased lipid peroxidation with the combination treatment was consistent with less lipofuscin deposits and less architectural damage as we observed through electron microscopic studies (see below).

*Effects of* B. monniera *on Impaired Antioxidant Status in Hippocampus*

Statistical analyses clearly indicated a negative impact of Al intoxication on the antioxidant enzyme status of the hippocampus. All primary antioxidant

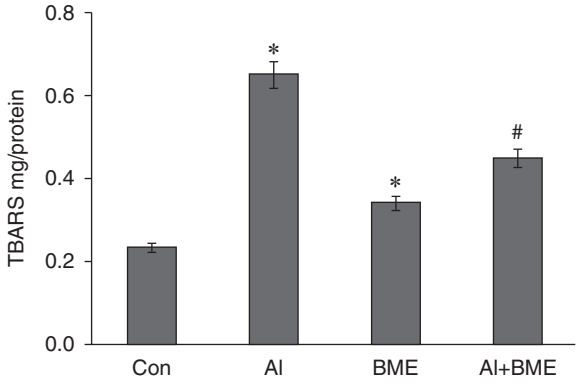


Fig. 1. Effects of *B. monniera* whole-plant extract (BME) on hippocampal TBARS levels in rats intoxicated with Al. Values are significant compared to control (*) and Al treated (#) groups at $P < 0.01$.

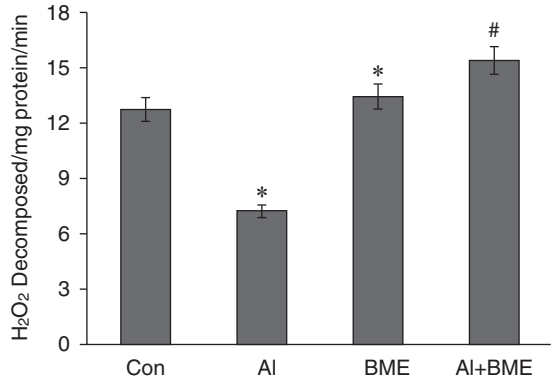


Fig. 3. Effects of *BME* on hippocampal CAT activity in rats intoxicated with Al. Values are significant compared to control (*) and Al treated (#) groups at $P < 0.01$.

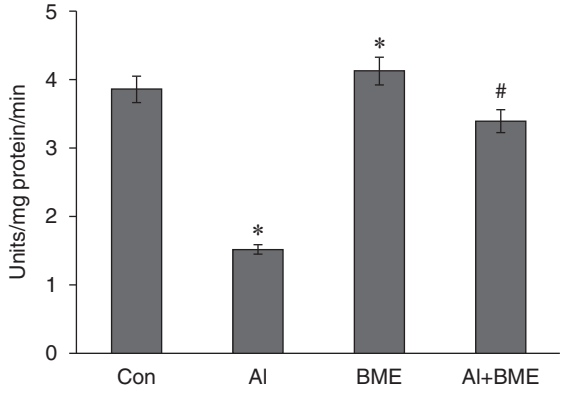


Fig. 2. Effects of BME on hippocampal SOD activity in rats intoxicated with Al. Values are significant compared to control (*) and Al treated (#) groups at $P < 0.01$.

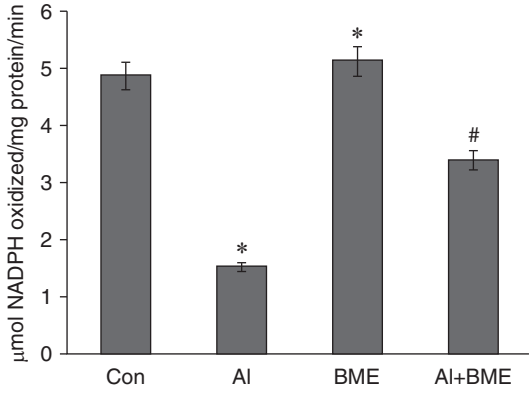


Fig. 4. Effects of BME on hippocampal GPx activity in rats intoxicated with Al. Values are significant compared to control (*) and Al treated (#) groups at $P < 0.01$.

enzyme activities, including SOD, CAT and GPx, were significantly ($P < 0.01$) decreased in the Al group compared to the control (Figs. 2, 3 and 4). It is noteworthy that the Al group treated with whole-plant extract of *B. monniera* for 30 days significantly ($P < 0.01$) restored the decreased antioxidant enzyme activities in the hippocampus. The restored CAT activity with *B. monniera* treatment was more prominent than the activities of other two enzyme, SOD and GPx.

*TEM Studies*

Ultrastructural changes in the hippocampus were evaluated by electron microscopic studies, and the tissue protective properties of BME were demonstrated against Al-induced damage. Evidence from electron micrographs clearly indicated that daily administration of Al for 30 days produced profound structural derangements in the hippocampus. The destructive effects of Al were evidenced by observing the several small cavities (vacuolation, V), pyramidal cell degeneration (PCD), lipofuscin (LP) deposition in the hippocampus (Fig. 5B). The most interesting finding of this study is that Al-induced architectural damages were lessened by treatment with the whole-plant extract. Electron micrograph of *B. monniera*-treated hippocampus showed less vacuolation, less degenerated neurons and less lipofuscin deposition compared to Al alone treated group, which indicated tissue protective properties of the whole-plant extract of *Bacopa* (Fig. 5D).

## Discussion

Exposure to Al toxicity for a period of time can ruin the antioxidant homeostasis and produce structural derangement in hippocampus that may contributes to the development of neurodegenerative diseases. In this study, we demonstrated that whole-plant extracts of *Bacopa monniera* was able to atten-

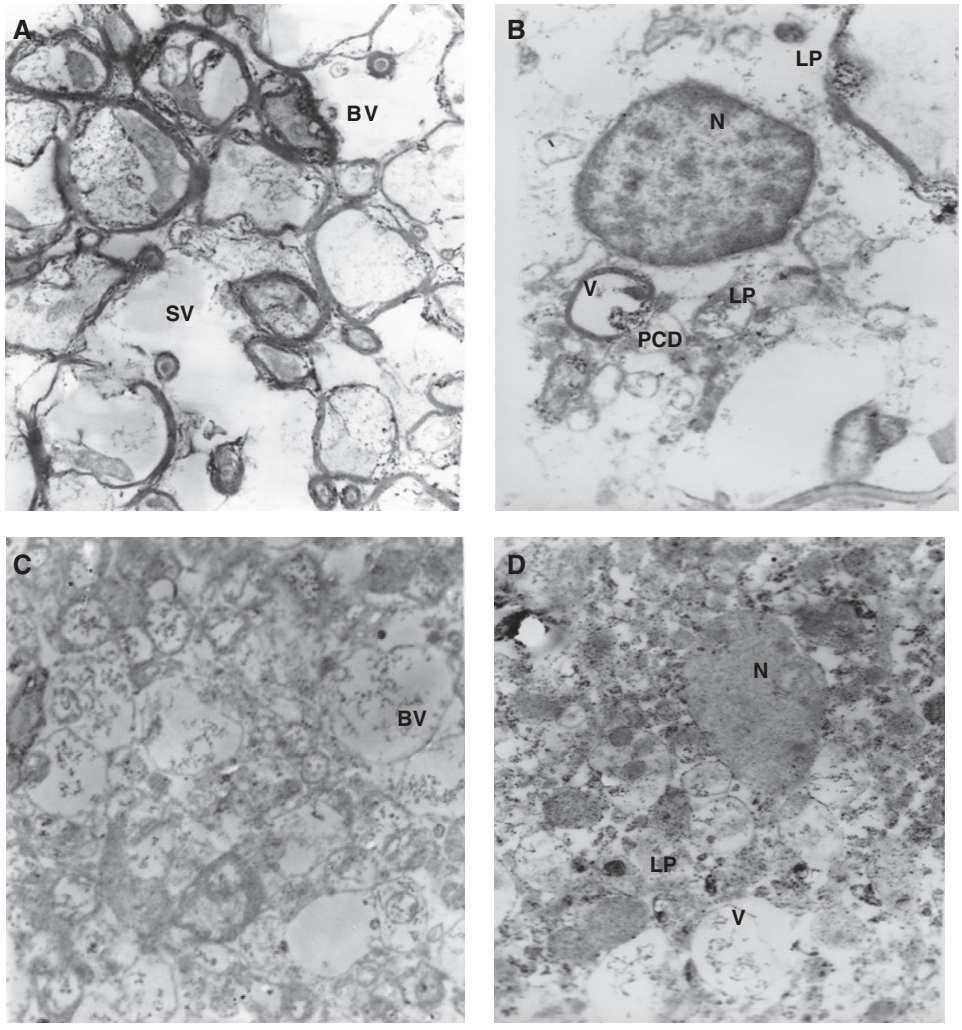


Fig. 5. Electron microscopic analyses of hippocampus damage and recovery. A. Electron micrograph of control rat hippocampus showing normal blood vessels (BV) and synaptic vessels (SV). B. Electron micrograph of Al intoxicated rat hippocampus showing nucleus (N), vacuolation (V), lipofuscin pigment (LP) and pyramidal cell degeneration (PCD). C. Electron micrograph of the hippocampus of BME-treated rat showing nucleus (N) and blood vessels (BV). D. Electron micrograph of the hippocampus of Al plus BME-treated rat demonstrating less vacuolation (V), pyramidal cell degeneration (PCD) and less lipofuscin pigments (LP). Magnification in all subfigures was 1,074X.

uate the Al-induced increased lipid peroxidation and restored the decreased activities of the antioxidant enzymes SOD, CAT and GPx in the hippocampus of rats. Strong supportive evidences from electron micrographs further indicated the tissue protective properties of *B. monniera* as the rescued pyramidal cell degeneration and less deposition of lipofuscin were observed. Since neurodegenerative diseases could be influenced by structural damages in hippocampus regions, preventing hippocampus damages by the administration of BME implies beneficial effects of BME against Al toxicity.

Lipid peroxidation is the crucial step in the pathogenesis of several diseases, which is fundamentally initiated by free radicals (24). Al administration has been shown to increase the excessive amount of free radicals/ROS (19), which destructively attack the polyunsaturated fatty acids of the fatty acid membrane, and initiate self-propagating chain reactions. The elevated TBARS with Al intoxication in our study clearly indicated increased membrane damage in the hippocampus of rats. The destruction of vital membrane lipids and increased end products of lipid peroxidation are harmful to cell and tissue viability (9, 26). Lipid peroxidation results in the loss of membrane fluidity, changes in membrane permeability and alteration in receptor functions (2, 16, 27). On the contrary, the reason behind slightly elevated TBARS with *B. monniera* alone treatment in our study remains unclear. It appears that some unknown compounds in the *B. monniera* extract may cause diverse effects. However, Al-induced increased lipid peroxidation was attenuated

by the combined administration of the *B. monniera* extract, as shown by decreased TBARS in the hippocampus. Our findings are in agreement with Jyoti and Sharma (21), who reported similar results with ethanolic extracts of *B. monniera*. Decreased lipid peroxidation by BME could be possible, because BME contains a variety of compounds, including triterpenoid saponins, mainly bacoside A & B, which may inhibit elongation of lipid peroxidation chain reaction (32). On the other hand, decreased lipofuscin and increased antioxidant enzyme activities by *B. monniera* treatment, as also reported in this study, may explain the decreased lipid peroxidation against Al exposure.

The enzymatic antioxidant system plays a frontline defense against ROS toxicity in brain cells and other tissues (2, 22, 36). The primary antioxidant enzyme, SOD, rapidly scavenges the superoxide anion radials to hydrogen peroxide. Hydrogen peroxide is an unstable byproduct, which is then converted into less toxic water and oxygen by CAT and GPx in a site-specific manner (16, 19). Decreases in these enzyme activities imply the level of oxidative stress in the tissues. In our study, chronic Al intoxication significantly decreased the hippocampal SOD, CAT and GPx activities. Decreased SOD activities may explain the pro-oxidant properties of Al, since Al is capable of formation of Al-superoxide semi-reduced radical ions ($AlO_2^{\bullet 2+}$) (12). Al-induced increased ROS production is a crucial step, where antioxidant enzymes act to eliminate excessive ROS to prevent oxidative damages. Thus, excessive utilization of enzymes may lead to decreases in the net enzymatic activities in the hippocampus. Interestingly, all primary antioxidant enzymes, such as SOD, CAT and GPx, were significantly restored on treatment with the BME. Increased antioxidant enzyme activities indicate effective elimination of toxic superoxide anion and $H_2O_2$ from the cells. Bhattacharya and colleagues reported that ethanolic extracts of *B. monniera* were able to increase SOD, CAT and GPx activities in different brain regions of the rat (5). Other previous studies also demonstrated neuroprotective properties of *B. monniera* extracts against Al-induced toxicity, which might be due to the improved antioxidant status (7, 20). The detailed mechanism involved in the regulation of antioxidant enzyme activities by treatment with different extracts of *B. monniera* remains to be elucidated. However, we speculate that unique ingredients present in BME may stimulate expression of antioxidant genes directed against Al-induced ROS, thereby preventing or reducing possible oxidative damages.

Another novel finding from this study is that Al-induced architectural changes in hippocampus were ameliorated by 30 days of treatment with *B. monniera* extract. Continuous exposure to Al alone produced mild to severe damages in the hippocampus as seen by vacuolation, lipofuscin deposition and degeneration of pyramidal cells through electron microscopic images. These structural derangements in the hippocampus may lead to tissue damage and impair cognitive functions. A recent study by Dede *et al*. (10) demonstrated that hippocampal damage impairs both component processes of recognition memory in humans. It has been well documented that hippocampus exhibits an increased peroxidation process followed by Al administration compared to other brain regions. Increased lipid peroxidation is one of mechanisms which renders cellular damages in a variety of neurological disorders, including aging. Furthermore, hippocampus is one of the brain regions in which Al deposition is most severe compared with other regions (11). Therefore, there may be more Al-induced structural damages in the hippocampus. Nevertheless, co-administration of BME along with Al showed its protective effects against Al-induced architectural damage. The lower extents of formation of cavities, lipofuscin deposition and pyramidal cells degeneration indicated tissue protective properties of the *B. monniera* extracts. Since ROS plays a key role in tissue damage, the beneficial outcome of *B. monniera* could be associated with enhanced antioxidant defense system in the hippocampus. Several naturally occurring compounds, including flavonoids, alkaloids and saponins (bacoside A and B), were identified in Bacopa extracts. These compounds have been claimed to be the responsible factors for most of the pharmacological efficacies of the extracts, such as antioxidant and neuroprotective properties and enhancing the memory and learning skills (7, 21, 32). The lack of statistical analysis for the electron micrographs is a limitation in this study, which need to be extended in further studies.

This study concludes that Al-induced increased lipid peroxidation was parallel with LP deposition and architectural damage in the hippocampus, which indicated membrane damage and structural derangement. However, treatment with BME for 30 days significantly decreased the lipid peroxidation, LP deposition and attenuated structural derangements in the hippocampus. Improved antioxidant enzymes activities by the whole-plant extracts against Al-induced loss possibly contributed to the inhibition of the elongation of lipid peroxidation process, thereby preventing the hippocampal damage in rats. These results suggest that BME can be used as pharmacological compounds to protect neurodegenerative diseases.

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