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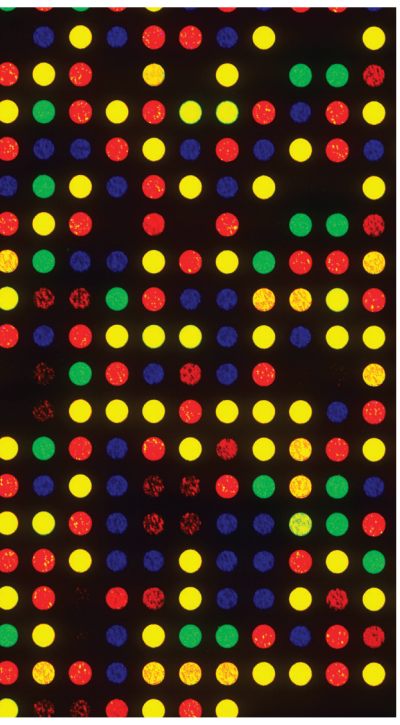
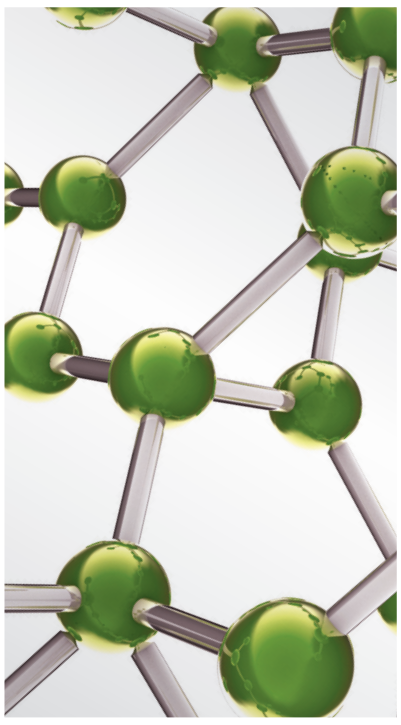
Guest Editors: Con Stough, Hemant Singh, and Andrea Zangara



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Editorial

Mechanisms, Efficacy, and Safety of *Bacopa monnieri* (Brahmi) for Cognitive and Brain Enhancement

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The plant *Bacopa monnieri* (water hyssop, Brahmi, thyme-leaved *Gratiola*, herb of grace, and Indian pennywort) is a perennial, creeping herb native to the wet lands of India, particularly northeast and southern regions. *Bacopa* is an important plant of Ayurveda, where it is named as Brahmi, after Lord Brahma, the mythological creator of the world and originator of the science of Ayurveda. *Bacopa* is frequently mentioned in the religious, social, and medical treatises of India since the time of *Vedic* civilization. Its antiquity can be traced to the time of *Athar Ved* (the science of well-being) written in 800 BC where *Bacopa* finds a mention in the very first verse of the third chapter of *Athar Samhita* (compilation on the factors promoting well-being).

More recently researchers have turned their attention to better understanding the mechanisms and efficacy of various extracts of *Bacopa monnieri* on human conditions. Although extracts of *Bacopa* have been studied and used to treat various disorders for centuries (pain, epilepsy, and inflammation, amongst many) perhaps the chief therapeutic claim concerning its benefits has been in improving memory. The Indian government has invested significant resources and conducted hundreds of studies examining the mechanisms of action on the brain and at a cellular level. Interestingly this research has uncovered a myriad of possible mechanisms relating to anti-inflammatory, antioxidant, metal chelation, amyloid, and cholinergic effects amongst many others. Although it is not unusual for plant based medicines to have multiple effects on cellular processes, *Bacopa monnieri* is perhaps one of the most scientifically studied in terms of mechanisms of action. Interestingly these mechanisms seem to comprehensively map on to the biological mechanisms that many researchers have argued underpin cognitive and memory processes. In 1996 a special extract of *Bacopa monnieri* was launched by the Indian Government's Central Drug Research Institute, Lucknow, termed CDRI 08. It was thought at the time that this particular standardised extract had been subjected to the most research and was the most promising extract for medical conditions. In 2010 the three editors for this special issue attended CDRI's 60th research anniversary where a special one-day symposium on research on CDRI 08 was held. It is this extract of *Bacopa monnieri* that is the focus of most of the papers in this special issue which reports studies relating to the safety, mechanisms, and efficacy of specific extracts of *Bacopa monnieri*.

Over the last ten years there have been growing scientific studies on this interesting terrestrial herb. As can be seen in Figure 1 the number of publications concerning *Bacopa monnieri* is steadily growing reflecting increasing scientific interest in this plant for human conditions. Most of these studies reflect scientific endeavours relating to cellular mechanisms. As such these studies are an excellent base to launch larger clinical trials in humans. Although much is known about the mechanisms of *Bacopa* extracts on the brain there are still significant gaps in our knowledge. For instance, long-term chronic trials in older people are now required to understand whether *Bacopa* extracts such as CDRI 08 can prevent age-related cognitive decline or even more insidious diseases such as Alzheimer's dementia. We note that a number of studies

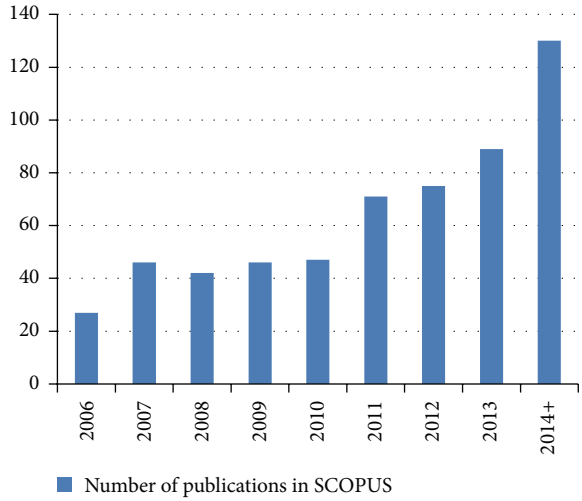

Figure 1

are also currently examining the effect of *Bacopa* extracts on improving cognitive and behavioural function in younger people. Clearly the next decade will focus on larger clinical trials in humans and expand upon the excellent animal and preclinical work mainly conducted in India.

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Research Article

Bacopa monniera (CDRI-08) Upregulates the Expression of Neuronal and Glial Plasticity Markers in the Brain of Scopolamine Induced Amnesic Mice

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Preclinical studies on animal models have discerned the antiamnesic and memory-enhancing potential of *Bacopa monniera* (Brahmi) crude extract and standardized extracts. These studies primarily focus on behavioral consequences. However, lack of information on molecular underpinnings has limited the clinical trials of the potent herb in human subjects. In recent years, researchers highlight plasticity markers as molecular correlates of amnesia and being crucial to design therapeutic targets. In the present report, we have investigated the effect of a special extract of *B. monniera* (CDRI-08) on the expression of key neuronal (BDNF and Arc) and glial (GFAP) plasticity markers in the cerebrum of scopolamine induced amnesic mice. Pre- and postadministration of CDRI-08 ameliorated amnesic effect of scopolamine by decreasing acetyl cholinesterase activity and drastically upregulating the mRNA and protein expression of BDNF, Arc, and GFAP in mouse cerebrum. Interestingly, the plant extract *per se* elevated BDNF and Arc expression as compared to control but GFAP was unaltered. In conclusion, our findings provide the first molecular evidence for antiamnesic potential of CDRI-08 via enhancement of both neuronal and glial plasticity markers. Further investigations on detailed molecular pathways would encourage therapeutic application of the extract in memory disorders.

1. Introduction

In the present era, medicinal efficacy of plant extracts mentioned in Ayurvedic literature with large variety of health promoting effects and minimal adversities is on the verge of gaining priority over conventional pharmacological approaches [1]. Among the plants mentioned in Ayurveda, *Bacopa monniera* (Brahmi) stands out in regard to its enormous potency to rejuvenate the central nervous system and improve mental health. The crude extracts of *B. monniera* as well its active principles, that is, bacosides, are being explored in a wide variety of central nervous system disorders, particularly impairment in memory or amnesia [2]. In this context, several studies on animal models [3] including the widely used cholinergic antagonist, scopolamine induced amnesia [4, 5] have revealed the efficacy of *B. monniera* extract in recovering memory impairment and exerting neuroprotection against cholinergic degeneration [6, 7]. The herbal extracts also facilitate short- and long-term memories and memory phases of acquisition, consolidation, and retrieval. However, the underlying molecular mechanisms that sustain the antiamnesic and memory-enhancing potential of *B. monniera* extract are largely unexplored. Consequently this lack of understanding in molecular details has limited the clinical application of *B. monniera* extracts in memory disorders.

Over the past decade, it is considered that the ability of brain to adapt or modify itself in response to intrinsic signals and external environment referred to as plasticity is integral to memory formation. It is a complex dynamic process involving adaptation of intrinsic properties of neurons and glia, reorganization of synaptic connections and neural network [8]. Accumulating evidences have substantiated the role of brain plasticity as a molecular correlate of memory processes [9]. Activation of gene expression associated with neuronal and glial plasticity is considered to be a key mechanism underlying the enduring modification of neural

networks required for the formation of memory [10, 11]. Amongst neuronal plasticity markers, brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, holds the most prominent place. BDNF regulates survival and differentiation of neurons and synaptic plasticity and is considered as a marker of memory decline during pathological conditions and brain aging [12, 13]. Memory acquisition and consolidation are associated with an increase in BDNF expression and activation of tyrosine receptor kinase (TrkB). In several behavioral paradigms including Morris water maze, contextual fear, and passive avoidance, memory formation is associated with a rapid and transient increase in BDNF mRNA expression in the hippocampus. Genetic as well as pharmacological deprivation of BDNF or TrkB impairs long-term potentiation (LTP) and hippocampus-dependent spatial memory [14–16]. BDNF regulates neuronal plasticity by stimulating gene transcription, activating protein synthesis, promoting neurotransmitter release, and modulating the activity and trafficking of postsynaptic receptors. Recently it has been reported that BDNF controls the transcription of neuronal immediate early gene Arc (activity regulated cytoskeletal-associated protein) which is strongly implicated in neuronal plasticity and memory [17, 18]. Several studies demonstrate involvement of Arc in AMPA receptor trafficking, LTP and consolidation of long-term memories [19]. Moreover, Arc knock-out mice showed significant impairment in long-term depression (LTD) and hippocampus-dependent spatial memory [20–22]. Our laboratory also demonstrated the involvement of hippocampal Arc in amnesia [23].

In addition to neuronal plasticity, astroglia and their resident protein glial fibrillary acidic protein (GFAP) are also important for memory function. GFAP is a specific astrocyte marker participating in the formation of cytoskeletal filaments of glial cells and processes of myelinization, cell adhesion, growth of neurites, and synaptogenesis. GFAP is a marker of glial plasticity regulating the astrocyte morphology, neuron-glia interactions, and mechanisms of memory formation [24, 25]. GFAP is drastically downregulated during amnesic conditions [26].

With this background, the present study was designed to investigate the molecular basis of recovery potential of *B. monniera* in scopolamine induced amnesic mouse model focusing on neuronal plasticity markers BDNF and Arc and glial marker, GFAP. The standardized bacoside-A enriched extract of *B. monniera* (CDRI-08) was used in the present study. Initially, antiamnesic potential of CDRI-08 was evaluated by analyzing its effect on scopolamine impaired cholinergic system via acetyl cholinesterase (AChE) activity assay in mouse cerebrum. Thereafter, molecular investigations were performed by examining the effect of CDRI-08 on the mRNA and protein expression of BDNF, Arc, and GFAP in the cerebrum of scopolamine injected amnesic mice.

2. Materials and Methods

2.1. Chemicals/Reagents. Random hexanucleotides, dNTPs, enhanced chemiluminescence (ECL) reagents, Taq polymerase, RNase inhibitor, and reverse-transcriptase enzymes were obtained from the New England Biolabs (USA); DTNB, acetyl thiocholine iodide, TRI reagent, monoclonal anti-β-actin-peroxidase (A3854) and rabbit polyclonal GFAP antibody (G4546), and mini protease inhibitor cocktail were purchased from Sigma-Aldrich (USA). Mouse monoclonal Arc antibody (sc-17839) and goat polyclonal BDNF antibody (sc-33904) were purchased from Santa Cruz Biotechnology, USA. Horse radish peroxidase conjugated secondary antibodies were purchased from Bangalore Genei, India, and polyvinyl difluoride (PVDF) membrane was procured from Millipore (Germany). CDRI-08 extract of *B. monniera* was generously provided by Lumen Marketing Company, Chennai. All other biochemicals were purchased from Merck (Germany).

2.2. Animals. Young (8 ± 2 weeks) male mice of Swiss albino strain were maintained at 12:12 h light/dark schedule with *ad libitum* standard mice feed and potable water in the animal house of Department of Zoology, Banaras Hindu University, Varanasi. All the animal experiments were approved by the Institutional Animal Ethical Committee, Banaras Hindu University, Varanasi, India. Animals were intraperitoneally injected with normal saline (vehicle) or 3 mg/kg BW scopolamine hydrobromide (dissolved in normal saline) whereas Tween-80 (vehicle) or 120 mg/kg BW CDRI-08 extract (suspended in 5% Tween-80) was administered orally through an oral gavage in the early hours of day (around 9 a.m.) daily for a week.

2.3. Treatment Schedule. All the drugs solutions were prepared just prior to use. In the initial experiments, mice were tested for ruling out any possible effect of treatment with vehicles alone and categorized into three groups: untreated (naïve), normal saline injected, and Tween-80 administered group. After obtaining the preliminary results from pilot study, we categorized mice into five groups: (a) SA-mice administered with normal saline, (b) SC-mice injected with scopolamine hydrobromide, (c) SC+CDRI-08 mice injected with scopolamine followed by CDRI-08 after 1 h, (d) CDRI-08-mice treated with CDRI-08 alone, and (e) CDRI-08+SC mice treated with CDRI-08 followed by scopolamine after 1 h. The drug treatment was continued for a week. On the seventh day, mice were sacrificed by cervical dislocation and cerebrum was removed quickly on ice. The biochemical assay of acetyl cholinesterase activity was performed after 3 h of the final drug administration to mice, whereas remaining brain samples were stored at −80°C for further use.

2.4. Acetyl Cholinesterase Assay. Acetyl cholinesterase (AChE) assay was done based on the principle and protocol as described earlier by Ellman et al. [27] with some minor modifications. Briefly, the cerebrum from each mouse was weighed and 20% homogenate was prepared in 0.1 M phosphate buffer (pH 8). Then 2.6 mL phosphate buffer (0.1 M, pH 8), 100 μL DTNB, and 0.4 mL homogenate were added in a glass cuvette and mixed thoroughly. A_{412} was measured using a UV-VIS spectrophotometer till the absorbance reached a constant value (basal reading). Finally, 20 μL of acetyl thiocholine iodide was added as substrate and change in absorbance was noted at the interval of 2 min to calculate the change in absorbance per minute. The AChE

Table 1: Primer sequences and PCR conditions.

Gene	Sequence of forward and reverse primers ($5' \rightarrow 3'$)	Condition of (a) denaturation (b) annealing (c) extension	Number of cycles
Arc [17]	FP-GGCGACCAGATGGAGCTGGACCATA- RP-CTGGCCCCTCTATTCAGGCTGGGTC-	(a) 94°C, 1 min (b) 59°C, 1 min (c) 72°C, 1.5 min	35
BDNF [20]	FP-TGCCAGAGCCCCAGGTGTGA- RP-CTGCCCTGGGCCCATTCACG-	(a) 94°C, 1 min (b) 63°C, 30 sec (c) 72°C, 45 sec	32
GFAP [20]	FP-TTCCTGTACAGACTTCTCC- RP-CCCTTCAGGACTGCCTTAGT-	(a) 94°C, 1 min (b) 52°C, 30 sec (c) 72°C, 45 sec	29
GAPDH [20]	FP-GTCTCCTGCGACTTCAG- RP-TCATTGTCATACCAGGAAATGAGC-	(a) 94°C, 1 min (b) 52°C, 30 sec (c) 72°C, 30 sec	26

activity was determined by following formula: $R = 5.74 \times 10^{-4} \times \Delta A/C$, where R = rate of enzymatic activity (in moles of acetyl thiocholine hydrolyzed/min/g tissue), ΔA = change in absorbance/min, and C = concentration of the tissue homogenate (mg/mL).

2.5. Semiquantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from the cerebrum of mice using TRI reagent kit. It was estimated by measuring absorbance at 260 nm and purity was checked by A_{260}/A_{280} ratio. Total RNA from different experimental groups was resolved on 1% agarose containing ethidium bromide to check the integrity of RNA by 18S and 28S rRNA. The total RNA was reverse-transcribed to cDNA and PCR-amplified using gene specific primers. The signals were scanned by Alpha Imager system and analyzed by AlphaEaseFC software (Alpha Innotech Corp., USA). The primer pairs and PCR conditions mentioned earlier are summarized in Table 1.

2.6. Immunoblotting. The cerebrum was removed from the mice of different experimental groups. The 10% protein lysate for Arc detection was prepared in RIPA buffer, whereas the same was prepared for BDNF and GFAP detection in another homogenizing buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 120 mM NaCl, and 10 μg complete mini protease inhibitor cocktail). All lysates were centrifuged at 10,000 ×g for 15 min. The supernatant was collected and protein was estimated by Bradford (1976) method. Protein was resolved by 10% SDS-PAGE, followed by semidry electroblotting onto PVDF membrane.

The membrane was blocked in 5% (w/v) fat-free skimmed milk in PBS (pH 7.4) at room temperature for 2 h, incubated overnight at 4°C with mouse anti-Arc polyclonal antibody (1 : 1000), goat anti-BDNF polyclonal antibody (1 : 5000), or rabbit anti-GFAP polyclonal antibody (1 : 1000); washed thrice in 1x PBS, incubated for 2 h at room temperature with HRP-conjugated goat anti-mouse IgG (1 : 1000) for Arc, rabbit anti-goat IgG (1 : 3000) for BDNF, and goat anti-rabbit IgG (1 : 2000) for GFAP; and washed twice in 0.1% PBST and signal was detected with ECL reagents on a X-ray film. For loading control, the same membrane was probed for β-actin with HRP-conjugated anti-β-actin antibody (1 : 10,000) for 3 h at room temperature.

2.7. Statistical Analysis. Each experiment was repeated thrice ($n = 3 \times 3$ mice/group). For AChE assay, the rate of enzymatic activity was measured as μmoles of acetyl thiocholine hydrolyzed/min./g tissue weight. For RT-PCR, the signal intensity of each candidate message was normalized against the signal intensity of internal control, that is, GAPDH. For immunoblotting, the signal intensity of each candidate protein was analyzed after normalization against the signal intensity of β-actin. The data are presented as a histogram with the mean (±SEM) of three values calculated as relative density values. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test through SPSS for Windows (standard version 16.0). P values ≤0.05 were considered significant.

3. Results

3.1. The Drug Vehicles Per Se Did Not Affect Expression of Plasticity Markers. In order to rule out the *per se* effect of vehicle controls for the drugs, we analyzed their effect on gene expression as compared to untreated naïve control. We observed no significant difference in the expression of BDNF, Arc, and GFAP mRNA among naïve (untreated), 0.9% saline (vehicle for scopolamine hydrobromide), and 5% Tween-80 (vehicle for CDRI-08) treated groups (Figure 1). Therefore, only saline control group was used for further experiments.

3.2. CDRI-08 Extract Attenuated Scopolamine Mediated Increase in AChE Activity in Mouse Cerebrum. The rate of degradation of acetylcholine was determined by AChE assay and we found that scopolamine hydrobromide increased the activity of AChE by 2-fold as compared to control. Pre- and posttreatment with CDRI-08 extract decreased AChE activity as compared to scopolamine (1.25-fold). However, with the *per se* treatment of CDRI-08, we observed insignificant

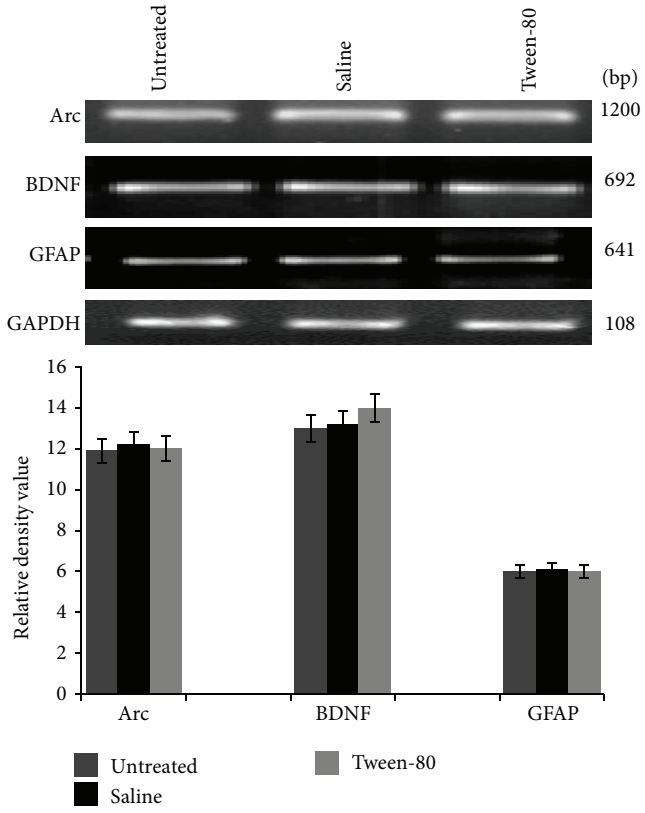

Figure 1: RT-PCR analysis of Arc, BDNF, and GFAP in vehicle control groups of male mice. Signal intensity for Arc, BDNF, and GFAP was normalized against the signal intensity for GAPDH individually. The data are presented as a histogram with the mean (±SEM) of three values calculated as relative density values of Arc/GAPDH, BDNF/GAPDH, and GFAP/GAPDH.

difference in the activity of AChE as compared to saline treated group (Figure 2).

3.3. Expression of BDNF, Arc, and GFAP mRNA Was Upregulated upon Administration of CDRI-08 Extract in the Cerebrum of Scopolamine Induced Amnesic Mice. Scopolamine drastically reduced BDNF mRNA level (6.25-fold) in mouse cerebrum. Pretreatment with CDRI-08 markedly attenuated the decrease by 5-fold while posttreatment attenuated the decrease by 3-fold as compared to scopolamine. The extract alone increased the level of BDNF mRNA by 1.3-fold as compared to saline (Figure 3(a)). Similar to BDNF, Arc mRNA was significantly downregulated by scopolamine (3.33-fold) and pre- as well as posttreatment with CDRI-08 showed 3-fold increase in Arc mRNA expression as compared to scopolamine (Figure 3(b)). The Brahmi extract alone also extensively upregulated (about 2-fold) the expression of Arc mRNA level as compared to control. Similar to neuronal markers, GFAP mRNA was significantly reduced by 4.16-fold by scopolamine treatment. Pre- and posttreatment with CDRI-08 equally attenuated the decrease by 4-fold as compared to scopolamine, whereas treatment with the extract alone did not show significant alteration as compared to saline control (Figure 3(c)).

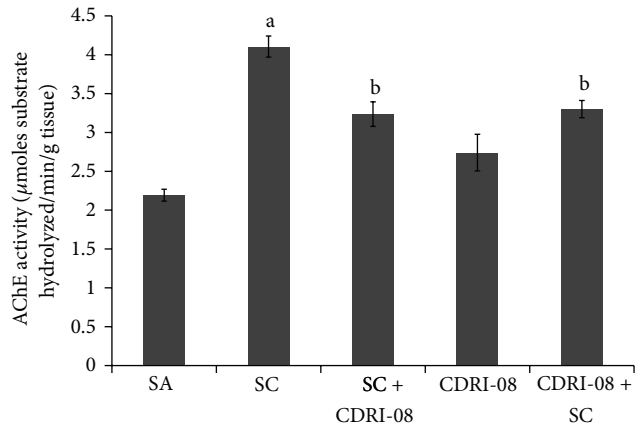

Figure 2: Effect CDRI-08 on AChE activity in the cerebrum of scopolamine (SC) administered amnesic mice. AChE activity plotted as mean AChE activity ± SEM (μmoles substrate hydrolysed/min./g tissue wt.) in three cerebrum samples in each experimental group. a denotes $P \leq 0.05$, significantly different from control (SA) group; b denotes $P \leq 0.05$, significantly different from scopolamine (SC) group.

3.4. Effect of CDRI-08 on the Protein Expression of BDNF, Arc, and GFAP Was Similar to That of mRNA. Scopolamine reduced the expression of 32 kDa BDNF protein (1.6-fold). Similar to mRNA, pretreatment with CDRI-08 extract attenuated the decrease of BDNF protein by 1.5-fold and posttreatment attenuated the decrease by 1.3-fold as compared to scopolamine. Treatment with CDRI-08 extract alone also upregulated the expression of BDNF protein (1.3-fold) (Figure 4(a)). Scopolamine administration significantly downregulated (1.75-fold) the expression of 55 kDa Arc protein in the cerebrum as compared to saline injected mice. Both post- and pretreatment with CDRI-08 augmented Arc protein level in a significant manner (2.8-fold) as compared to scopolamine injected group. The administration of extract alone significantly upregulated (3.3-fold) the expression of Arc protein (Figure 4(b)). Corresponding to neuronal markers, expression of 45 kDa GFAP protein was reduced by 2-fold by scopolamine treatment. Pre- and posttreatment with CDRI-08 extract attenuated the decrease by 1.8- and 1.6-fold, respectively. The extract *per se* did not alter GFAP protein expression (Figure 4(c)).

4. Discussion

B. monniera is recognized as a memory-enhancing agent in the centuries-old Ayurvedic literature and its beneficial effects have been endorsed to the active constituent saponin, as bacosides A, bacoside B, and bacosaponins. However the molecular details are still undefined.

In the present study, we have explored the recovery potential of bacoside-A enriched alcoholic extract CDRI-08 [28] in scopolamine induced amnesic mouse model emphasizing molecular markers of brain plasticity. Our study showed that CDRI-08 extract significantly attenuated scopolamine induced downregulation of neuronal and glial

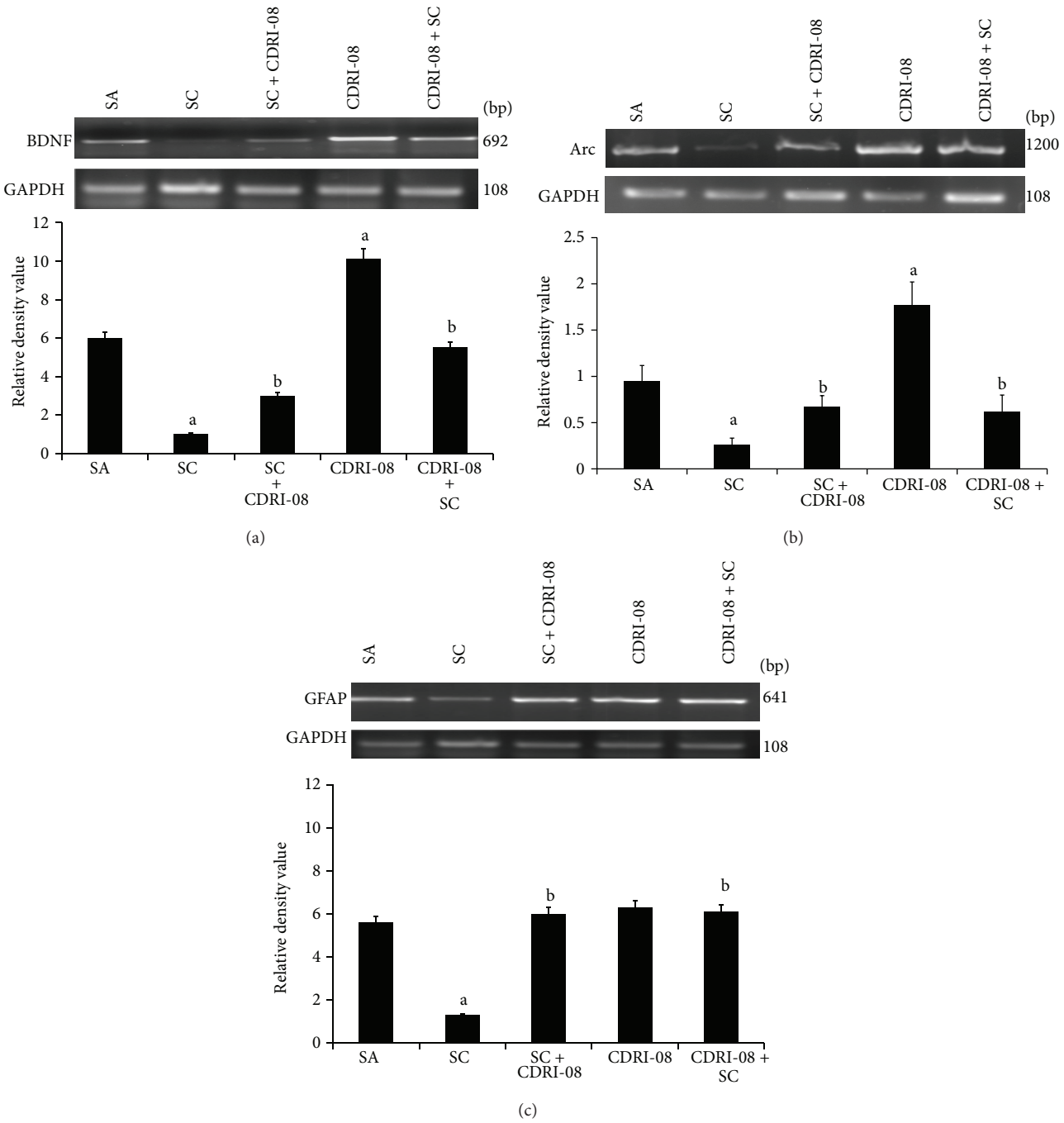

Figure 3: Effect of CDRI-08 on the mRNA expression of (a) BDNF, (b) Arc, and (c) GFAP in the cerebrum of SC administered amnesic mice. The data are presented as a histogram with the mean (±SEM) of three values calculated as relative density values of BDNF/GAPDH, Arc/GAPDH, and GFAP/GAPDH. a denotes $P \leq 0.05$, significantly different from control (SA) group; b denotes $P \leq 0.05$, significantly different from SC group.

plasticity markers and markedly elevated their expression level, indicating enhancement of brain plasticity.

Scopolamine is an acetylcholine (ACh) receptor antagonist blocking cholinergic neurotransmission and causing memory impairment. The concentration of ACh is regulated by a serine hydrolase known as acetyl cholinesterase (AChE), which hydrolyses and inactivates acetylcholine at the synapse [29]. Therefore, we first analyzed the effect of scopolamine on AChE activity and assessed the influence of CDRI-08 treatment in mouse cerebrum. Scopolamine significantly increased the activity of AChE, implying rapid degradation of available ACh and blockade of downstream signaling. Increased AChE levels were also found in the brain of neurodegenerative transgenic mice with impaired memory [30], supporting our result of elevated AChE levels during scopolamine induced amnesia. We observed that CDRI-08

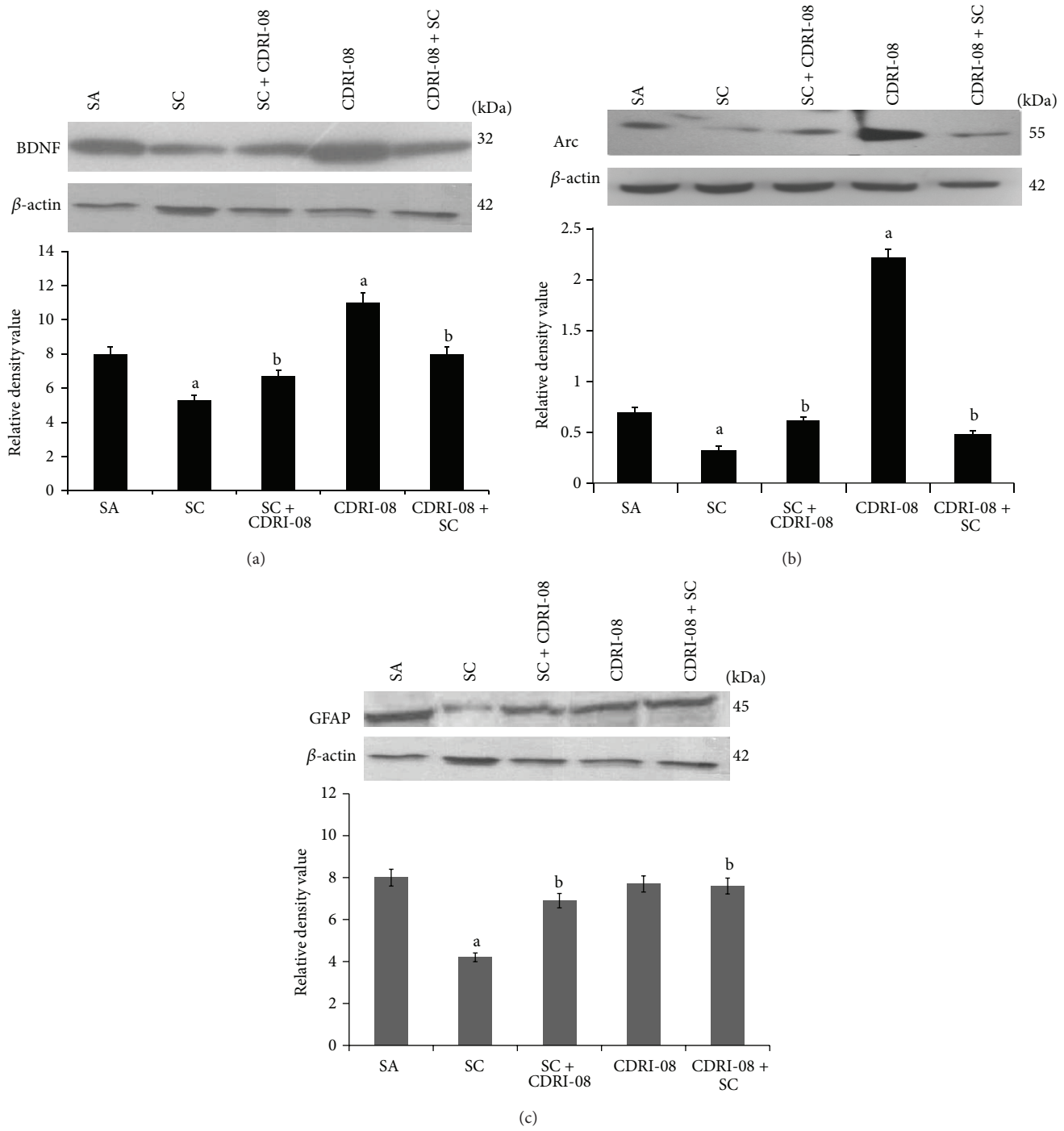

Figure 4: Effect of CDRI-08 on the protein expression of (a) BDNF, (b) Arc, and (c) GFAP in the cerebrum of SC administered amnesic mice. The data are presented as a histogram with the mean (±SEM) of three values calculated as relative density values of BDNF/β-actin, Arc/β-actin, and GFAP/β-actin. a denotes $P \leq 0.05$, significantly different from control (SA) group; b denotes $P \leq 0.05$, significantly different from scopolamine (SC) group.

extract decreased AChE activity when administered orally before or after the scopolamine administration. The anticholinesterase activity of the extract might be attributed to its antiamnesic potential as also reported for other *B. monniera* extracts [31].

However, we did not find any significant effect of the extract *per se* on AChE activity as compared to control. As herbal extracts elicit their action slowly with prolonged effects, it is likely that chronic treatment of Brahmi extract with longer duration might deliver anticipated results.

CDRI-08 extract significantly enhanced the expression of plasticity markers in the cerebrum of scopolamine administered amnesic mice, the effect being more pronounced for neuronal genes BDNF and downstream effector Arc than astrocytic GFAP. CDRI-08 administered alone or in combination with scopolamine upregulated both BDNF mRNA and protein level. It has been established that BDNF is controlled by Ca^{2+} regulated transcription factor CREB that remains bound to BDNF promoter in an inactive form. Phosphorylation of CREB by calcium-regulated kinase cascades stimulates the recruitment of components of the basal transcription machinery to BDNF promoter, and then new BDNF mRNA is synthesized [32, 33]. Earlier reports on *B. monniera* root extract reveal that it influences kinase-CREB pathway to ameliorate memory impairment. Recently, Preethi et al. [34] reported that CDRI-08 extract improves hippocampus-dependent contextual memory with concomitant activation of ERK-CREB signaling cascade.

We also noted a prominent increase in the cerebrum expression of Arc mRNA and protein by CDRI-08 extract during scopolamine induced amnesia. The induction of the IEG, Arc, is strongly implicated in synaptic plasticity [35, 36]. Overexpression of Arc resulted in enhanced AMPA receptor endocytosis and increased LTP and memory consolidation [37]. However, the regulatory mechanisms underlying the activity-dependent transcription of Arc and related neuronal functions remain largely unknown. Recent investigations demonstrate that BDNF-dependent Arc transcription is crucial for neuronal plasticity [38–40]. Therefore, upregulation of BDNF by CDRI-08 might activate Arc transcription and translation during scopolamine induced amnesia.

Interestingly, CDRI-08 extract also attenuated scopolamine mediated decrease in astrocytic plasticity marker, GFAP. However, GFAP expression was unaltered upon *per se* treatment with the extract. GFAP regulates metabolism and activity of the neurons and maintains neuron-glia interactions and plastic rearrangement of the synaptic connections [41]. The excessive increase in GFAP expression resulting in astrogliosis implicates inflammation, increase in reactive oxygen species, and transition to neurodegenerative state [42]. Therefore, our findings suggest the amazing neuroprotective property of CDRI-08 extract which is balanced enough to maintain glial plasticity providing neuronal support but not exceeding the threshold such that it becomes neurotoxic. GFAP gene regulation is also relatively less studied and few reports [43] propose the involvement of transcription factors pCREB and NFκB in regulation of transcription of GFAP. Overall, our data suggest that CDRI-08 extract might activate cholinergic signaling, downstream kinase cascades, and eventually CREB mediated basal transcriptional machinery of memory linked neuronal and glial plasticity markers.

5. Conclusion

Our findings provide the first evidence on molecular basis of recovery potential of CDRI-08 extract in scopolamine induced amnesia in relation to neuronal and glial plasticity markers. Further investigations on detailed pathways and morphological analysis of neurons and glia are warranted to establish the extract as a potential therapeutic target in memory disorders.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Arpita Konar and Akash Gautam have contributed equally in this paper.

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Review Article

Molecular and Functional Characterization of *Bacopa monniera*: A Retrospective Review

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Over the last 50 years, laboratories around the world analyzed the pharmacological effect of *Bacopa monniera* extract in different dimensions, especially as a nerve tonic and memory enhancer. Studies in animal model evidenced that *Bacopa* treatment can attenuate dementia and enhances memory. Further, they demonstrate that *Bacopa* primarily either acts via antioxidant mechanism (i.e., neuroprotection) or alters different neurotransmitters (serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA), acetylcholine (ACh), γ-aminobutyric acid (GABA)) to execute the pharmacological effect. Among them, 5-HT has been shown to fine tune the neural plasticity, which is a substrate for memory formation. This review focuses on the studies which trace the effect of *Bacopa* treatment on serotonergic system and 5-HT mediated key molecular changes that are associated with memory formation.

1. Introduction

Bacopa monniera (L.) Wettst., which belongs to the family Scrophulariaceae, is an annual creeping plant found in wet, damp, and marshy areas. The leaves and stem of the plant are used for medicinal purposes traditionally [1]. In the ancient Indian system of medicine, namely, Ayurveda, *B. monniera* known as "Bhrami" has been classified under Medhya Rasayana and described in ancient ayurvedic medical encyclopedias, namely, *Charaka Samhita*, *Sushrutha Samhita,* and *Astanga Hrdaya,* as cure for mental disorders and loss of intellect and memory. It has been tested in different animal models to understand its effect on memory [2, 3] and antiamnesic activity [4–9]. These pharmacological properties lead to clinical trial of *B. monniera* extract in elderly persons to improve cognitive performance and memory [10–15]. In parallel, *Bacopa* is a main constituent in the preparation of ayurvedic medicine prescribed for cognitive dysfunction. In addition, several research groups and pharmaceutical companies formulated *Bacopa* for clinical use in different countries including India, New Zealand, Australia, and United States of America. Earlier, many reviews have discussed pharmacological property of *B. monniera* in a broad perspective; however, no comprehensive article has yet shown its effect on molecular level. In this review, we summarize the *in vivo* experiments that suggest that *B. monniera* treatment enhances cognitive function by altering the molecular targets through serotonergic system.

2. Bioactive Compounds in *B. monniera* Leaf Extract

Series of biochemical studies identified different pharmacological compounds from ethanolic extracts of *Bacopa*, which include alkaloids (brahmine, nicotine, and herpestine), saponins (monnierin, hersaponin), sterols (b-sitosterol, stigma-sterol), d-mannitol, acid A, and betulinic acid [16–18]. The principal constituents of *B. monniera* are triterpene saponins of the dammarane class, which have been named bacosides and bacopasaponins. There are two types of

saponins, jujubogenin and pseudojujubogenin, which differ only in the nature of the sugar units in the glycosidic chain and the position of the olefinic side chain in the aglycone. These saponins are complex mixture of closely related structures, namely, bacosides A_1 [19] and A_3 [20] and bacopasaponins A–G [21–23]. Two new dammarane-type jujubogenin bisdesmosides, bacopasaponins E and F [24], pseudojujubogenin glycosides, bacopasides I and II [25], phenylethanoid glycosides, namely, monnierasides I–III with the known analogue plantainoside B [26], and bacopasides III, IV, and V [27] have also been identified. The major chemical entity shown responsible for neuropharmacological effects of *B. monniera* is bacoside A (64.28%) and bacoside B (27.11%); the latter differs only in optical rotation. The bacoside A (bacogenins A1, A2, A3, and A4) derives from two triterpenoid saponins: pseudojujubogenin and jujubogenin on acid hydrolysis [16–18, 28]. All these bacogenins (especially A4) are rich in the standardized extract of *Bacopa* which is termed as bacosides-enriched standardized extract of *Bacopa* (BESEB CDRI-08) that contains 55 ± 5% bacosides (Lumen Marketing Company, Chennai, India), and BESEB CDRI-08 is mentioned as BME in this paper.

3. Neuropharmacological Activity of BME

3.1. Learning and Memory. Bacopa treatment has been reported to improve behavior of different laboratory animal models under variety of experimental conditions. Oral administration of BME improved spatial learning of rats and mice in Morris water maze [4, 5, 29–31]. Interestingly, several other studies demonstrated that it also improved spatial working memory in different mazes like plus maze [32, 33], Y-maze [34, 35], radial arm maze [34, 36], Barnes maze [36], T-maze [37], Hole board [35], and modified Y maze [38]. In addition, it also improved negative reinforcement (foot-shock motivated brightness discrimination task, conditioned avoidance response) and positive reinforcement (conditioned taste aversion) based memory [2, 39]. Similarly, in passive avoidance task and fear conditioning task *Bacopa* treatment increased the transfer latency and freezing response [33, 35, 37, 38, 40–42], whereas, in contextual cues associated with odor, BME treated rats showed less latency to retrieve the reward [43] and exhibited improved discrimination of novel object [38, 44, 45]. In addition, it has been stated that *Bacopa* treatment induced dendritic arborization of neurons in hippocampal and basolateral amygdala [46, 47], which possibly enhanced neural plasticity.

4. *B. monniera* Extract Treatment Ameliorates Chemicals Induced Dementia

Interestingly, several studies investigated the pharmacological effect of BME against different chemical compounds that induce anterograde/retrograde amnesia by targeting different neuronal system. These studies reported that BME effectively attenuated anterograde/retrograde amnesia induced by chemical compounds such as scopolamine, an acetylcholine receptor antagonist [2, 6, 7, 22, 36, 40, 48, 49], diazepam, a positive allosteric modulators of γ-aminobutyric acid (GABA) type A receptor [4], N_ω-nitro-l-arginine (L-NNA), a nitric oxide synthase inhibitor [8, 9], BN52021, a receptor antagonist for platelet activating factor [48], and sodium nitrite, a anticholinergic drug [48]. In addition, memory impairments caused by Okadaic acid, a selective inhibitor of protein phosphatase [31], aluminium-chloride which causes oxidative damage [50], autistic symptoms induced by sodium valproate, a weak blocker of sodium ion channels, and inhibitor of GABA transaminase [51] were also ameliorated by *Bacopa* treatment.

5. Uptake of Bacosides

We have learned from pioneering works about different active compounds in *B. monniera* extract [16–18]. As a first step to validate the effect of BME on the reported behavioral improvements, Charles et al. [35] confirmed that orally treated BME was uptaken into the system. HPLC analysis showed the presence of bioactive compound bacoside A in the serum of BME treated rats. The bioactive compounds in the BME could directly or indirectly interact with neurotransmitter systems to enhance learning and memory. Since the bacosides present in the BME are nonpolar glycosides [25–27], they can cross the blood-brain barrier (BBB) by simple lipid-mediated passive diffusion [52], and its bioavailability in brain has been confirmed by the biodistribution of radiopharmaceuticals [53] effectively activating the cascade which participates in the memory enhancing mechanism.

6. Activation of Neurotransmitter Systems by Bacoside

The balanced functions of various neurotransmitters such as acetylcholine (ACh) [2, 40], serotonin (5-hydroxytryptamine, 5-HT) [2, 54], catecholamine [55], γ-aminobutyric acid (GABA) [56], and glutamate (Glu) [8] were all altered by BME treatment. It has been reported that the BME treatment increased the 5-HT level in the hippocampus, hypothalamus, and cerebral cortex [54], and also modified the ACh concentration directly/indirectly through other neurotransmitter systems. As a first step, Rajan et al. [41] estimated the level of neurotransmitters to understand the effect of BME treatment. They found that BME treatment during postnatal period significantly upregulated the level of 5-HT, ACh, GABA, and Glu. In contrast, it reduced the level of dopamine (DA). Notably, the reported inhibitory effects of cholinesterase activity of BME may possibly increase the level of ACh and enhance memory [33, 40]. On the other hand, 5-HT receptors present in the GABAergic neuron [57] may activate the GABAergic neurons [58, 59], which enhances the release of GABA. In fact, increased GABA level in hippocampus could activate the inhibitory GABA receptors on cholinergic system that leads to inhibition of ACh release [60, 61], but 5-HT receptors may directly act on the cholinergic system and increase release of Ach [62]. These proceedings and the observed trend in the 5-HT level have drawn the attention to analyse the effect of BME on 5-HT system. Further, studies were designed to test the pathway associated with 5-HT system (Figure 1). Observed effect of BME on neurotransmitter systems and

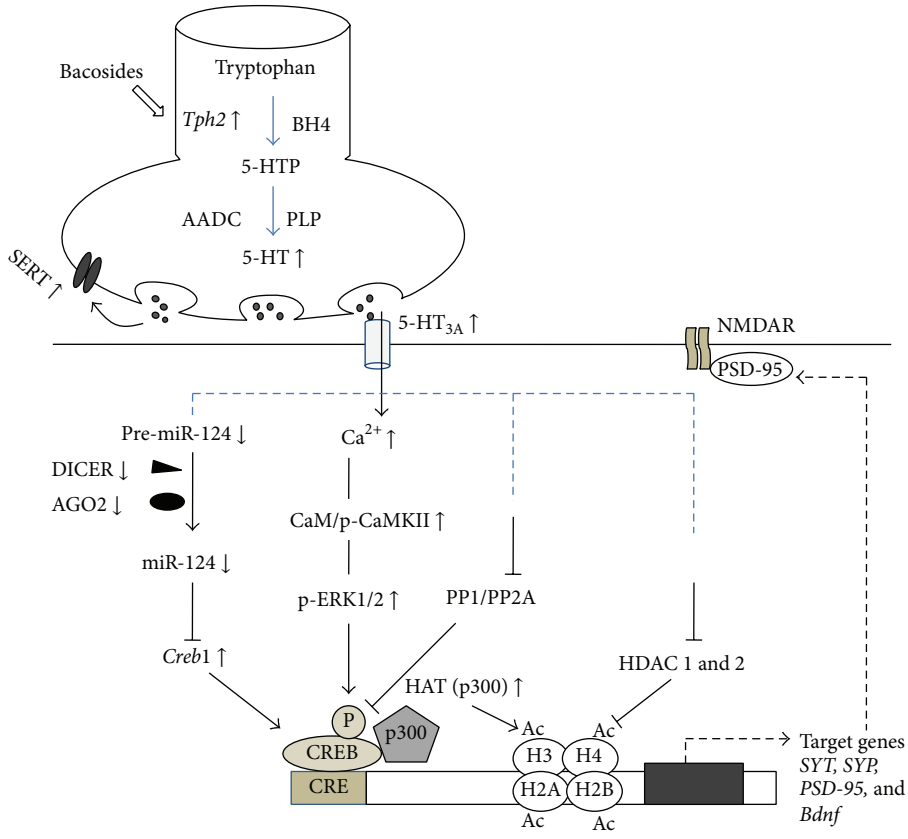

FIGURE 1: Diagram showing the possible mechanism of serotonin mediated signaling pathway activated by BME during learning. (↑: increase; ↓: decrease).

the molecules involved in the signaling pathway are shown in Table 1.

7. BME Treatment Regulates the Synthesis of Serotonin

Earlier studies demonstrated that increasing level of tryptophan hydroxylase (TPH) mRNA expression elevated TPH activity and 5-HT metabolism, which profoundly could influence the synaptic 5-HT activity [63, 64]. Further, serotonin transporter (SERT) is known to critically uptake the 5-HT by transport across presynaptic membrane [65]. The upregulated level of 5-HT by BME raises the question, does it alter the level of TPH2 and SERT? Interestingly, Charles et al. [35] showed that TPH2, SERT mRNA expression was upregulated and the level persisted even a week after the BME treatment [35]. The upregulated SERT expression could regulate the reuptake of released 5-HT and control the duration and intensity of serotonergic activity at the synapse. This could be one of the mechanisms that enhance the learning and memory processing and it fits well into established concept in different models [66, 67]. In addition to these studies, *in silico* analysis suggested that interaction of bacosides (A, A_3) with TPH2 possibly alters the activity of TPH2 that could be one of the mechanisms for increased 5-HT synthesis [68].

8. Activation of 5-HT Receptor by BME Treatment

Previously, it has been found that synaptically released 5-HT exerts its function through their diverse receptors [69]. Activated receptors either positively or negatively regulate the downstream signaling cascade that is involved in regulation of synaptic plasticity [70–72]. In view of these reports, expression of 5-HT receptors (5-HT_{1A}, 5-HT_{2A}, 5-HT_4, 5-HT_{5A}, 5-HT_6, and 5-HT_7) after BME treatment was examined. Notably, 5-HT_{3A} receptor expression was increased compared to all other receptors. It is the only metabotropic receptor, and its expression could be stimulated by endogenous 5-HT which may facilitate the hippocampal-dependent task [73, 74]. Hence, the role of 5-HT_{3A} in hippocampal-dependent learning could be tested by using 5-HT_3 antagonist 1-(*m*-chlorophenyl)-biguanide (*m*CPBG), which effectively impairs the retention of the conditioned response [75] in both short- and long-term memories [76]. The 5-HT_3 antagonist *m*CPBG has facilitated gaining insight into the BME induced 5-HT_{3A} receptor mediated role in

Table 1: Summary of *Bacopa monniera* treatment effects on serotonergic system and its associated pathway.

Neurotransmitters	Effects	Genes (mRNA)	Effects	Genes (Protein)	Effects	References
Serotonin	↑	*Tph2*	↑			[35]
		SERT	↑			
Serotonin	↑	*5-HT1$_A$*	—			[41]
Dopamine	↓	*5-HT2$_A$*	↑			
Acetylcholine	↑	*5-HT3$_A$*	↑			
GABA	↑	*5-HT4*	—			
Glutamate	↑	*5-HT5*	—			
		5-HT6	—			
		5-HT7	↓			
		Nrf2	↑	SYP	↑	[43]
				SYT	↑	
				t-αCaMKII	↑	
				p-αCaMKII	↑	
				PSD-95	↑	
		Dicer	↓	DICER	↓	[39]
		Ago2	↓	AGO2	↓	
		miR-124	↓	t-CREB1/2	↑	
		Creb1	↑	p-CREB1/2	↑	
		Bdnf	↑	t-ERK1/2	↑	[42]
		PP1α	↓	p-ERK1/2	↑	
				t-CREB1/2	↑	
				p-CREB1/2	↑	
				Ac-H3	↑	
				Ac-H4	↑	
				HDAC1	↓	
				HDAC2	↓	
				p300	↑	
				PP2A	↓	

↓: decrease; ↑: increase.

hippocampal-dependent learning and its regulation of other neurotransmitters. Interestingly, treatment of BME ameliorated the antagonistic effect of *m*CPBG. The combination of *m*CPBG and BME treatment recorded improvement in behavioural task accompanying the upregulation of 5-HT$_{3A}$ receptor. Considering the interaction of multiple neurotransmitters involved in learning and memory network [77–80], it could be interesting to know the interaction of 5-HT$_3$ receptor in activation/inhibition of other neurotransmitter systems.

The upregulated 5-HT$_{3A}$ receptor might regulate serotonergic system and may interact with other neurotransmitters that are involved in learning and memory [58, 67, 81]. It should be noted that 5-HT$_{3A}$ is a heteroreceptor; its stimulation by means of *m*CPBG has been reported to enhance GABA and DA levels and inhibit the release of ACh [74]. The activation of 5-HT$_3$ receptors in dopaminergic neuron could facilitate the release of DA [82, 83], and *m*CPBG inhibits dopamine uptake by binding with dopamine transporter [84], thereby increasing the synaptic dopamine level. On the other hand, the anticholinesterase activity of BME [40] and other regulatory mechanisms of BME are also involved in the regulation of ACh level and memory enhancement [33, 85].

A noteworthy point is that it did not alter the level of Glu. This suggests that glutamate neurons in the hippocampus may not colocalize with 5-HT$_{3A}$ receptor [59]. The observed changes are indication of the facilitatory effect of BME on long-term and intermediate forms of memory through 5-HT$_{3A}$ receptor.

9. Activation of Protein Kinases-CREB Pathway

A pioneering study in 1976 described that serotonin stimulation increases the level of cyclic adenosine monophosphate (cAMP) by the adenyl cyclase in the neuronal cells [86]. Subsequent study by Castellucci et al. [87] established that activation of cAMP mediates downstream signaling process through phosphorylating proteins, namely, cAMP-dependent protein kinase or protein kinase A (PKA). Upon activation, cAMP-dependent PKA dissociates into regulatory and catalytic subunits. The catalytic subunit of

PKA drives to activate mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) [88, 89]. It has been shown that activation of protein kinases (MAPK/ERK) can induce the phosphorylation of the key transcription factor CREB, which is a positive regulator of memory consolidation [90–93]. These proceedings triggered us to test whether the BME treatment induced activation of 5-HT_{3A} receptor regulated synaptic plasticity through protein kinase and cAMP response element binding (CREB) protein signaling pathway. It is noteworthy to mention that treatment of BME increased the phosphorylation of ERK1/2 and provides a physiological and functional meaning for the observed different forms of memory [42]. If the p-ERK activity is decreased/increased, one would expect concomitant changes in the CREB and CREB targeted gene expression and functional consequences [94–97]. It should be noted that the induction of p-CREB1 is involved in the regulation of synaptic proteins synthesis, which are known to be involved in synaptic plasticity related events in hippocampus [98] and their synthesis is necessary for the consolidation of long-term memory (LTM) [99–102]. Preethi et al. [39] found that level of both total and phosphorylated CREB protein was increased in the BME treated individuals. When BME treated before *m*-CPBG treatment, the *m*CPBG mediated suppression of CREB phosphorylation was attenuated by BME, thus adding additional support to the effect of BME in regulation of PKA-CREB pathway.

10. Activation of CREB Regulation through MicroRNA-124 by BME

Long-term memory formation requires synthesis of new proteins [103, 104], which is regulated by mRNA transport and translation [105]. At this point, several studies proposed that microRNAs (miRNAs) are one of the factors that regulate expression of gene [106, 107] which could be regulated by level of miRNA/biosynthesis of miRNA. There are two molecules, Dicer and Ago2, involved in the regulation of miRNA biosynthesis [108]. It is noteworthy to mention that there is an interaction between miR-124 and 5-HT, because the stimulation of the latter has been shown to downregulate the expression of miR-124 during 5-HT-induced synaptic facilitation [109]. Thus, we thought that BME treatment might alter the level of miR-124 expression and the molecules involved in its biosynthesis pathway. Subsequently, we found that BME treatment reduced the level of Dicer, Ago2 mRNA, and protein [39]. Reduction in Dicer has been known to enhance synaptic plasticity [110]; the formation of miRNA-induced silencing complex (miRISC) requires the activation of Ago2 [111]. Further, this study revealed that reduction of Dicer and Ago2 directly downregulated miR-124 level in BME treated individuals. Conversely, inhibition of 5-HT activity by treating with *m*CPBG showed upregulated Dicer, Ago2, and miR-124 [39]. It has been postulated that the downregulation of miR-124 would lead to the upregulation of CREB [109]. Though it is well established that 5-HT can upregulate *Creb1* mRNA level [112], recent studies claimed that miR-124 might directly bind to *Creb1* 3′UTR and regulates the expression of CREB [109, 113]. Indeed, upregulated CREB reciprocally regulates the miRNA [109, 114]. This in turn regulates the activation of immediate early genes that ultimately facilitates synaptic plasticity [115–118]. These cellular events demonstrate that BME possibly regulates the transcriptional regulators to fine tune transcription factors.

11. Phosphorylation of CREB Regulated by BME Treatment

Contrary to the protein kinases, protein phosphatases (PPs) act as dephosphorylating enzymes that dephosphorylate the molecules like CREB [119]. PPs critically regulate the phosphorylation events that favor forgetting [120], cognitive decline in ageing [121, 122], and suppress learning and memory. In brain, several PPs are known to be expressed. Among them, Ser/Thr phosphatases (PP1, PP2) are the most likely candidates that negatively act on the phosphorylation of CREB [123–125] and thereby downregulate the transcription of CREB targeted genes [120, 126, 127]. BME treatment significantly reduced the PP1α and PP2A level in hippocampus, which appears to be responsible for observed BME mediated enhanced memory [42]. This study revealed the contribution of BME in regulation of CREB phosphorylation that favors the transcription of CREB targeted genes to memory formation. Moreover, it supported the earlier reports which showed inhibition of PPs to enhance memory formation [120, 124, 125, 128–130], but the exact mechanism that inhibits PPs is not yet studied.

12. Chromatin Modifications Differentially Regulated by BME Treatment

Studies in memory highlighted chromatin alteration and epigenetic changes that are associated with CREB activation. Contribution of histone tail acetylation and deacetylation in chromatin are widely known to be involved in the formation of long-term memory and synaptic changes [131–133]. Histone deacetylase (HDAC) inhibitors are known to induce acetylation of histones (H3, H4). It has been reported that HDAC inhibitors repress the HDAC-PP1 complex and thus block dephosphorylation of CREB [134, 135]. On the other hand, *in vitro* and *in vivo* studies claimed that transcriptional induction of CREB occurred by pSer133, which requires histone acetylase (HAT)—CREB binding protein (CBP/p300) [136, 137]. P300 contains intrinsic HAT activity and it has been shown to interact with CREB [138–140]. Manipulation in p300 leads to reduction in the histone acetylation and impairs hippocampus dependent memory [141–143]. These reports prompted us to examine the potential role of BME in chromatin modifications especially with histone acetylation and deacetylation.

An earlier study reported significant enhancement of p300 level in hippocampus of BME treated groups, but not in control groups after training [42]. These reports suggest that BME plays an agonistic role for p300 in hippocampus; further it may acetylate H3 and H4 histones [144–146]. Accordingly, we found that BME treatment induced marked increment in the level of Ac-H3 and Ac-H4 in hippocampus [42]. These results agree with the earlier studies, in which HDAC

inhibitors have been found to induce acetylation of histones (H3, H4) and improve memory [147–150]. In addition, the level of HDAC 1 and HDAC 2 in the hippocampus of BME treated group was decreased compared to control group. The reduction in HDAC 1 and HDAC 2 levels together with increased acetylation of histones in BME groups added additional evidence to the mechanism of BME [42].

13. BME Treatment Activates the Synaptic Proteins to Induce Synaptic Plasticity

Behavioural response to the stimuli is basic functional circuit formation between the neuronal cells. The molecular mechanism underlying the circuit (synaptic plasticity) is likely to provide insight to role of molecules/molecular complexes. The communications between the neuronal cells are initiated by the recruitment of adhesion molecules in pre-post synaptic neurons [151, 152]. Synaptic plasticity depends on activity strength, which leads to release of neurotransmitters to the synaptic cleft. However, the release of neurotransmitters is critically regulated by synaptic proteins synaptotagmin-I (SYT-1) and synaptophysin (SYP). SYT-1 is sensitive to Ca^{2+} and conserved at least in vertebrates [153]. This synaptic vesicle protein is exclusively involved in synaptic vesicle docking and regulating release of neurotransmitter [154]. Another key synaptic protein SYP is playing important role in regulation of synaptic vesicle association by protein-protein interactions [153]. It is a vesicle-associated regulatory protein which is involved in plasticity related changes in the hippocampus [155, 156]. The levels of SYT-1 and SYP were upregulated after BME treatment which possibly established the synaptic communication and synaptic function [43]. BME treatment upregulated the synaptic proteins (SYT-1, SYP), which is possibly by the elevated level of 5-HT. The level of signaling components is essential for neurotransmission and synaptic plasticity. The upregulated synaptic proteins could enhance neurotransmission and synaptic plasticity. However, this should be transferred to postsynaptic neurons. There are two key postsynaptic proteins (post synaptic density protein 95 (PSD-95) and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII)) distributed densely. Acute phosphorylation and localization of PSD-95 and CaMKII is fundamental to synaptic function [157]. They are critical for long-term potentiation (LTP) and information storage [158, 159]. Translocation of CaMKII to postsynaptic region by autophosphorylation is necessary for early phase of memory formation, where it controls the phosphorylation of different postsynaptic proteins [160]. The induction and phosphorylation of CaMKII depends on the release of 5-HT [161]. Genetic manipulation and pharmacological studies pointed out the critical role of CaMKII in synaptic plasticity and memory formation [161, 162]. BME treatment upregulated the induction and phosphorylation of CaMKII; it could be by the level of 5-HT, thus the improved memory recorded. PSD-95 is a core component in the architecture of synapses [163, 164] involved in localization of receptors, clustering of synaptic signaling proteins, and synapse stabilisation [164–166]. The level of PSD-95 increases at synapses during learning/learning-induced plasticity [167, 168]. Earlier, we demonstrated that PSD-95 was upregulated after BME treatment [43]; upregulated PSD-95 may increase the interaction between PSD proteins and enhances synaptic transmission [169–172]. These results suggest that BME treatment activates the synaptic proteins; thus neurotransmission and synaptic plasticity are enhanced between the neurons.

14. Conclusion

Taken together, bacosides present in the *Bacopa* extract has been known to improve cognitive function by modulating different neurotransmitters. However, this review focused on the studies which provide much attention to the serotonergic system, in which, starting from *in silico* approach to alternation in 5-HT levels, their receptors and associated signaling cascades known to be involved in synaptic plasticity and memory enhancement were discussed. These studies provide molecular evidence to possible mechanism of BME on serotonergic system and its associated pathway.

Abbreviations

5-HT:	5-Hydroxytryptamine
ACh:	Acetylcholine
BESEB CDRI-08:	Bacosides-enriched standardized extract of *Bacopa*
CaMKII:	Ca^{2+}/calmodulin dependent protein kinase II
cAMP:	Cyclic adenosine monophosphate
CBP:	CREB binding protein
CREB:	Cyclic adenosine monophosphate (cAMP) response element binding
DA:	Dopamine
ERK1/2:	Extracellular signal-regulated kinase
GABA:	γ-Amino butyric acid
Glu:	Glutamate
HAT:	Histone acetylase
HDAC:	Histone deacetylase
L-NNA:	N_ω-nitro-l-arginine
LTM:	Long-term memory
LTP:	Long-term potentiation
MAPK:	Mitogen activated protein kinase
*m*CPBG:	1-(*m*-Chlorophenyl)-biguanide
miRISC:	miRNA-induced silencing complex
miRNAs:	MicroRNAs
PKA:	Protein kinase A
PPs:	Protein phosphatases
PSD-95:	Postsynaptic density protein 95
SERT:	Serotonin transporter
SYP:	Synaptophysin
SYT1:	Synaptotagmin I
TPH:	Tryptophan hydroxylase.

Conflict of Interests

The authors have declared that no conflict of interests exists.

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Research Article

A Special Extract of *Bacopa monnieri* (CDRI-08) Restores Learning and Memory by Upregulating Expression of the NMDA Receptor Subunit GluN2B in the Brain of Scopolamine-Induced Amnesic Mice

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In the present communication, we have investigated effects of the CDRI-08, a well characterized extract of *Bacopa monnieri*, on expression of the GluN2B subunit of NMDAR in various brain regions of the scopolamine-induced amnesic mice. Our behavioral data reveal that scopolamine-treated amnesic mice exhibit significant decline in the spatial memory compared to the normal control mice. Our RT-PCR and immunoblotting data revealed that the scopolamine treatment resulted in a significant downregulation of the NMDAR GluN2B subunit expression in prefrontal cortex and hippocampus. Our enzyme assay data revealed that scopolamine caused a significant increase in the acetylcholinesterase activity in both the brain regions. Further, oral administration of the CDRI-08 to scopolamine-treated amnesic mice restored the spatial memory which was found to be associated with significant upregulation of the GluN2B subunit expression and decline in the acetylcholinesterase activity in prefrontal cortex as well as hippocampus towards their levels in the normal control mice. Our study provides the evidence for the mechanism underlying role of the *Bacopa monnieri* extract (CDRI-08) in restoring spatial memory in amnesic mice, which may have therapeutic implications.

1. Introduction

Amnesia is characterized by deficit in memory caused by either brain damage, neurological disorders, psychological trauma [1], use of sedative/hypnotic drugs [2], or alcohols [3] due to alterations in the excitatory glutamatergic synaptic strength, which is dependent on the activation of the ionotropic glutamate receptors, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) or NMDA (N-methyl-D-aspartic acid) receptors (AMPAR or NMDAR), and metabotropic glutamate receptors (mGluRs) [4]. Studies on the NMDAR mutant mice have shown that activity dependent hippocampal CA1 synaptic plasticity is abrogated due to the absence of NMDAR [5]. Large body of evidences suggests that expression of calcium/calmodulin-dependent protein kinase II (CaMKII), brain-derived neurotrophic factor (BDNF), and calcineurin is differentially altered in the hippocampus, basolateral amygdala (BLA), and medial prefrontal cortex (mPFC) stress-induced amnesia [6]. However, information on alterations in the expression of NMDA receptor or its particular subunit in the drug-induced amnesic animal model is less studied.

N-Methyl-D-aspartate (NMDA) receptors, a heterotetrameric structure, consist of two GluN1 subunits and two additional GluN2 or GluN3 subunits which together confer the functionality to the receptor. Each subunit possesses the N-terminal domain containing binding sites for allosteric regulators such as Zn^{2+}, the agonist binding domain for glycine/D-serine (GluN1) and glutamate (GluN2) where competitive antagonists bind [4]. GluN1 has eight splice variants whereas GluN2 subunit consists of four splice variants (NR2A-D). GluN1 subunit is an obligatory component of

the NMDA receptors whereas variation takes place at the level of GluN2 subunit types. The GluN2 subunit type present in NMDA receptor complex critically determines its biophysical, pharmacological, and physiological properties including sensitivity to Zn^{2+}, H^{+} and polyamines, single channel conductance, and interactions with intracellular signaling molecules [7, 8]. GluN2A and GluN2B are associated with higher brain functions [9, 10] and they are predominantly expressed in hippocampus and cortex [4, 11]. Recent evidence indicates that NMDA receptor activity is correlated with learning, memory, and cognition by modulating dendritic spine density, synaptic plasticity, and synaptic strength [12, 13]. Also, GluN2A and GluN2B subunits of NMDARs are implicated in the development of LTP in the hippocampus-dependent spatial and fear memory, and they have been correlated with overexpression of GluN2B subunit in order to enhance the above memory forms in adult mice [14]. Recent studies have implicated possible role of NMDA receptors in various neurological disorders like epilepsy, Alzheimer disease, and Huntington chorea and mild cognitive impairment (MCI) [7, 15]. Pharmacological and knockout studies have demonstrated that mice lacking GluN2A/B subunits exhibit impaired LTP and thereby deficient spatial memory [16]. GluN2B subunit of the NMDA receptor has been shown to be associated with altered synaptic plasticity in Parkinson's disease [17, 18]. Therefore, investigation on alterations in the expression of the GluN2B in the amnesic mice may provide an important support to involvement of NMDAR in learning and memory and which may also serve as a measure to evaluate the molecular mechanism of effects of herbal neuromodulator drugs such as *Bacopa* extract. Therefore, we have analyzed alterations in the expression of the NMDA receptor subunit GluN2B in prefrontal cortex and hippocampus of the scopolamine-treated amnesic mice [19, 20] and investigated the potential effects of *Bacopa monnieri* extract on its alterations during experimental amnesia.

Bacopa monnieri is a traditional herbal plant and its extract has been used in Indian medicine system since ancient period as a nerve tonic for the treatment of varieties of neurological diseases and memory related disorders [21, 22]. CDRI-08 is a well characterized extract of *Bacopa monnieri* and it contains several active phytochemical constituents such as bacosides A and B, alkaloids, and saponins. Bacosides A and B [23–25] have been used in the treatment of neurological disorders like insomnia, depression, anxiety, psychosis, and stress [26–28]. Several studies have demonstrated their (bacosides A and B) antiamnesic, antiepileptic, neuroprotective, and memory enhancing effects [21, 29–31]. The number of studies carried out on the mechanisms of antiamnesic action of *Bacopa monnieri* extract indicates that it improves the working memory and cognition in elderly human subjects by reducing plasma acetylcholinesterase activity (AChE) [32]. Evidence from studies on the effects of *Bacopa monnieri* extract on the scopolamine-induced amnesic mice suggests that it reverses the state of amnesia by significantly improving calmodulin level and by partially attenuating the protein kinase C and pCREB activities [33]. However, literatures on the effects of the *Bacopa monnieri* extract particularly the CDRI-8 on alterations in the AChE activity and its possible correlation with expression of the NMDA receptor subunit GluN2B especially in various brain regions in animal models of experimental amnesia are lacking.

To investigate above, amnesic mice model was developed by intraperitoneal injection of scopolamine (2 mg/kg BW), examined the mice for their spatial memory impairment by eight-arm radial maze test and studied its possible correlations with altered expression of NMDAR GluN2B subunit in the prefrontal cortex and hippocampus using Western blotting and semiquantitative RT-PCR techniques. Further, to examine the neuroprotective effects of CDRI-08 via NMDA receptor, the amnesic mice were treated with standardized dose of CDRI-08 compared with vehicle-treated normal and CDRI-08-treated control mice, separately. Since, scopolamine is a known nonselective muscarinic acetylcholine receptor antagonist, which in turn blocks the effects of acetylcholine, impairs LTP, and induces amnesia in mammals [34], and it has been widely used to induce amnesia in animal models, we also examined alterations in the activity of acetylcholinesterase to validate the scopolamine's anticholinergic effects which might lead to accumulation of the synaptic acetylcholine content and thereby increase in the activity of acetylcholinesterase. Here, we report that scopolamine-induced amnesia is associated with decline in the expression of NMDA receptor GluN2B subunit in both the brain regions and the CDRI-08 reverses the memory loss by upregulating its expression close to the vehicle-treated normal control mice.

2. Materials and Methods

2.1. Animals. Male Swiss strain albino mice of 20 ± 2 weeks were used throughout the experiment and they were maintained in the animal house at 24 ± 2°C with 12 hr light/dark cycle and fed with standard mice feed and water *ad libitum*. Mice were used as per norms set by animal ethical clearance committee of Banaras Hindu University.

2.2. Chemicals, Drugs, and Antibodies. Chemicals used in experiments were of molecular biology grades and were purchased from Sigma, USA, or Merck, India. The specialized extract of *Bacopa monnieri* extract containing bacosides A and B (CDRI-08) was obtained from Mr. S. Selvam, Lumen Research Foundation, Chennai, India, as a gift and was suspended in Tween 80 (0.5% v/v). Scopolamine was purchased from Sigma-Aldrich, New Delhi, and was dissolved in normal saline. Drugs solutions were freshly prepared at the time of use. Anti-GluN2B primary antibody was obtained from Antibodies Incorporated (Neuromab, UC Davis, USA) and HRP-conjugated secondary antibody raised in goat against anti-mouse primary antibodies was purchased from Genie, Bangalore, India.

2.3. Animal Groups and Drug Treatment Schedule for Acquisition Study. Mice were divided into four groups as shown in Table 1. Each group comprised of 7 mice. *Group I* (control): mice in this group received oral administration of 0.5%

Table 1: Schedule for the vehicle/drug treatment and training on Radial Arm Maze paradigm.

Experimental Set	Group ($N = 7$)	Treatment Schedule		
		Day 1–7	Day 8–15	Day 16–21
Control	I	0.5% Tween 80 (150 min before) + NS (120 min before)		
B. monnieri extract (CDRI-08)	II	0.5% Tween 80 (150 min before) + NS (120 min before)	*B. monnieri* extract (200 mg/Kg) in 0.5% Tween 80 (150 min before) + NS (120 min before)	
Scopolamine	III	0.5% Tween 80 (150 min before) + NS (120 min before)		Scopolamine (2 mg/Kg) in NS (120 min before) + 0.5% Tween 80 (150 min before)
Scopolamine + *B. monnieri* extract (CDRI-08)	IV	0.5% Tween 80 (150 min before) + Scopolamine (2 mg/Kg) in NS (120 min before)	*B. monnieri* extract (200 mg/Kg) in 0.5% Tween 80 (150 min before) + NS (120 min before)	

Note: NS-Normal saline; before-duration of time before behavioral recording.

Tween 80 in normal saline medium followed by intraperitoneal injection of normal saline and were subjected to radial arm maze test daily for three weeks; *Group II* (*Bacopa monnieri* extract (CDRI-08) treated): mice in this group were treated daily for one week period as in the control group and subjected to radial arm maze tests. They were further treated by oral administration of CDRI-8 (200 mg/Kg BW) in 0.5% Tween 80 diluted with normal saline as a medium for two weeks and subjected to radial arm maze test daily two hours after the treatment. This group was prepared to study the effects of CDRI-08 on the normal control mice; *Group III* (scopolamine-treated): mice in this group were treated, with normal saline and 0.5% Tween 80, as in the control group and subjected to radial arm maze test daily for two weeks. Thereafter, these mice were treated with intraperitoneal injection of scopolamine (2 mg/Kg BW in normal saline) followed by oral treatment of 0.5% Tween 80 diluted with normal saline for the third week. These mice were subjected to radial arm maze test daily two hrs after the treatments; *Group IV* (scopolamine and CDRI-08-treated): mice in this group were treated orally with 0.5% Tween 80 and with intraperitoneal injection of scopolamine as in Group III daily for one week. Thereafter, these mice were treated orally with CDRI-08 (200 mg/Kg BW) daily as in Group II for two weeks and each mouse in the group was subjected to radial arm maze test two hrs after treatments. The time gap of two hrs between drug treatment and the radial arm maze was chosen to avoid any possible alterations in the motor activities of mice compared to 30–90 min time gaps reported in literatures wherein researchers have used 0.5–1.0 mg scopolamine/Kg BW. Thus mice belonging to all four groups were subjected to three weeks of behavioral test on the eight-arm radial maze paradigm equally.

The final tests were performed on the 22nd day as has been described in Section 3.

3. Methodology

3.1. Eight-Arm Radial Maze Test. Each behavioral test session for studying acquisition and memory after the vehicle or drug treatment was conducted in standard eight-arm radial maze (RAM) equipment consisting of a central platform of a 25 cm diameter with eight arms of 70 cm (length) × 10 cm (width) × 15 cm (height) each, radiating at equal angle from the central platform. The maze was placed at a fixed position to reduce the variability of each test. In the present study, baited and unbaited arms were fixed throughout the tests. The 1st, 3rd, 5th, and 7th arms were baited (with food) while the 2nd, 4th, 6th, and 8th arms were unbaited (without food). At the very beginning of each test session, each mouse was placed in the central platform of the equipment at the position facing towards the 1st arm. Food-deprived mice were expected to seek specific arms with rewards and subsequently register and retain the memory of each entered arm where food was present. Each mouse was allowed to freely explore and consume food rewards for 3 minutes or until all food rewards of the four baited arms were eaten, which ever occurred first. An entry was recorded every time when the mice placed all four paws into the initial part of the arm. The maze was then thoroughly cleaned with 70% alcohol prior to the next test session in order to minimize the effect of residual odors of food from previous tests [35]. The first entry into never-baited arms was scored as a reference memory error (RME), reentry into arms where the food reward had already been eaten was scored as a working memory error (WME), and reentry into unbaited arm is considered reference-working memory errors (RWE) [36, 37].

3.2. Brain Tissue Harvesting and Processing. After the radial arm maze tests were completed on every individual mouse of each experimental set separately, mice of each group were sacrificed by cervical dislocation. Whole brain was immediately removed and washed with ice cold normal saline. Prefrontal cortex and hippocampus were dissected out on ice and blotted dry quickly within the folds of blotting paper and pooled and used directly for the neurochemical studies or stored frozen at −70°C.

3.3. Assay of Acetylcholinesterase (AChE) Activity. AChE activity was measured using modified Ellman's colorimetric method [38, 39]. Briefly, hippocampal and prefrontal cortical tissue of brain were quickly homogenized in 0.1 M phosphate buffer, pH 7.4, separately. The acetylcholinesterase activity was measured by adding an artificial substrate analog of acetylcholine, acetylthiocholine (ATC) for every two min.

Thiocholine released because of the cleavage of ATC by AChE is allowed to react with the –SH group of the reagent 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB), which is reduced to thionitrobenzoic acid, a yellow colored anion with an absorbance maxima at 412 nm. The molar extinction coefficient of the thionitrobenzoic acid was taken as 1.36×10^4/M/cm. The concentration of thionitrobenzoic acid was determined using a UV-Vis spectrophotometer and the AChE activity was calculated using the formula: $(R = 5.74 \times 10^4\ \mathrm{XA})/\mathrm{CO}$, where R = rate in moles of substrate hydrolyzed/min/gm wet wt of tissue; A = change in absorbance/min; and CO = original concentration of the tissue (mg/mL).

3.4. Prefrontal Cortex and Hippocampal Lysate Preparation. The prefrontal or hippocampus tissue was homogenized in TEEN buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl) supplemented with 2 mM PMSF and 1 μg/mL protease inhibitor cocktail. Thereafter, the homogenate was centrifuged at 5000 ×g. The resulting supernatant was collected and aliquoted in small fractions. The total protein content in the lysate was estimated by Bradford method [40]. Aliquots were directly used for further experiment or stored at −70°C.

3.5. Western Blotting. The prefrontal or hippocampal lysate was boiled at 100°C for 5 min in SDS containing sample loading buffer (10 mM Tris-HCl pH 6.8, 0.2% β-mercaptoethanol, 2% SDS, 20% glycerol) and centrifuged at 10000 ×g at 4°C for 20 min. The supernatant was carefully collected. 50 μg total protein was resolved on 7.5% SDS-polyacrylamide gel as described earlier [41]. Thereafter, proteins from the gel were immobilized onto polyvinylidene difluoride (PVDF) membrane by wet transfer method. To ensure the transfer of proteins, the membrane was stained with Ponceau-S. The PVDF membrane was washed in 1x phosphate buffer saline (PBS) and was blocked with 5% nonfat milk powder dissolved PBS for 4 h at RT. Thereafter, the membrane was incubated with anti-GluN2B antibody (1 : 2000) overnight and washed for 5 min in PBST (PBS containing 10 mM Tris-HCl, pH 7.0). The blots were also processed with rabbit monoclonal anti-β-actin antibody (1 : 25,000, Sigma-Aldrich, USA) in parallel in order to examine the level of β-actin as internal control. Thereafter, membranes were incubated with goat anti-mouse HRP-conjugated secondary antibody (1 : 2500 in PBS containing 5% nonfat milk) for 4 h and then washed with PBST at RT. The specific protein-antibody complex on the membrane was detected by enhanced chemiluminescence (ECL) method following the manufacturer's protocols. Resulting signals on the X-ray film were densitometrically scanned individually and quantified by computer-assisted densitometry (Alpha imager 2200). Scan data of individual proteins were normalized with that of the β-actin to obtain relative density value (RDV) for GluN2B.

3.6. Isolation of Total RNA. Total RNA from the prefrontal cortex or hippocampus was isolated using TRI reagent (Sigma, USA) following the suppliers' manual. The aqueous phase was collected and mixed with equal volume (v/v) of isopropanol and precipitated at −70°C. The RNA pellet was collected, washed with ice-chilled 70% ethanol, and dissolved in diethylpyrocarbonate- (DEPC-) treated water. Extracted RNA was treated with DNase-I (DNA-free, Ambion) according to the manufacturer's guidelines to remove any DNA contamination. RNA content was determined by measuring the absorbance at 260 nm using UV-Vis spectrophotometer. Integrity of the RNA samples was checked by 1% formaldehyde agarose gel electrophoresis [41].

3.7. Semiquantitative RT-PCR. cDNA from total RNA was synthesized by mixing 2 μg of the DNA-free total RNA and 200 ng random hexamer primers (MBI Fermentas, USA) in 11 μL reaction volume and incubating the whole mix at 70°C for 5 min. Thereafter, 2 μL of 5x reaction buffer, 2 μL of 10 mM dNTP mix, and 20 U of RNase inhibitor (Ribolock, MBI Fermentas, USA) were added, and the volume was made up to 19 μL. The tube was incubated for 5 min at 25°C, and 200 U of M-MuLv reverse transcriptase (RT) (New England Biolabs) was added. Further, the tube was incubated for 10 min at 25°C initially and then at 42°C for 1 h in the Thermal Cycler (G-Storm, UK). The reaction was terminated by heating the reaction mix at 70°C for 10 min followed by its incubation at 4°C. PCR reactions were carried out in a 25 μL reaction mixture containing 2 μL cDNA, 1x Taq polymerase buffer with $MgCl_2$, 0.2 mM of each dNTP (MBI Fermentas, USA), 1.0 unit of Taq DNA polymerase (Banglore Genei, India), and 10 pmol of appropriate primers for GluN2B (F-CTGGATTCTGCATTGTGAGC, R-CACGAGGATGACAGCGATG) and β-actin (F-ATCGTGGGCCGCTCTAGGCACC, R-CTCTTTGATGTCACGATTTC) in Thermal Cycler (G-Strom, UK) for 28 cycles. Each PCR amplified product was individually mixed with 6x loading dye (30% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol) and were resolved separately by 2% agarose gel electrophoresis using a tank buffer 1x TAE buffer (40 mM Tris, 40 mM acetic acid, and 1 mM EDTA) containing ethidium bromide. The DNA bands were visualized in UV transilluminator and images of the gel were captured. The image was densitometrically scanned separately and quantified using Fluorchem software, version 2.0 (Alpha Innotech, USA). Integrated density value (IDV) of the GluN2B specific DNA band was normalized with IDV of the β-actin DNA band to obtain relative density value (RDV).

3.8. Statistical Analysis. All the neurochemical experiments were repeated at least three times taking a batch of 6-7 mice per experimental group. The RDV data was presented as bar diagram showing mean ± SEM and the data were analyzed by one-way ANOVA between experimental groups followed by post hoc Bonferroni multiple comparison tests using two-tailed P values with SPSS-16. The P values < 0.05 were taken as significant. Performance of mice from each experimental group on the radial arm maze test for the analysis of various memory types during retention test was analyzed by one-way ANOVA followed by Bonferroni tests and learning curves for percent correct memory were analyzed as trial blocks for three trials (one trial block/day).

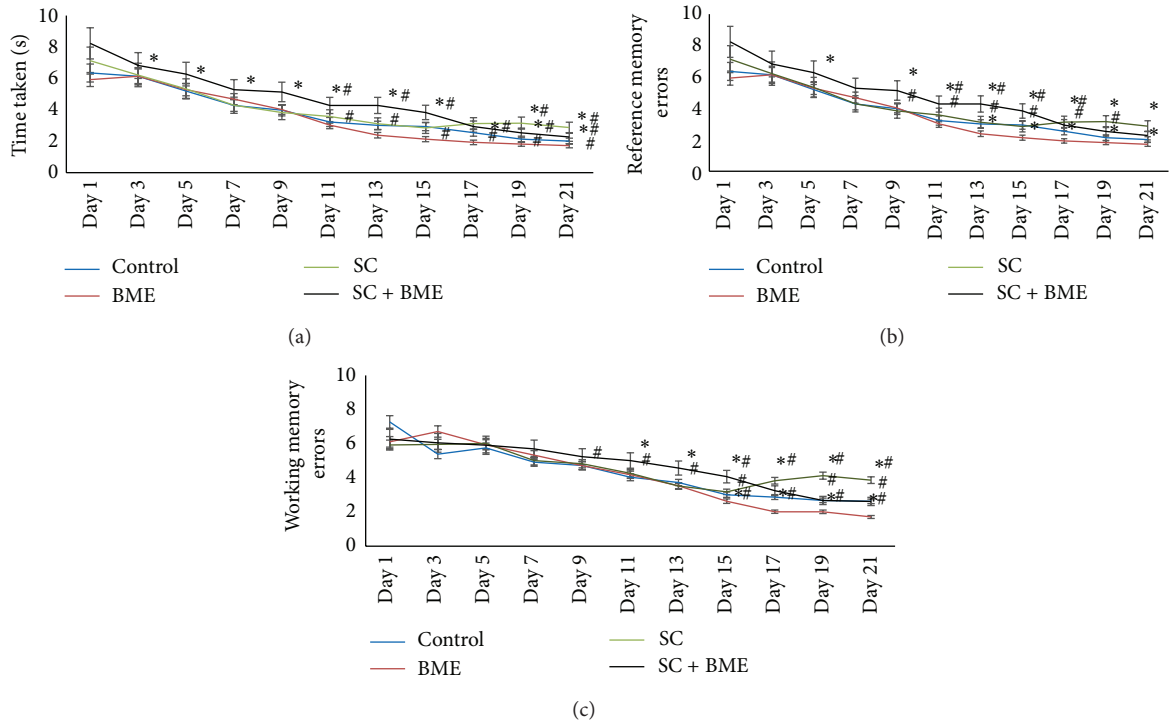

FIGURE 1: Radial arm maze analysis of acquisition by mice during training period. Mouse of each experimental group was individually trained on the maze for searching food and time spent; reference memory errors, working memory errors, and reference-working memory errors were recorded. Graphs represent average value ± SEM of above parameters during acquisition trials. Data were analyzed by repetitive measures ANOVA followed by Dunnett's post hoc tests. ∗, $P < 0.05$ for mice groups in comparison to control within same day; #, $P < 0.05$ for mice groups on a particular day in comparison to that mice within groups on day 1. (a) Time taken to retrieve hidden food in radial arm maze. (b) and (c) Reference memory errors and working memory errors.

4. Results

4.1. Acquisition Processes Is Not Affected by Tween 80 and Normal Saline. To understand whether Tween 80 or normal saline per se have any impact on the acquisition and memory processes, the control mice were subjected to radial arm maze test. Figure 1 reveals the results of acquisition and memory during vehicle or the drug treatment period at the level of latency time in general, and reference and working memory at the levels they incorporate errors while entering in the nonbaited and baited arms of the RAM, respectively. As is evident from Figure 1(a), treatment of mice with the drug vehicle, that is, Tween 80 or normal saline for various durations, did not affect the learning and memory processes. The behavioral analysis data reveal that all the mice in control set were able to learn during training period from day 1 in the first week till day 21 in the third week. Tween 80 and normal saline did not affect their abilities of learning and memory at the level of latency time and incorporation of errors.

4.2. Bacopa monnieri Extract Reverses Learning Defects and Corrects the Scopolamine-Induced Spatial Memory Loss. It is evident from the behavioral data obtained from radial arm maze test as shown in Figures 1(a), 1(b), and 1(c) that scopolamine treatment leads to significant decline in the acquisition and *Bacopa monnieri* extract (CDRI-08) treatment significantly improved it towards that in the normal control mice.

Figure 2(a) shows track plot report of mice of different experimental groups. It reveals that CDRI-08-treated mice showed fewer errors and were able to track the food more accurately as compared to control group. However, the scopolamine-treated mice experienced problems in locating the hidden food pellet with significantly more errors in above process. CDRI-08-treated mice significantly improved their performance with fewer errors nearer to the normal control. The CDRI-08 treatment to amnesic mice restored the memory for locating food pellets compared to scopolamine-treated mice. Our Any Maze Software Analysis data on the eight-arm radial maze test reveal that scopolamine-treated mice exhibit significant impairment in memory retrieval (amnesia) compared to that in the normal control mice ($P < 0.01$). *Bacopa monnieri* extract CDRI-08 treatment reverses the condition of scopolamine-induced amnesia ($P < 0.01$). It was also observed that mice treated with CDRI-08 alone also showed significant improvement in the memory retention ($P < 0.01$) (Figures 2(a) and 2(b)).

Figure 3 reveals alterations in various memory forms such as working memory, reference memory, and reference-working memory on the 22nd day based on producing errors during entries in the baited arms (in which the food reward

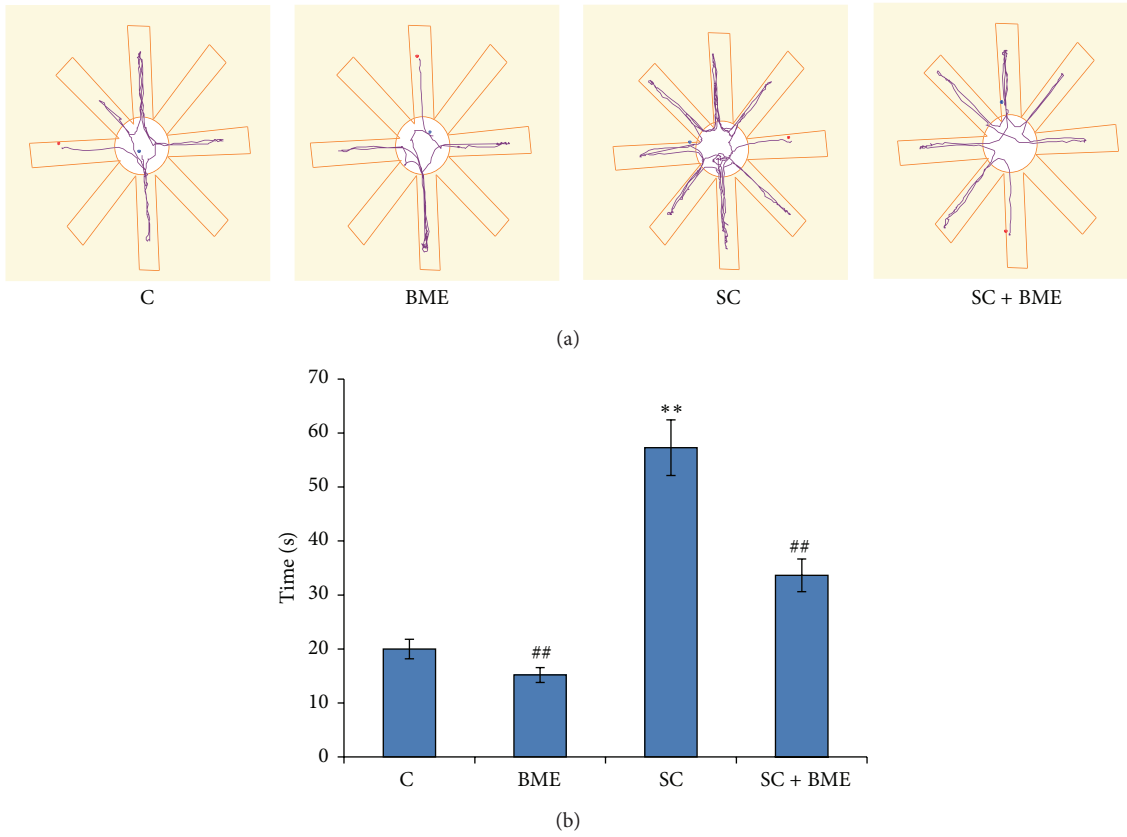

FIGURE 2: Radial arm maze analysis of spatial memory of mice of control and experimental groups: track record of movement mice in radial arms (a). Bar diagram showing the latency time for retrieving the hidden food (b). Mouse of each group was individually subjected to radial arm maze test and the time taken for retrieving food was recorded. Data represents mean ± SEM. C, vehicle-treated control; BME, *Bacopa monnieri* extract (CDRI-08) treated (200 mg/Kg/BW); SC, scopolamine-treated (2 mg/Kg BW); SC + BME, scopolamine-treated mice treated with CDRI-08. ∗ and #, $P < 0.05$ and ## and ∗∗, $P < 0.01$ were considered significant. ∗, comparison between control and other groups, and #, comparison between SC and other groups.

was kept) or nonbaited arms (where there was no food reward). Figure 3(a) shows errors in reference memory shown by the mice of different groups. Our observations reveal that the scopolamine-treated amnesic mice commit significantly more errors in retrieving the reference memory as compared to that in the normal control mice ($P < 0.05$). It suggests that these mice lose the ability to remember the exact location of food in respect of the arms which were not baited. Mice of control group treated with CDRI-08 produced significantly fewer reference memory errors in comparison to normal control mice ($P < 0.01$). The scopolamine-treated mice (amnesic) after the treatment with CDRI-08 produced significantly less number of errors while retrieving the reference memory during location of food ($P < 0.05$). Scopolamine was found also to significantly reduce the working memory in mice compared to normal control ($P < 0.05$). CDRI-08 alone was found to improve the working memory significantly compared to its effect on the normal control mice ($P < 0.05$). Scopolamine-treated amnesic mice after the treatment of CDRI-08 showed significantly improved performance on the maze test ($P < 0.05$) (Figure 3(b)).

Figure 3(c) shows alterations in reference-working memory in mice belonging to different experimental groups. Our data suggest that the scopolamine treatment of mice leads to significant decline in the RWM compared to that in the normal control mice. Significant improvement in the reference-working memory (RWM) was observed in amnesic mice after treatment with CDRI-08 ($P < 0.05$). It was observed that the CDRI-08 treatment alone was also able to boost the RWM compared to that in the normal control mice ($P < 0.01$). Thus the quantitative analysis of RAM behavior data reveals that *Bacopa monnieri* extract (CDRI-08) does have positive effects on the normal control mice at above scales of learning and memory and it has altogether neuroprotective effects. Further, the treatment of scopolamine-induced mice with *Bacopa monnieri* extract (CDRI-08) has a precognitive effect on learning and memory lost due to scopolamine-induced amnesia.

4.3. Scopolamine Enhances the AChE Activity in Prefrontal Cortex and Hippocampus and Bacopa monnieri Extract (CDRI-08) Reverses This Effect. Our observations on the assay of AChE activity reveal that its activity is significantly

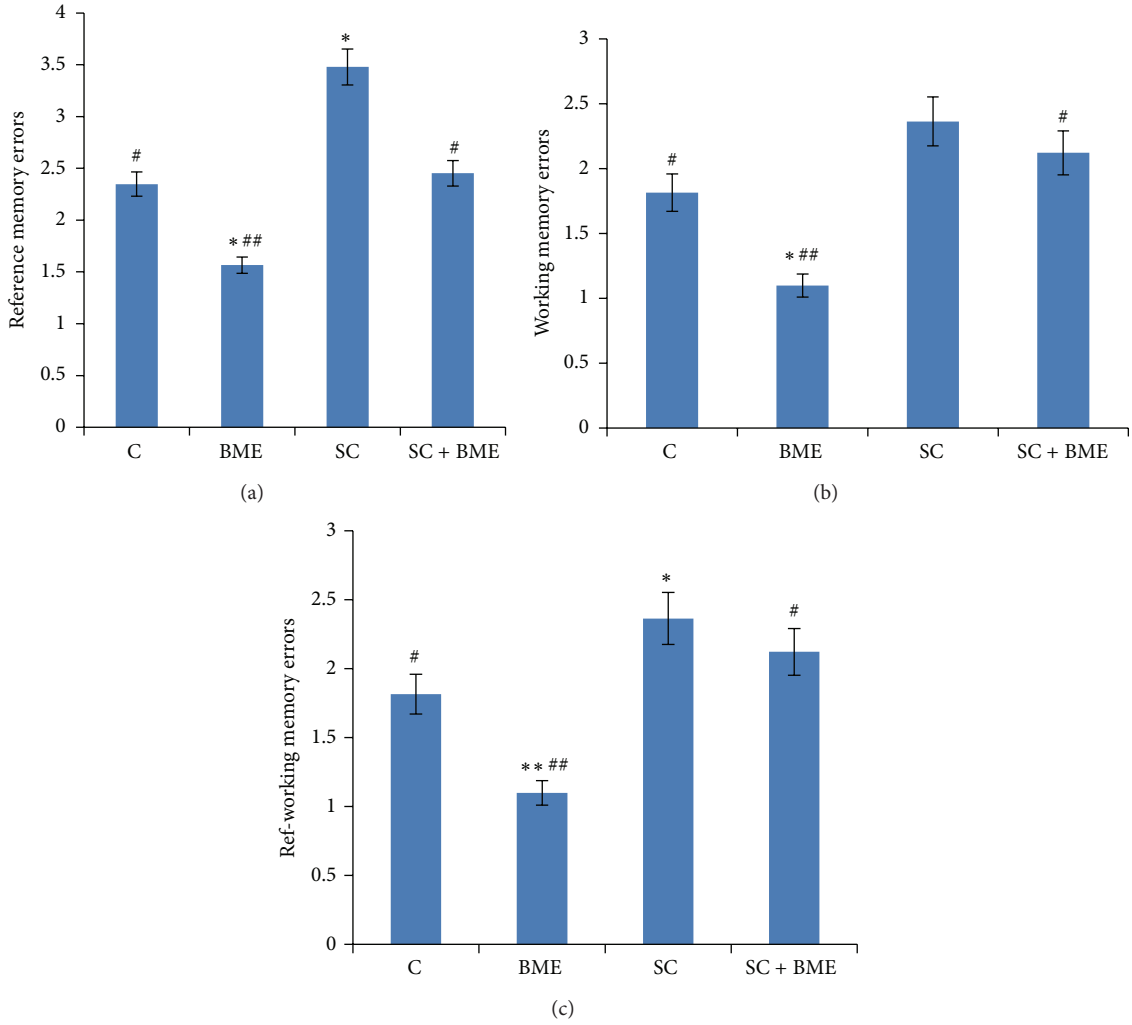

Figure 3: Radial arm maze tests for reference memory error (a), working memory error (b), and reference-working memory error (c). Mouse of each group was individually subjected to radial arm maze test for recording the errors. Data represents mean ± SEM. C, control; BME (*Bacopa monnieri* extract), CDRI-08-treated; SC, scopolamine-treated; SC + BME, scopolamine-treated mice treated with CDRI-08 as in Figure 2. ∗ and #, $P < 0.05$ and ## and ∗∗, $P < 0.01$ were considered significant. ∗, comparison between control and other groups. #, comparison between SC and remaining groups.

increased in the prefrontal cortex of scopolamine-treated amnesic mice when compared with that in the normal control mice ($P < 0.05$). CDRI-08 treatment to amnesic mice results in significant decline in the AChE activity and the CDRI-08 treatment when given to normal control mice; the AChE activity is significantly reduced ($P < 0.05$) (Figure 4(a)). Figure 4(b) shows the patterns of AChE activities in the hippocampus of mice of different experimental groups. Scopolamine-treated mice exhibited significant increase in the AChE activity ($P < 0.05$) compared to that in the hippocampus of the normal control mice. CDRI-08 treatment of the amnesic mice significantly decreases the AChE activity toward that in the normal control mice ($P < 0.05$). Also, the CDRI-08 alone significantly decreases the activity in the hippocampus when compared to that in the normal control mice ($P < 0.01$).

4.4. Scopolamine Downregulates the Expression of GluN2B in Prefrontal Cortex and Hippocampus and Bacopa monnieri Extract (CDRI-08) Recovers It towards Normal. Our immunoblot data reveals that the scopolamine treatment significantly downregulates the level of GluN2B subunit expression in the prefrontal cortex ($P < 0.05$) (Figure 5(a)) and the hippocampus when compared to that in the normal control mice ($P < 0.05$) (Figure 6(a)). CDRI-08 treatment to scopolamine-treated (amnesic mice) significantly upregulates the expression of GluN2B subunit toward that in the normal control mice in both the brain regions. Further, the CDRI-08 alone also is found to significantly upregulate the expression of the GluN2B in the prefrontal cortex as well as hippocampus ($P < 0.01$) (Figures 5(a) and 6(a)). Our semiquantitative RT-PCR data on the expression of GluN2B transcript largely resembles

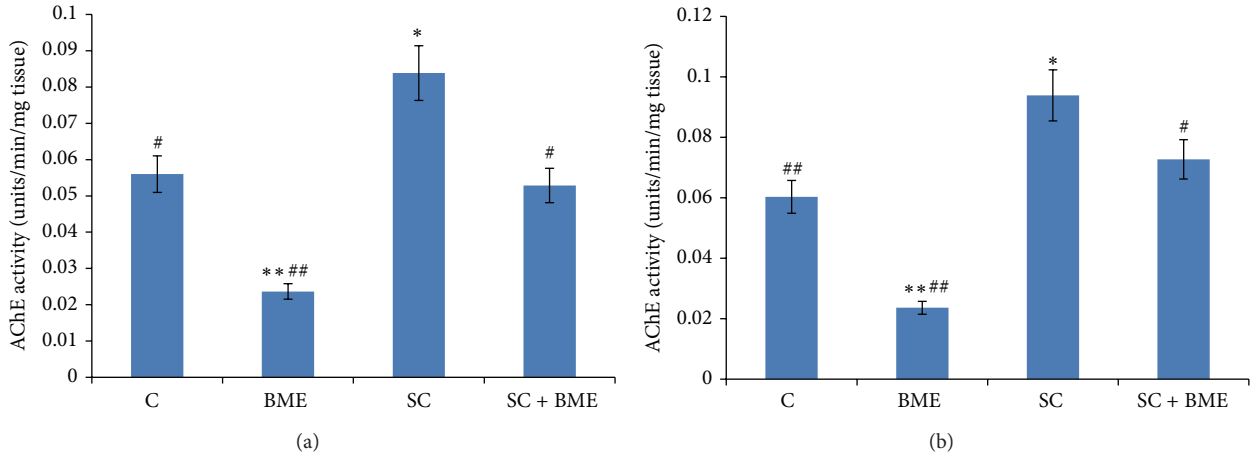

FIGURE 4: Acetylcholinesterase activity in prefrontal cortex (a) and hippocampus (b). Tissues obtained from 6-7 mice of each group were pooled and AChE activity was assayed. The AChE activity was expressed as unit/min/mg tissue. Data represents mean ± SEM. C, control; BME (*Bacopa monnieri* extract), CDRI-08-treated; SC, scopolamine-treated; SC + BME, scopolamine-treated mice treated with CDRI-08 as in Figure 2. ∗ and #, $P < 0.05$ and ## and ∗∗, $P < 0.01$ were considered significant. ∗, comparison between control and other groups. #, comparison between SC and remaining groups.

with those of our immunoblot data for both the prefrontal cortex and the hippocampus (Figures 5(b) and 6(b)).

5. Discussion

We have studied whether expression of GluN2B in the prefrontal cortex and hippocampus of amnesic mouse model is altered in order to understand the mechanisms of scopolamine-induced amnesia involving one of the ionotropic glutamate receptors such as NMDA receptors and effects of *Bacopa monnieri* extract CDRI-08 during recovery of memory loss in mice. Also, we have investigated the effects of CDRI-08 on the expression on this subunit of the NMDA receptor as it plays important role in synaptic plasticity which underlies learning and memory. Since CDRI-08 has been in use as memory booster drug, we checked its role in the recovery of impaired learning and memory due to amnesia using eight-arm radial maze paradigm. We have used scopolamine for inducing amnesia in mice in the current study. As the scopolamine is a muscarinic acetylcholine receptor antagonist, it may lead to accumulation of acetylcholine (ACh) in the cholinergic synapse and this in turn is likely to increase the activity of the acetylcholinesterase and thereby it might affect the neuronal transmission which may in turn affect the memory processes. Therefore, to validate this, we assessed the AChE activity in the prefrontal cortex and the hippocampus. Since CDRI-08 is in use for boosting the memory in normal human subjects, we also examined its direct effects on the normal control mice in addition to above.

Our radial arm maze (RAM) data from the acquisition experiment suggest that mice were able to learn well during the training period (Figure 1) and scopolamine treatment leads to decline in the learning for reference, working, and reference-working memory. Also, it suggests that mice treated with scopolamine may develop impairments in both working and reference memory by affecting the prefrontal cortex, which may later on form defective long-term memory in the hippocampus. This could be attributed to defective synaptic plasticity due to inactivation of acetylcholine receptor activity and/or altered NMDA receptors on the postsynaptic density in prefrontal cortex or the hippocampus. Mode of scopolamine action is known to block the muscarinic receptors acetylcholine receptors [42, 43] which ultimately lead to profound deficits in attention and memory by inhibiting cholinergic neurotransmission. Our data are consistent with the scopolamine-induced deficiency in the spatial memory [29]. The other possibility of memory deficit may be due to scopolamine-induced oxidative stress in the brain [44]. Available evidence also suggests that the memory deficiency might be due to effects of scopolamine in blocking NMDA receptors [34]. However, our data do not directly support this function of the scopolamine but it indicates their association.

Acetylcholine level is under the dynamic regulation of an enzyme AChE at the synapse [45]. Numerous studies have implicated the importance of acetylcholine in higher brain functions like learning and memory [46–48]. Alterations in the acetylcholine metabolism are also involved in various neuropathological conditions like mild cognitive impairment (MCI), Alzheimer's disease (AD), and dementia [49, 50]. Decreased levels of acetylcholine at the synaptic cleft have been implicated in the loss of synaptic architecture leading to state of amnesia in rodents [51]. In our study, activity of AChE was found to be elevated in scopolamine-treated group which suggests a rapid breakdown of the acetylcholine which might lead to decline in its level in the synapse which may further be correlated with decline in various memory types. The CDRI-08 treatment was found to reverse the level of AChE which further was correlated with recovery of memory close to that in the normal control mice. The CDRI-08 alone also leads to a significant decline in the AChE activity. This suggests that CDRI-08 effect on memory improvement is via its action on the AChE. Our study confirms the neuroprotective role of

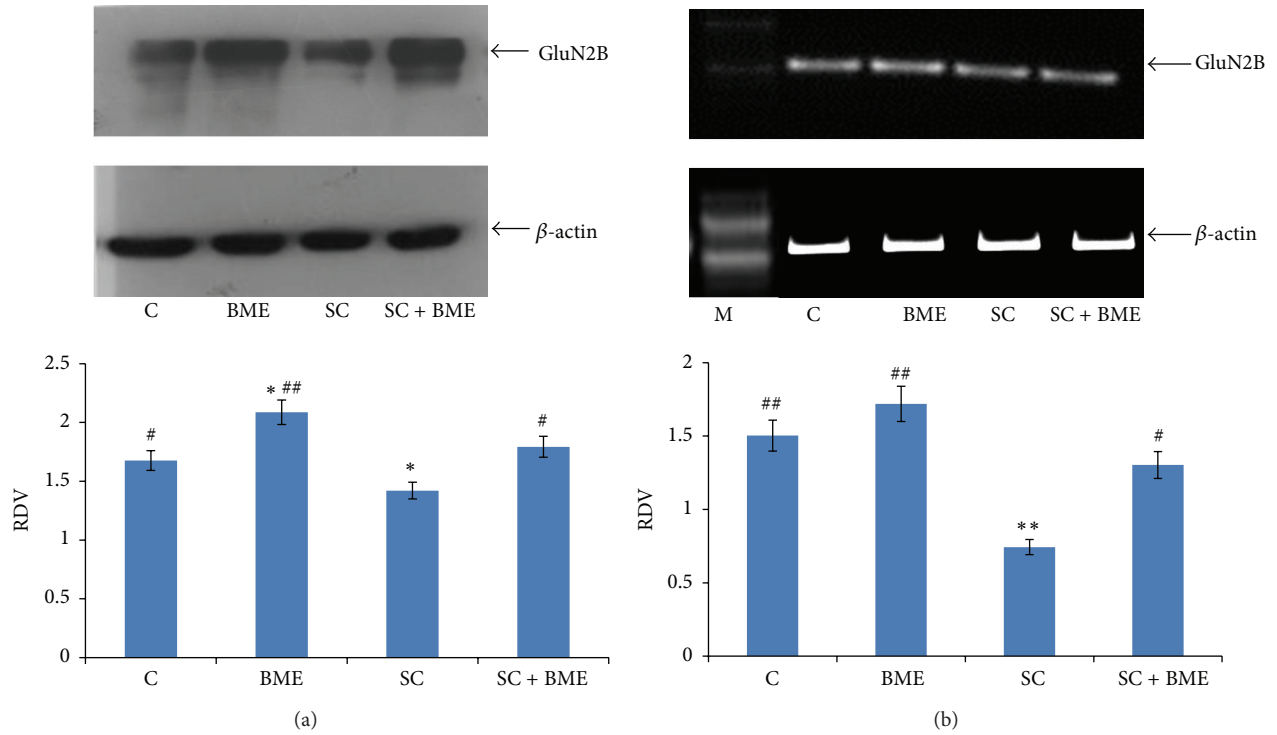

Figure 5: Western blot (a) and semiquantitative RT-PCR (b) analysis of GluN2B expression in prefrontal cortex. Prefrontal cortex from 6-7 mice of each group was pooled; lysates were prepared and detected for presence of GluN2B by ECL. X-ray film was scanned and the data was expressed as relative density value (RDV) by dividing the integrated density value of GluN2B by IDV of the β-actin. The data represents mean ± SEM. C, control; BME (*Bacopa monnieri* extract), CDRI-08-treated; SC, scopolamine-treated; SC + BME, scopolamine-treated mice treated with CDRI-08 as in Figure 2. $*$ and #, $P < 0.05$ and ## and $**$, $P < 0.01$ were considered significant. $*$, comparison between control and other groups. #, comparison between SC and the remaining groups.

CDRI-08 in recovering the memory loss due to amnesia induced by scopolamine treatment. The precise mechanism of CDRI-08 effects on the regulation of AChE and memory formation as well as memory recovery process, however, is yet to be understood. Nonetheless, recent study on schizophrenic human subjects supports our finding on the modulation of cholinergic neuronal activities by CDRI-08 [32]. Over the past several decades, plethora of natural products like coumarins, flavonoids, and stilbenes have been in use for the treatment of cognitive dysfunctions by inhibiting AChE [52–54]. Our findings on the recovery of memory loss by *Bacopa monnieri* extract CDRI-08 also suggest that the CDRI-08 protects the memory loss or improves the cognitive functions of the brain abrogated by scopolamine by inhibiting the AChE activity in the prefrontal cortex and hippocampus. The CDRI-08 effect may also be brought by altering the choline acetyltransferase (CAT) activity and thereby the level of acetylcholine in the synapse. Our study requires to be further supplemented with a close examination of alterations in the CAT activity due to CDRI-08 which may provide an insight into its mechanism of action on the improvement of memory in either normal or amnesic subjects.

NMDA receptors are widely concentrated in the cortical region of brain and in hippocampus. Recent studies have correlated NMDA receptor GluN2B subunit with varieties of learning and memory functions in the hippocampus-dependent spatial memory [9, 55]. Loss of NMDA receptors (NMDARs) has been implicated in long-term depression (LTD), loss of synaptic plasticity, learning, memory, and progression of various neuropathological conditions [56, 57]. In our study, we observed that scopolamine-induced forgetfulness in mice, as evidenced by our study on behavioral analysis by radial arm maze test, is correlated with decline in expression of the GluN2B subunit in the prefrontal cortex as well as hippocampus. This may further be associated with decline in cholinergic function along with alterations in the GluN2B expression resulting into loss of memory reflected by significant increase in the reference, working, and reference-working memory errors by amnesic mice while performing on the radial arm maze. Further, treatment of scopolamine-induced amnesic mice with CDRI-08 upregulates the expression of GluN2B in both the brain structures which is associated with enhancement of various memory types. Also, the CDRI-08 alone has ability to elevate the expression of GluN2B subunit compared to the normal control mice. In a recent study, Krishnakumar et al. also demonstrated a significant decline in the expression of GluN2B in the cerebral cortex of pilocarpine-induced epileptic rats which was reversed by the treatment of *Bacopa monnieri* extract [58]. Our data do suggest that CDRI-08 is highly effective in improving

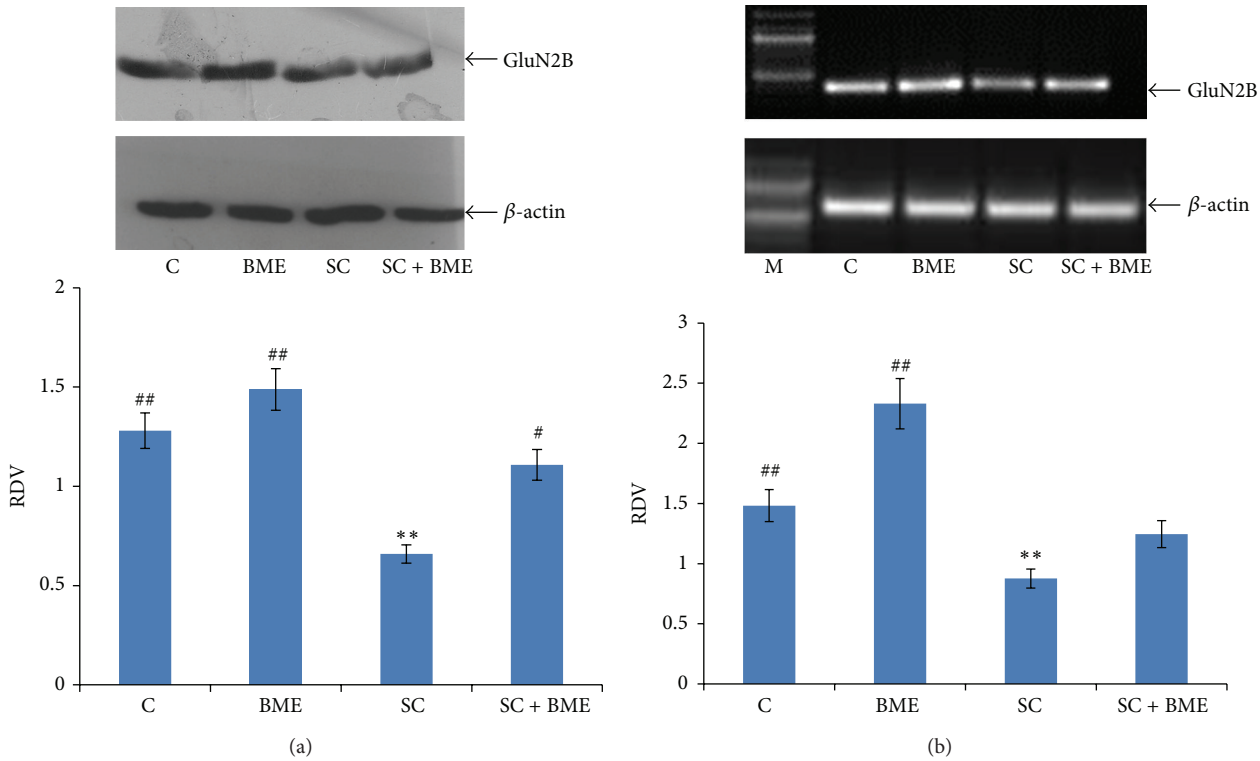

Figure 6: Western blot (a) and semiquantitative RT-PCR (b) analysis of GluN2B expression in hippocampus. Hippocampus from 6-7 mice of each group was pooled; lysates were prepared and detected for presence of GluN2B by ECL. X-ray film was scanned and the data was expressed as relative density value (RDV) by dividing the integrated density value of GluN2B by IDV of the β-actin. The data represents mean ± SEM. C, control; BME (*Bacopa monnieri* extract), CDRI-08-treated; SC, scopolamine-treated; SC + BME, scopolamine-treated mice treated with CDRI-08 as in Figure 2. ∗ and #, $P < 0.05$ and ## and ∗∗, $P < 0.01$ were considered significant. ∗, comparison between control and other groups. #, comparison between SC and the remaining groups.

and recovering the memory loss/deficiency which might be by positively regulating the synaptic plasticity, which would have been otherwise damaged by scopolamine leading to memory loss. Our expression study data, which corresponds to behavioral data on the performance of mice for various memory types, clearly suggests the use of CDRI-08 with its possible mechanism of action in restoration of memory loss. However, *Bacopa monnieri* extract has been shown to reverse the memory loss by decreasing the density of NMDAR in the prefrontal cortex and CA1 neuronal region of the hippocampus in phencyclidine-induced cognitive deficit rat model where its density was elevated during memory impairment [59]. Thus it is evident that CDRI-08 induced mechanisms for the recovery of memory loss (amnesia) involves alterations in the level of NMDAR. Role of CDRI-08 during recovery of memory loss due to scopolamine-induced amnesia by regulating the other neuronal systems such as GABAergic system [60] and involvement of various glutamate transporters and their regulation by CDRI-08 may also be importantly involved [59].

Based on the behavioral and molecular analysis, our study suggests that scopolamine-induced amnesia in mice is mediated via increase in the acetylcholinesterase activity and decrease in the population of NMDA type glutamate receptor in the glutamatergic synapse. Also, our study provides a molecular basis of the possible therapeutic action of a special *Bacopa monnieri* extract, CDRI-08, in the recovery of the scopolamine-induced memory deficit and its role in enhancing the levels of learning and memory in mice. However, to ascertain the precise role of NMDA receptor types and AChE in CDRI-08-mediated modulation of synaptic plasticity, neuronal cell density, dendritic arborization, dendritic spine density and their morphological aspects, and so forth is required to be thoroughly addressed. A thorough examination of the expression of other subunits of NMDAR such as GluN1, GluN2A, their trafficking [61], and assay of ChAT activity will be required to ascertain the mechanisms of action of the bacosides A and B rich CDRI-08 on the glutamatergic and cholinergic system, respectively, during its action on improvement of memory.

Conflict of Interests

The authors declare that there is no conflict of interests in respect of communication of this paper.

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Research Article

CDRI-08 Attenuates REST/NRSF-Mediated Expression of NMDAR1 Gene in PBDE-209-Exposed Mice Brain

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CDRI-08 is a standardized bacoside enriched ethanolic extract of *Bacopa monnieri*, a nootropic plant. We reported that CDRI-08 attenuated oxidative stress and memory impairment in mice, induced by a flame retardant, PBDE-209. In order to explore the mechanism, present study was designed to examine the role of CDRI-08 on the expression of NMDAR1 (NR1) and the binding of REST/NRSF to NR1 promoter against postnatal exposure of PBDE-209. Male mice pups were orally supplemented with CDRI-08 at the doses of 40, 80, or 120 mg/kg along with PBDE-209 (20 mg/kg) during PND 3–10 and frontal cortex and hippocampus were collected at PND 11 and 60 to study the expression and regulation of NR1 by RT-PCR and electrophoretic mobility shift assay, respectively. The findings showed upregulated expression of NR1 and decreased binding of REST/NRSF to NR1 promoter after postnatal exposure of PBDE-209. Interestingly, supplementation with CDRI-08 significantly restored the expression of NR1 and binding of REST/NRSF to NR1 promoter near to the control value at the dose of 120 mg/kg. In conclusion, the results suggest that CDRI-08 possibly acts on glutamatergic system through expression and regulation of NR1 and may restore memory, impaired by PBDE-209 as reported in our previous study.

1. Introduction

CDRI-08 is a standardized bacoside enriched ethanolic extract of *Bacopa monnieri* (Linn.) (BM). BM has been classified as a nootropic drug in the traditional system of Ayurvedic medicine. Preliminary study indicated that a neuropharmacological effect of *Bacopa* was due to two active saponin glycosides, bacosides A and B [1, 2]. It has been put forwarded that BM treatment enhances the cognitive functions via modulating various neurotransmitters such as acetylcholine (ACh), serotonin (5-hydroxytryptamine, 5-HT), gamma amino butyric acid (GABA), glutamate, and dopamine [3–6]. Furthermore, CDRI-08 is found to attenuate the diazepam, Nω-nitro-L-arginine (L-NNA) and 1-(m-chlorophenyl)-biguanide (mCPBG)-induced memory impairments [7–9]. Another study suggested that CDRI-08 reduces hypobaric hypoxia-induced spatial memory impairment [10]. Recently, we have reported that CDRI-08 significantly attenuates alterations in the oxidative status in frontal cortex and hippocampus and spatial memory behaviour following postnatal exposure of 2,2′,3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether (PBDE-209) in male and female mice [11, 12]. However, molecular mechanism of action of CDRI-08 against PBDE-209-induced memory impairment is unexplored.

PBDE-209, a highly brominated congener of polybrominated diphenyl ether (PBDE), containing 10 bromine atoms, is the most widely used congener of the PBDEs. It has good thermal stability and thus requires in smaller amount to be used as flame retardant in different types of industrial and consumer products [13]. It is a persistent, lipophilic and bioaccumulates in wildlife and humans and biomagnifies up the food chain [14–16]. It has been detected not only in the environment and certain foods but also in human tissue, such as adipose tissue, serum, and the breast milk with increasing levels in rapidly developing countries such as China and India [17, 18]. Recent studies indicate that

in both the environment and organisms, PBDE-209 can be debrominated to lower congeners, which have higher risks of bioaccumulation and toxicity [19, 20]. Our previous study has demonstrated that exposure to PBDE-209 causes developmental neurotoxicity by interfering in the oxidant and antioxidant homeostasis that results into impaired learning and memory performances in Morris water and radial arm maze tasks [21]. Learning and memory are majorly governed by N-methyl-*D*-aspartate receptors (NMDARs), glutamate-gated cation channels that belong to a class of ionotropic glutamate receptors (iGluRs) [22]. NMDAR is a heteromeric complex containing NMDAR1 (NR1) (GluRζ in mouse) subunits in various combinations with NMDAR2A-D (NR2A-D) (GluRε1–4 in mouse) and NMDAR3A-B (NR3A-B) subunits [23]. Though all the subunits play crucial role in learning and memory, NR1 subunit is obligatory for NMDAR function; therefore at least one NR1 subunit is always incorporated into the receptor complex for channel activation [24, 25]. Moreover, NR1 is found in almost all neurons of the central nervous system [26] and unlike other subunits, it is expressed consistently throughout the brain development. Genetic enhancement of NR1 expression is implicated in the formation of long-term memory [27]. However, genetic knockout of the NR1 gene blocks initiation of long-term potentiation (LTP) in both hippocampus and neocortex [28]. The expression and regulation of NR1 are challenged during several neurodegenerative pathologies, though the regulation of NR1 expression during PBDE-209-induced memory deficit is not fully understood.

Proper regulation of NR1 expression and function is required for normal physiological process within the central nervous system. Deletional and mutational analyses of the rat NR1 promoter reveal the presence of highly conserved 21–23 bp DNA sequence called Repressor Element 1 (RE1)/Neuron-Restrictive Silencer Element (NRSE) cis-regulatory site on the 5′-upstream region of the NR1 gene [29]. It modulates NR1 gene expression by binding with Repressor Element Silencing Transcription Factor (REST)/Neuron-Restrictive Silencer Factor (NRSF), a 116 kDa GLI-Krüppel class C2H2 zinc finger protein expressed in all tissues and required for proper development of vertebrates. REST/NRSF is comprised of an N-terminal repressor domain, a cluster of eight zinc fingers that functions as a DNA-binding domain, a highly basic region, a repeat region, and a C-terminal repressor domain with a single zinc finger motif [30]. The binding of REST/NRSF to RE1/NRSE represses multiple neuronal target genes in nonneuronal tissues and also in undifferentiated neural precursors of the central nervous system to control the proper timing of neuronal gene expression during neurogenesis. Conversely, disruption of REST/NRSF during embryogenesis results in cellular apoptosis, aberrant differentiation, lethality, and delayed development [31]. REST/NRSF-mediated deregulation is causative factor for several pathological conditions, such as Huntington's disease [32], cancer [33], ischemia [34], seizure activity [35], and neuropathic pain [36]. Surprisingly, it is not known whether REST/NRSF-mediated dysregulation of NR1 expression has a causative mechanism in PBDE-209-induced memory impairment and CDRI-08 has capability to revert the effects of PBDE-209 via acting on the same target. Therefore, in the light of these observations, we were interested to evaluate the role of CDRI-08 on the expression of NR1 and its transcriptional regulation mediated by REST/NRSF against PBDE-209 in the frontal cortex and hippocampus of male mice.

2. Materials

2.1. Animals. Male and female adult Swiss albino mice, weighing 25–30 g, were maintained in animal house as per the recommendations from Central Animal Ethical Committee (number Dean/11-12/CAEC/257) of the Banaras Hindu University, Varanasi, India, for care and use of the laboratory animals. They were maintained in dedicated mice colony at 12-hour light and dark schedule at 24 ± 2°C with standard mice feed (pellets) and drinking water supply *ad libitum*. Two females were housed with one male in a cage for breeding. Females were examined every morning to observe the formation of a vaginal plug. The vaginal plug-positive females were caged individually. The day of litter born from each female was designated postnatal day (PND) 0. The size of the litter was adjusted as much as possible in order to obtain litters of the same size (6–8 pups). The newborn pups were kept with the mother till the age of PND 40.

2.2. Chemicals. PBDE-209 (98%, CAS number 1163-19-5) was purchased from Aldrich-Chemie while the corn oil from Sigma (St. Louis, MO, USA). The standardized ethanolic extract of BM (CDRI-08), containing 58.18% bacosides, developed by the Central Drug Research Institute, Lucknow, India, was generously gifted by Professor Singh, ex-Deputy Director [37]. The primers and oligos were obtained from IDT, USA, and Eurofins Genomics India Pvt. Ltd, India. Radiolabelled αP^{32}-dCTP was purchased from the Board of Radiation and Isotope Technology, Hyderabad, India. Analytical grade chemicals were used for all the experiments. Molecular biology grade chemicals were used wherever necessary. Chemicals and enzymes were stored at specific temperatures, diluted, and used as per manufacturers' instructions. The stock solution of PBDE-209 was prepared by mixing the compound with corn oil and sonicated for 30 minutes at room temperature. The stock solution of BM was prepared by uniformly suspending it in 5% tween 80.

2.3. Experimental Design. At PND 0, male pups within the same litter were randomly assigned to five treatment groups of fourteen each and treated as follows:

Group I: control (vehicle),

Group II: PBDE-209 (20 mg/kg),

Group III: PBDE-209 (20 mg/kg) + CDRI-08 (40 mg/kg),

Group IV: PBDE-209 (20 mg/kg) + CDRI-08 (80 mg/kg),

Group V: PBDE-209 (20 mg/kg) + CDRI-08 (120 mg/kg).

The doses of PBDE-209 and CDRI-08 were selected according to Rice et al. [38] and Saraf et al. [39]. All the treatments were given orally via a micropipette with 100 μL microtip at a volume of 5.0 μL/gm body weight (bw) of pups from PND 3–10. The pups were sacrificed at PND 11 (neonate) by decapitation and at PND 60 (young) by cervical dislocation. Meninges and white matter were removed carefully as much as possible. Left side of each tissue was processed for RT-PCR while that of right side for electrophoretic mobility shift assay (EMSA). Frontal cortex and hippocampus were collected and stored at −80°C as these two brain regions are involved in spatial memory function [40].

2.4. Semiquantitative RT-PCR Analysis of NR1

2.4.1. RNA Extraction. Total RNA from the frontal cortex and hippocampus of mice at neonate and young age was extracted using TRI reagent (Sigma-Aldrich) according to its user guidance and dissolved in diethylpyrocarbonate- (DEPC-) treated water. RNA was stored at −80°C until use [41, 42]. To make sure the RNA preparation free from DNA contaminants for further experiments, the total RNA preparation was subjected to DNA-free (Ambion) treatment using supplier's manual. The RNA content was estimated by measuring the absorbance at 260 nm taking 1 Unit A_{260} value equivalent to 40 μg of RNA. The total RNA was separated on agarose gel containing formaldehyde as denaturing agent in order to check the quality of the preparation. The major RNA bands were visualized under UV light and captured the images using Alpha imager software to quantify the ratio between the 28S and 18S rRNA bands.

2.4.2. Reverse Transcription. For reverse transcription of total RNA, about 2.0 μg of total RNA digested with DNase I was incubated with 200 ng of random hexamers, dNTPs, and M-muLV reverse transcriptase (RevertAid H Minus, 200 units, NEB) in 1X RT buffer, supplied with the enzyme, at 42°C for 1 hour. During incubation, degradation of RNA was prevented by adding 5 units of human placental RNase inhibitor. The reaction was inactivated by heating at 70°C for 10 min and after chilling on ice, the tube was stored at −80°C or directly used for the PCR reaction.

2.4.3. Polymerase Chain Reaction. Expression of NR1 and β-actin was assessed by amplification reaction by using the following gene-specific primers 5′-CAAGTGGGCATCTAC-AATGG-3′ and 5′-CCCCGTACAGATCACCTTCT-3′ for NR1; 5′-ATCGTGGGCCGCTCTAGGCACC-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-3′ for β-actin. The PCR reactions were carried out in amplification reaction system for each gene in a 25 μL reaction volume containing 2.0 μL of cDNA, 2.5 μL of 10X Taq DNA polymerase buffer containing 15 mM $MgCl_2$, 0.6 mM dNTPs mix, 3 units of Taq DNA polymerase (Bangalore Genei), and 10 pmols of forward and reverse primers. The samples were denatured at 94°C for 5 min and amplification reactions were carried out with following amplification parameters: denaturation at 94°C for 1 min, primer annealing at 53°C (for NR1) or 57°C for β-actin for 1 min, elongation at 72°C for 1 min per cycle. PCR amplification was performed for 32 cycles for NR1 and β-actin. Various amplification parameters such as Mg^{2+} and primer concentrations, temperature for denaturation, primer annealing, elongation, and the number of cycles were determined by a pilot PCR reaction for each gene separately. Further, the number of PCR cycles was optimized such that it falls in the exponential phases of each amplification reaction (data not shown). The PCR amplified products of NR1 and β-actin genes were separately resolved by 1.5% agarose gel electrophoresis and the gel was visualized in UV transilluminator and photographed.

2.5. Electrophoretic Mobility Shift Assay (EMSA)

2.5.1. Preparation of Nuclear Extract. Nuclear extract was prepared from the frontal cortex and hippocampus of mice belonging to various experimental sets following the procedure of Dignam et al. [43] with minor modifications [44]. The nuclear proteins (extracts) were estimated by Bradford method [45]. In order to quantify the sample, 0.1 mL of the suitably diluted protein extract was mixed with 0.9 mL of the stain, mixed well, and incubated for 1 min at room temperature. The absorbance was measured at 595 nm wavelength. The standard curve was plotted by mixing varying concentrations of bovine serum albumin (BSA) and Bradford reagent and the corresponding A_{595} was measured to find out the $1A_{595}$ value (standard value). Using the standard value obtained from above standard curve, the protein content in the experimental samples was calculated. The nuclear proteins were analyzed on 10% SDS-polyacrylamide gel electrophoresis and stained the gel in silver staining medium.

2.5.2. Annealing, Labelling, and Purification of Oligonucleotides. The complementary oligos for RE1 (−140 bp to −250 bp) 5′-GCGGAGGGTGATTCAGAGGC AGGTGC-3′and 3′-CTCCCACTAAGTCTCCGTCCACGACG-5′ were annealed in a 50 μL reaction volume having equimolar concentrations of each oligo in 1X TNE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA). Further, the oligos were denatured at 95°C in a water bath for 15 min and allowed to cool down gradually. It was incubated at room temperature overnight and stored the annealed oligos at −20°C for further use in EMSA experiments. The annealed double stranded (ds) oligos were labelled by end filling technique. The 5′ overhangs were filled in 20 μL volume. The reaction mixture contained 50 ng of ds-oligos, 2.0 μL of 10X reaction buffer, 2.5 μL of 2 mM dNTPs mix (except dCTP), 50 μCi αP^{32}-dCTP, and 1 unit of Klenow fragment (exo-) at 30°C for 15 min. The reaction was stopped by incubation of the reaction tubes at 70°C for 10 min. Thereafter, the labelled oligos were separated from unlabelled and free nucleotides by Sephadex G-50 spun column equilibrated with 0.19 TE buffer (pH 8.0). The first eluate after centrifugation was collected and its radioactivity content was measured by Beckman LS-100 liquid scintillation counter and stored at −70°C.

2.5.3. Electrophoretic Mobility Shift Assay. To analyze the interactions of transcription factors present in the nuclear extract to their corresponding promoter sequences,

the electrophoretic mobility shift assay technique was used. Approximately 5,500 cpm (corresponding to 0.1–0.2 ng) of the labelled ds oligos was used for each interaction reaction. The interaction reaction was carried out in 20 μL volume. The reaction mix constituted 20 μg of total nuclear protein, binding buffer, 1.0 μg of *Escherichia coli* sheared DNA and 0.1 ng (or 0.2 ng) of radiolabelled oligos. After that, the tubes were incubated at 22°C for 20 min for allowing the DNA-protein binding. The reaction was terminated by adding 5.0 μL of 5X loading dye (6% sucrose, 2 mM Tris-Cl, pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The interaction reaction products (samples) from various experimental sets were electrophoresed on a prerun (1 hour at 50 V) 6% nondenaturing polyacrylamide gel (acrylamide: bisacrylamide, 19 : 1) containing 0.5X TBE (Tris-borate, EDTA) buffer at 100 V (Constant) for 1 hour. At the end of the electrophoresis, the gel was transferred onto Whatman 1 M filter paper and fixed in a medium containing 10% acetic acid and 10% methanol for 15 min at room temperature. It was then covered with saran wrap and dried for 45 min at 80°C. The gel was exposed to the intensifying screen in cassette and signals for the radiolabelled DNA-protein complexes were captured in the Phsophor Imager. Later, the signal images were scanned using Alpha Imager Software for quantitation of the interactions.

2.6. Statistical Analysis. All the experiments were repeated three times (7 mice/age group/set). PCR amplified DNA bands and signals of complexes were quantitated using computer-assisted densitometry (Alpha-Ease FCTM software, Alpha Innotech Corporation, CA). Results represent the mean ± SEM of data obtained from three different sets of experiments. The mean ± SEM values were analyzed by SPSS (16.0) Software. All the data were evaluated with two-way analysis of variance (ANOVA) between subject factors age and treatment followed by Tukey HSD post hoc test. A difference of $P < 0.05$ was considered statistically significant for main effects; however, difference of $P < 0.1$ was considered significant for interactions.

3. Results

3.1. Analysis of NR1 in Frontal Cortex and Hippocampus. For expression of NR1 transcript in the frontal cortex of male mice, two-way ANOVA indicated the significant main effects of age ($F_{1,8}$ 52.591, $P = 0.000$), treatment ($F_{1,8}$ 240.154, $P = 0.000$), and the interaction of age × treatment ($F_{1,8}$ 5.564, $P = 0.046$) by comparing the PBDE-209-exposed group with control. Further, comparison of BM-supplemented groups with PBDE-209-exposed group indicated significant main effects of age ($F_{1,16}$ 43.291, $P = 0.000$) and treatment ($F_{3,16}$ 16.106, $P = 0.000$); however, the interaction of age × treatment ($F_{3,16}$ 0.760, $P = 0.533$) was not significant. RT-PCR data revealed that the mRNA expression of NR1 was significantly upregulated in the frontal cortex of PBDE-209-exposed neonate and young mice as compared with their respective controls ($P < 0.05$). However, following administration of 40, 80, and 120 mg/kg bw dose of BM in PBDE-209-exposed mice, a significant downregulation ($P < 0.05$) attaining the values of control in the expression of NR1 was found only at the maximum dose of BM (120 mg/kg) in the frontal cortex (Figure 1).

Similarly, in the hippocampus, main effects of age ($F_{1,8}$ 42.237, $P = 0.001$), treatment ($F_{1,8}$ 129.276, $P = 0.000$), and the interaction of age × treatment ($F_{1,8}$ 2.997, $P = 0.122$) were significant on comparison of PBDE-209-exposed group with control. Further, comparison of BM-supplemented groups with PBDE-209-exposed group indicated significant main effects of age ($F_{1,16}$ 53.709, $P = 0.000$) and treatment ($F_{3,16}$ 17.055, $P = 0.000$), whereas not in age × treatment ($F_{3,16}$ 0.501, $P = 0.687$) interaction. Similarly, in the hippocampus of neonate and young mice, a significant restoration ($P < 0.05$) was noticed at 120 mg/kg bw dose of BMagainst PBDE-209-induced upregulation in the mRNA expression of NR1 (Figure 2).

3.2. EMSA of REST/NRSF with Its Cognate NR1 Gene Promoter Sequences in Frontal Cortex and Hippocampus. In the frontal cortex of male mice, two-way ANOVA indicated the significant main effects of treatment ($F_{1,8}$ 126.221, $P = 0.000$), whereas age ($F_{1,8}$ 0.195, $P = 0.670$) and the interaction of age × treatment ($F_{1,8}$ 2.281, $P = 0.169$) were not significant as compared to PBDE-209-exposed group with control on binding of REST/NRSF to NR1 promoter. Further, comparison of BM-supplemented groups with PBDE-209-exposed group on same indicated significant main effects of treatment ($F_{3,16}$ 90.153, $P = 0.000$); however, the main effects of age ($F_{1,16}$ 0.330, $P = 0.573$) and the interaction of age × treatment ($F_{3,16}$ 1.347, $P = 0.295$) were not significant. The binding of REST/NRSF to NR1 promoter was significantly decreased in frontal cortex of PBDE-209-exposed neonate and young mice as compared with their respective controls ($P < 0.05$). Furthermore, supplementation with BM, at 120 mg/kg dose bw in PBDE-209-exposed mice, caused significant restoration ($P < 0.05$) in the binding of REST/NRSF (Figure 3).

In the hippocampus, main effect of treatment ($F_{1,8}$ 43.892, $P = 0.000$) was significant, while age ($F_{1,8}$ 0.475, $P = 0.510$) and the interaction of age × treatment ($F_{1,8}$ 2.071, $P = 0.188$) were not significant as compared to PBDE-209-exposed group with control on binding of REST to NR1 promoter. Further, comparison of BM-supplemented groups with PBDE-209-exposed group indicated significant main effect of age ($F_{1,16}$ 0.545, $P = 0.510$), treatment ($F_{3,16}$ 11.910, $P = 0.000$), and the interaction of age × treatment ($F_{3,16}$ 0.133, $P = 0.939$). However, a significant restoration ($P < 0.05$) was noticed at 120 mg/kg bw dose of BM against PBDE-209-induced decreased binding of REST/NRSF to NR1 promoter in the hippocampus of neonate and young mice (Figure 4).

4. Discussion

PBDE-209, a developmental neurotoxicant, causes behavioral impairments after exposure from PND 3 to PND 10 [46] which is a critical period for brain development, called brain growth spurt period [47]. In rats and mice, the brain growth reaches its peak at PND 10; however, it continues till the first 3-4 weeks of neonatal life. This period includes

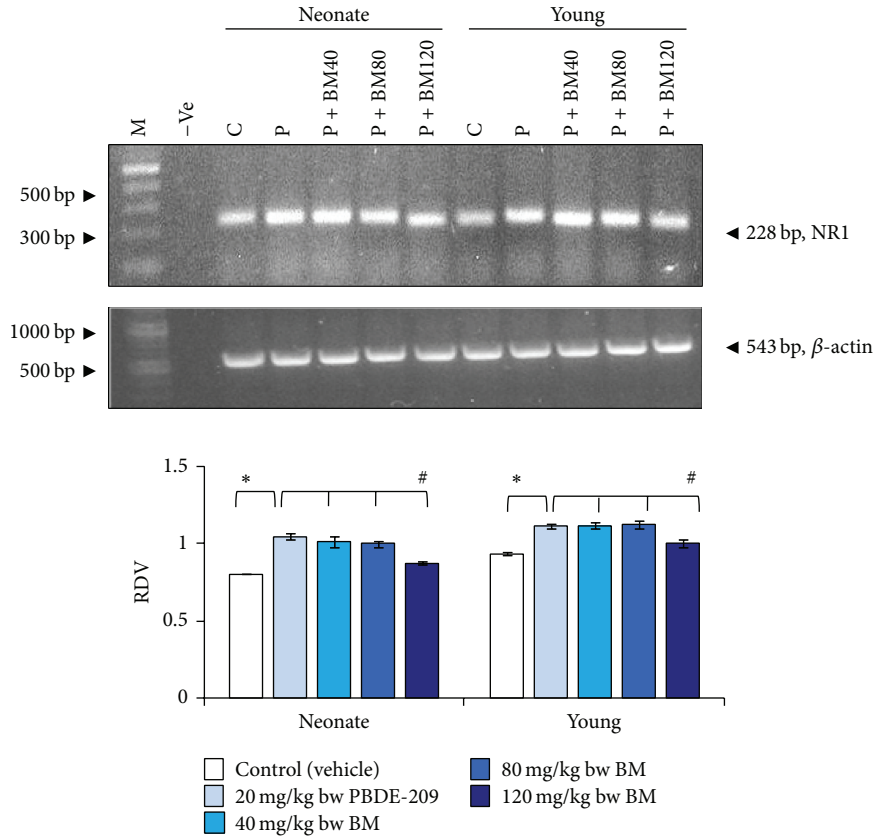

Figure 1: The prophylactic role of CDRI-08 (BM) (40, 80, and 120 mg/kg) against PBDE-209 (20 mg/kg) on mRNA expression of NR1 in frontal cortex of male mice at neonate and young age. M: marker (1000 bp DNA ladder), −Ve: negative control. Histograms represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments. $^{*}P < 0.05$, PBDE-209 versus control groups and $^{\#}P < 0.05$, *B. monnieri* doses versus PBDE-209 groups. RDV: relative densitometric value; C: control (vehicle); P: PBDE-209 (20 mg/kg); BM40: *B. monnieri* (40 mg/kg); BM80: *B. monnieri* (80 mg/kg); BM120: *B. monnieri* (120 mg/kg).

axonal and dendritic outgrowth, establishment of neuronal connections, synaptogenesis, and proliferation of glia cells with accompanying myelinisation [48]. Therefore, in the present study, we have investigated the protective role of CDRI-08 against PBDE-209-intoxicated mice pups from PND 3–10, acting through REST/NRSF-mediated expression and regulation of NR1 in the frontal cortex and hippocampus. From the present findings, it is postulated that it may be one of the mechanisms of CDRI-08 to improve memory, impaired by postnatal exposure of PBDE-209 in our previous report. It is well known that the glutamatergic system mediates activity-dependent processes in both the developing and the mature brain [49]. In particular, activation of the NMDAR subtype of glutamate receptor is required for the modulation of learning and memory functions and synaptic plasticity processes, such as LTP. Although it has been suggested that spatial learning and hippocampal LTP may be associated with a differential expression of NMDAR subunits, NR1, an obligatory subunit of NMDAR complex, is required for the proper functioning of NMDAR channel [24, 25]. On the contrary, the overstimulation and pronounced activation of NMDARs by excess glutamate binding cause an immense Ca^{2+} influx and a subsequent rise in the production of reactive oxygen species (ROS) which can weaken cellular antioxidation and conduct oxidative stress [50]. In the present study the increased expression of NR1 in frontal cortex and hippocampus of neonate and young male mice following postnatal exposure of PBDE-209 (20 mg/kg) may be attributed to increased ROS levels that results into neuronal damage and impaired learning and memory by PBDE-209 [21]. Subsequently, upregulated expression of NR1 was significantly restored by supplementation with CDRI-08 at the dose of 120 mg/kg bw in the present study. The current findings are consistent with the report of Paulose et al. [51]. According to them BM plays an important role in the alteration of glutamate receptor binding and gene expression of NR1 in hippocampus of pilocarpine-induced epilepsy in rats. Bacosides present in the CDRI-08 are non-polar glycosides; lipid-mediated transport may facilitate the bacosides to cross the blood–brain barrier by passive diffusion, which possibly act on the neurotransmitter system [52]. Considering the interaction of multiple neurotransmitters involved in learning and memory network, CDRI-08 also acts on the serotonergic system and the elevated level of 5-HT and upregulates the expression of 5-HT3A receptor, which possibly interacts with the cholinergic system [9, 53, 54]. Therefore, in the present study, it is hypothesized that CDRI-08 may act through glutamatergic system by supplementing CDRI-08 at the dose of 120 mg/kg. From the data of Zhou et al. [55],

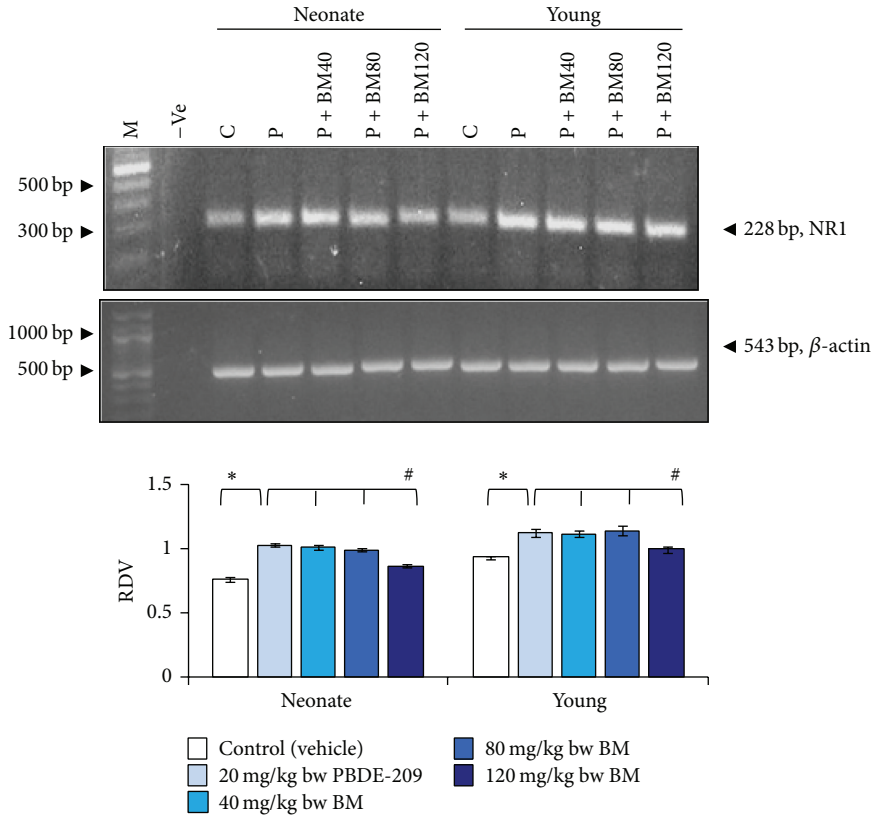

Figure 2: The prophylactic role of CDRI-08 (BM) (40, 80, and 120 mg/kg) against PBDE-209 (20 mg/kg) on mRNA expression of NR1 in hippocampus of male mice at neonate and young age. M: marker (1000 bp DNA ladder), −Ve: negative control. Histograms represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments. $^{*}P < 0.05$, PBDE-209 versus control groups and $^{\#}P < 0.05$, *B. monnieri* doses versus PBDE-209 groups. RDV: relative densitometric value; C: control (vehicle); P: PBDE-209 (20 mg/kg); BM40: *B. monnieri* (40 mg/kg); BM80: *B. monnieri* (80 mg/kg); BM120: *B. monnieri* (120 mg/kg).

it is concluded that CDRI-08 also significantly attenuated the L-NNA-induced anterograde amnesia and partially reversing L-NNA-induced retrograde amnesia.

In concurrence with the expression of NR1, binding of REST/NRSF respond differentially to the exogenous exposures as the present study suggested decreased binding of REST/NRSF to cognate promoter sequence of NR1 in response to postnatal exposure to PBDE-209 in the frontal cortex and hippocampus of neonate and young male mice. It was significantly restored following supplementation with CDRI-08 (120 mg/kg) in PBDE-209-exposed mice indicating the steady level of NR1 expression. It has been reported that REST/NRSF dysregulation is implicated in some pathological disorders of the nervous system, such as global ischemia and epilepsy [34, 35]. To our knowledge, we provide here the first evidence that REST/NRSF is involved in PBDE-209-induced neurotoxicity. REST/NRSF functions as a transcriptional repressor of neuronal genes [56, 57]. The REST/NRSF binding motif termed RE1/NRSE is located approximately −140 to −250 bp 5′ of the initiation site of NR1 mRNA [58].

Recently, studies of neuronal gene expression have revealed a negative regulatory mechanism by which the RE1/NRSE element interacts with the active suppressor REST/NRSF. Since REST/NRSF mRNA is expressed at a high level in the embryonic brain and becomes reduced in the developing brain during the neonatal period, it is proposed that regulation of this protein plays a key role in activation of the neuronal genes during the brain development [56, 57]. REST/NRSF blocks transcription of a gene when the RE1/NRSE is located upstream or downstream of the open reading frame in either orientation. REST/NRSF is identified as a silencing element of the genes encoding SCG10 and type II sodium channel [56, 57]. Contrary to REST/NRSF pattern, the expression of NR1 mRNA remains at a low level in embryonic brain and undergoes a robust increase in the brain during the neonatal brain development [59]. This suggests that the increased binding of REST/NRSF to NR1 promoter causes reduced expression of NR1 and vice versa. REST/NRSF silences the expression of its target genes by its two independently acting repressor domains at the N- and C-termini. The N-terminal repressor domain of REST/NRSF has been shown to recruit some corepressors such as mSIN3 and histone deacetylases (HDACs) into the vicinity of the promoter. Histone deacetylation leads to a more compact chromatin that prevents accessibility of transcription factors. The C-terminal repressor domain (CTRD) of REST/NRSF has been shown to interact with

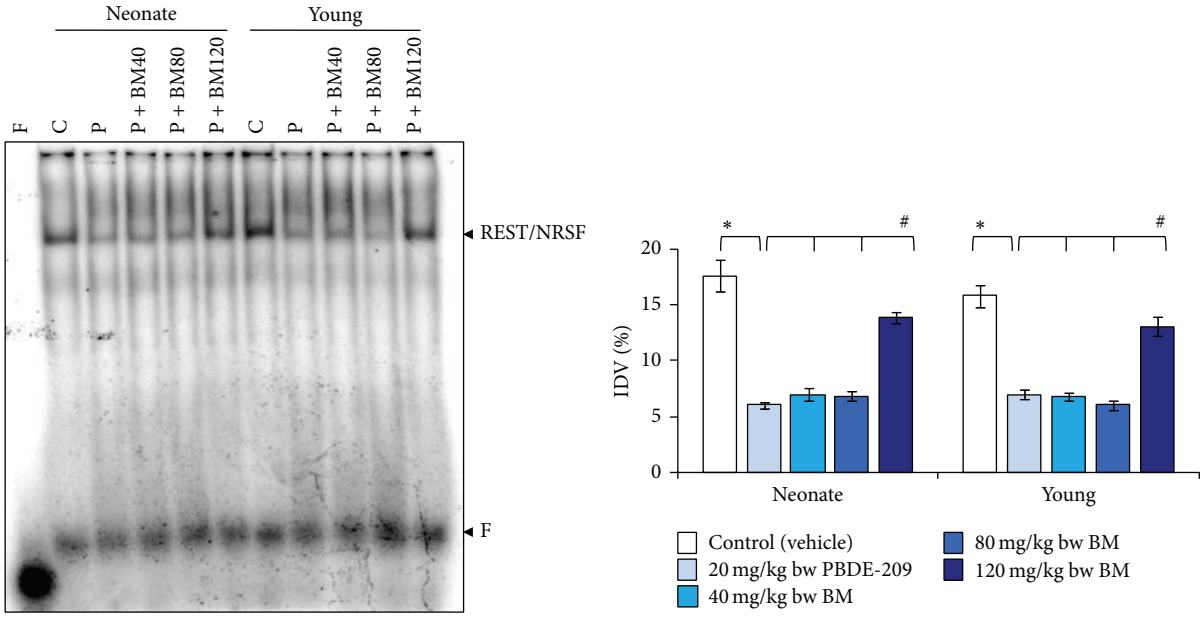

Figure 3: The prophylactic role of CDRI-08 (BM) (40, 80, and 120 mg/kg) against PBDE-209 (20 mg/kg) on the binding of REST/NRSF to their cognate promoter site on NR1 in frontal cortex of male mice at neonate and young age. Histograms represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments. $^{*}P < 0.05$, PBDE-209 versus control groups and $^{\#}P < 0.05$, *B. monnieri* doses versus PBDE-209 groups. IDV: %integrated density value; C: control (vehicle); P: PBDE-209 (20 mg/kg); BM40: *B. monnieri* (40 mg/kg); BM80: *B. monnieri* (80 mg/kg); BM120: *B. monnieri* (120 mg/kg).

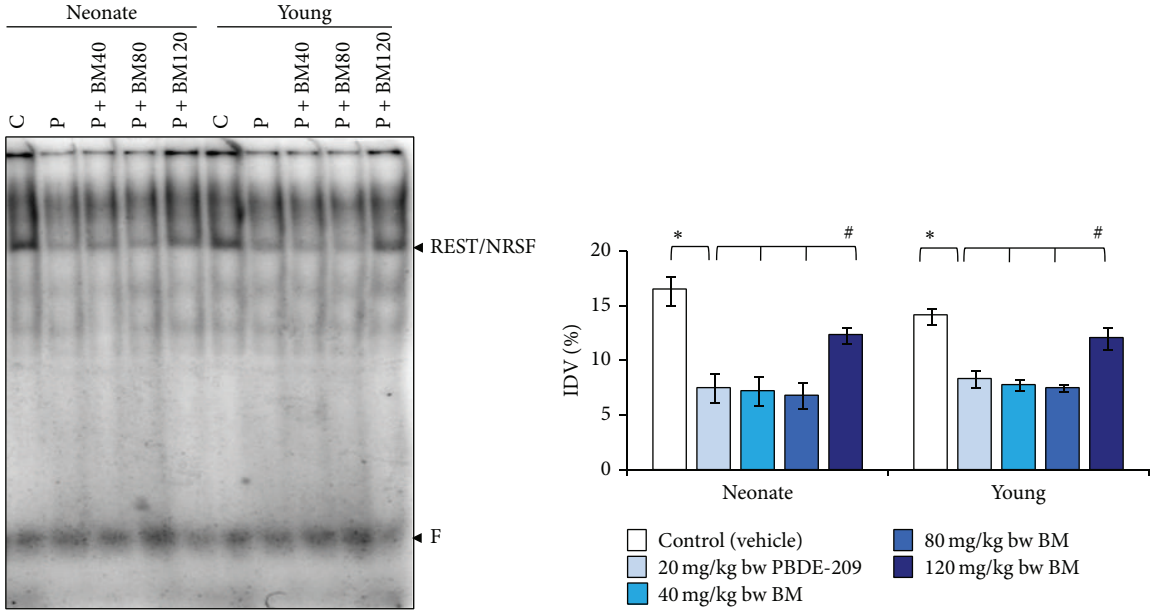

Figure 4: The prophylactic role of CDRI-08 (BM) (40, 80, and 120 mg/kg) against PBDE-209 (20 mg/kg) on the binding of REST/NRSF to their cognate promoter site on NR1 in hippocampus of male mice at neonate and young age. Histograms represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments. $^{*}P < 0.05$, PBDE-209 versus control groups and $^{\#}P < 0.05$, *B. monnieri* doses versus PBDE-209 groups. IDV: %integrated density value; C: control (vehicle); P: PBDE-209 (20 mg/kg); BM40: *B. monnieri* (40 mg/kg); BM80: *B. monnieri* (80 mg/kg); BM120: *B. monnieri* (120 mg/kg).

at least one factor, the transcriptional corepressor CoREST that may serve as a platform protein for the recruitment of molecular machinery that imposes silencing across a chromosomal interval [60, 61].

The findings of the current study and our previous reports suggest that the REST/NRSF-mediated increase in NR1 expression observed in this study could indeed underlie the damaging effect of PBDE-209 on learning and memory. Furthermore, CDRI-08 has capability to improve memory possibly via acting through NR1 and its regulation especially by REST/NRSF-mediated regulation. This might be one of the mechanisms of CDRI-08 for the enhancement of memory.

In conclusion, our present findings provide insights of a molecular mechanism of bacosides enriched ethanolic extract of BM (CDRI-08) in improvement of memory against PBDE-209-induced impairment and suggest REST/NRSF as an attractive molecular target in CDRI-08-mediated therapy. Since REST/NRSF acts as negative regulator of NR1 gene, it maintains the steady level of expression of NR1 by binding of REST/NRSF transcription factor to cognate sequence of NR1 promoter. Thus CDRI-08 has capability to reinstate the expression and regulation of NR1, impaired by postnatal exposure of PBDE-209. Future study may need investigations on the role of CDRI-08 on downstream signaling mechanisms of memory consolidation.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Bacopa monnieri Extract (CDRI-08) Modulates the NMDA Receptor Subunits and nNOS-Apoptosis Axis in Cerebellum of Hepatic Encephalopathy Rats

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Hepatic encephalopathy (HE), characterized by impaired cerebellar functions during chronic liver failure (CLF), involves N-methyl-D-aspartate receptor (NMDAR) overactivation in the brain cells. *Bacopa monnieri* (BM) extract is a known neuroprotectant. The present paper evaluates whether BM extract is able to modulate the two NMDAR subunits (NR2A and NR2B) and its downstream mediators in cerebellum of rats with chronic liver failure (CLF), induced by administration of 50 mg/kg bw thioacetamide (TAA) i.p. for 14 days, and in the TAA group rats orally treated with 200 mg/kg bw BM extract from days 8 to 14. NR2A is known to impart neuroprotection and that of NR2B induces neuronal death during NMDAR activation. Neuronal nitric oxide synthase- (nNOS-) apoptosis pathway is known to mediate NMDAR led excitotoxicity. The level of NR2A was found to be significantly reduced with a concomitant increase of NR2B in cerebellum of the CLF rats. This was consistent with significantly enhanced nNOS expression, nitric oxide level, and reduced Bcl2/Bax ratio. Moreover, treatment with BM extract reversed the NR2A/NR2B ratio and also normalized the levels of nNOS-apoptotic factors in cerebellum of those rats. The findings suggest modulation of NR2A and NR2B expression by BM extract to prevent neurochemical alterations associated with HE.

1. Introduction

The patients with liver cirrhosis develop a serious nervous system disorder known as hepatic encephalopathy (HE) [1]. It is characterized by wide spectrum of neuropsychiatric symptoms related to motor dysfunction, cognitive impairment, and disturbed sleep wake cycle [2–4]. Most of the liver cirrhotic patients have been found to show minimal to overt HE symptoms [5, 6], characterized mainly by the impaired motor functions [2, 7–9], which is considered to be associated with deranged cerebellar functions [10, 11]. Some recent findings from our lab also suggest that cerebellum shows greater susceptibility to undergo neurochemical changes in the models of chronic type HE [12–14]. Therefore, cerebellum was selected for the present study.

Based on the studies conducted to understand pathophysiology of HE, it has been suggested that the level of glutamate, an important excitatory neurotransmitter, increases in the synaptic cleft due to the increased blood and brain ammonia level during liver dysfunction, resulting into overactivation of the ionotropic N-methyl-D-aspartate receptors (NMDAR) [1]. And there is a general agreement that activation of glutamate-NMDA receptor-nNOS pathway constitutes main neurochemical aberrations associated with HE [1, 14].

Functional NMDAR, a tetrameric protein complex, comprises of two subunits of a constitutive glycine binding NR1 and remaining two of glutamate binding NR2 from amongst NR2A, NR2B, NR2C, and NR2D subunits. Importantly, combination of different NR2 subunits is suggested to confer unique electrophysiological properties to this neurotransmitter receptor [15, 16]. For example, alterations in the ratio of NR2A versus NR2B of NMDAR have been found to be associated with the changes in long term potentiation (LTP) and long term depression (LDP) functions during hippocampal plasticity involved in memory consolidation [17]. In addition, NR2A dominating combination of NMDAR is demonstrated to provide neuroprotection but NR2B rich

NMDAR is known to drive the postsynaptic neuron towards apoptosis during glutamate excitotoxicity [14]. Thus, regulating NMDAR function by altering its composition, without pharmacological blockage of the channel, could be a unique and novel cerebral mechanism to prevent NMDAR overactivation led neurological disorders.

It is now evident that neuronal nitric oxide synthase (nNOS), via modulating NO level in the brain cells, plays critical roles in transmitting NMDAR led neurophysiological changes under different pathophysiological conditions [18, 19]. A threshold level of NO is essential to activate NO-cGMP signaling to maintain NMDAR dependent memory consolidation and cognition functions [18]. However, excess of NO is known to induce apoptosis and neuronal death [20, 21]. Such multimodal roles of NMDAR-nNOS axis in brain cells are orchestrated by a molecular link between NMDAR and nNOS protein. The NR2 subunits of NMDAR complex, through their tSXV motifs, connect with the postsynaptic density protein-95 (PSD-95), which in turn, via its PDZ domain, interacts with the nNOS in the postsynaptic neurons [19, 22]. This constitutes the main mechanism of NMDAR activity led NO production and subsequent changes in the neuronal functions. Particularly during HE, activation of NMDAR leads to the increased calcium influx which in turn activates nNOS and thereby overproduces NO in the postsynaptic neurons [1, 9, 13]. Thus increased glutamate led NMDAR activation, via activation of nNOS, is considered critically involved in developing HE associated neuropsychiatric problems in the patients/animals [13, 23, 24].

Obviously NMDAR becomes choice of an important therapeutic target for the neurobiologists for HE management [25, 26]. Some earlier studies conducted *in vivo* and *in vitro* using NMDAR antagonists indeed demonstrated desirable results; however, this approach was found to produce undesirable neurological complications during the clinical trials [25, 27]. This is not surprising as NMDAR activity is critical for maintaining normal neurophysiology including higher order brain functions and memory consolidation mechanisms [17]. Therefore, instead of blocking NMDAR channel, modulation of NMDAR activity by alterations in its functional composition and downstream signaling seems to be of special scientific merit. However, this evolving concept needs to be examined in the animal models with excitotoxic neurological problems. Since development of HE is related with NMDAR led excitotoxicity [1] and that herbal formulations are now evident to modulate brain chemistry in many ways, the present work was undertaken to evaluate whether *Bacopa monnieri* (BM) extract, a known neuroprotectant, is able to modulate NMDAR composition and related downstream events in cerebellum of the CLF induced HE rats.

Amongst a good number of herbal drugs available, *Bacopa monnieri* extract has been widely evaluated as a memory enhancer, adaptogenic, anti-inflammatory, analgesic, antipyretic, sedative, and antiepileptic agent [28]. Also, some studies suggest its neuroprotective roles against epilepsy (neuroexcitotoxic outcome) by modulating serotonergic receptor [29], and against Parkinson's and Alzheimer's disease via altering dopaminergic signaling [30] and cholinergic [31] receptors, respectively. Moreover, molecular mechanisms underlying these BM extract effects remain largely unexplored.

Importantly, though information is limited, efficacy of BM extract has also been shown against glutamate toxicity via modulating NMDAR1 gene expression and in turn affecting glutamatergic signaling [32]. In our previous reports, we have observed a direct association between overexpression of the constitutive NR1, nNOS activation, and enhanced NO production in cerebellum of the CLF rats exhibiting HE characteristics [2, 13, 14]. Importantly, we could also observe reciprocal expression of NR2A and NR2B in the cerebellum of those rats (data from this paper). This tempted us to investigate whether administration of BME is able to alter this unusual NR2A/2B composition and thus NMDAR-nNOS pathway in the cerebellum of the HE rats.

2. Materials and Methods

2.1. Chemicals. Chemicals used were of analytical grade supplied by E-Merck and Sisco Research Laboratory (India). Acrylamide, *N,N′*-methylenebisacrylamide, N,N,N′N′-tetramethylethylenediamine (TEMED), phenyl methyl sulphonyl fluoride (PMSF), bromophenol blue, and Ponceau were purchased from Sigma-Aldrich, USA. Primary antibodies used were procured from the following companies: rabbit monoclonal β-actin from Sigma Aldrich, rabbit monoclonal anti-NR2B from Invitrogen, rabbit monoclonal NR2A from Epitomics, rabbit polyclonal Bcl2 and Bax from Cell Signaling Technology, and rabbit polyclonal nNOS from Santa Cruz Biotechnology. Rabbit and mouse horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Genei. ECL western blotting detection kit was purchased from Thermo Scientific.

The ethanolic extract of *Bacopa monnieri* extract (BM/CDRI-08), containing 64.28% bacoside A and 27.11% bacoside B, was obtained from the Lumen Research Foundation, Chennai, India.

2.2. Animals. Inbred adult female albino rats weighing 150–160 g were used in this study. The rats were kept in separate cages, fed with the recommended diet, and maintained at standard conditions of 12 h light and dark period at room temperature ($25 \pm 2^{\circ}$C) in an animal house. The use of animals for the present study was approved by the Institutional Animal Care and Use Committee (IACUC); Animal Ethical Committee (AEC) of the Banaras Hindu University, Varanasi.

2.3. Induction of Chronic Liver Failure (CLF)/HE and Treatment Schedule. The CLF/HE model of neuroexcitotoxicity in adult albino rats was induced by the administration of thioacetamide (TAA) as standardized previously [2]. For this, rats were randomly divided into three groups with 6 rats in each. Group A: control (C), administered with 0.9% saline i.p, once daily for 14 days; Group B: CLF/HE group, administered with 50 mg/Kg bw TAA i.p once daily for 14 days; Group C: CLF + Bacopa extract (CLF + BM). Rats in Group C were orally administered with ethanolic extract of BM extract (CDRI-08; 200 mg/Kg bw), suspended in 1%

gum acacia, once daily starting from 8th day onwards till 14th day, and were administered 4 h after the TAA treatment. The dose of BM was selected which was able to recover TAA induced neurobehavioural deficit in the rats. All the rats were sacrificed on 15th day. The cerebellum was dissected out and stored at −80°C for further experiments.

2.4. Preparation of Cerebellar Extracts. As described earlier [13], mitochondria free cerebellar extracts were prepared in an extraction medium consisting of 400 mM sucrose, 1 mM EDTA, 0.2 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.02% heparin. The extracts were centrifuged initially at 12000 ×g for 15 min and finally at 19000 ×g for 45 min at 4°C. The supernatant obtained was collected as the cytosolic fractions. The protein content in the tissue extract was measured by Lowry method [33] using BSA as standard.

2.5. Western Blotting. As described previously [13], the cytosolic fractions containing 60 μg protein/lane were separated on 10% SDS-PAGE and electrotransferred to nitrocellulose membrane at 50 mA and run overnight at 4°C. Protein transfer was checked via Ponceau staining. The membrane was then placed in a blocking solution of 5% skimmed milk in 1X PBS for 2 h followed by washing in PBS 3 times. The membranes were then separately processed for immunodetection of NR2A, NR2B, nNOS, Bcl2, and Bax using monoclonal/polyclonal anti-NR2A (1 : 1000), anti-NR2B (1 : 1000), anti-nNOS (1 : 500), anti-Bcl2 (1 : 1000), and anti-Bax (1 : 500), respectively. HRP conjugated secondary antibody was used for final detection of the proteins using ECL western blotting detection kit. β-actin, used as loading control, was detected using a monoclonal anti-β-actin peroxidase antibody. The bands were quantified and analyzed using gel densitometry software AlphaImager 2200. The photographs in the figure are representatives of the three western blot repeats.

2.6. Nitric Oxide (NO) Estimation. Nitric oxide level was measured by estimating total nitrite (NO_2) and nitrate (NO_3) content in the tissue extracts as described earlier [13] using the method of Sastry et al. [34]. Briefly, tissue fractions (100 μL), $NaNO_2$, and KNO_3 standards (0.1 mM each) were mixed separately with 400 μL of 50 mM carbonate buffer (pH 9). For NO_3 estimation, activated copper-cadmium alloy (150 mg) was added and incubated for an hour at 37°C. The reaction was stopped using 100 μL each of 0.35 M NaOH and 120 mM zinc sulphate. After centrifugation, 400 μL supernatant was incubated with the Griess reagent: 200 μL 1% sulphanilamide prepared in 2.5% H_3PO_4 and 200 μL 0.1% N-(1-naphthyl)-ethylenediamine. Absorbance was recorded at 545 nm. For NO_2 estimation, similar procedure was followed except addition of the copper-cadmium alloy.

2.7. Statistical Analysis. The data have been expressed as mean ± SD. For two group comparisons, statistical analysis was performed using unpaired Student's t test. A probability of $P < 0.05$ was taken as a significant difference between the groups. Each of the experiments was repeated thrice.

3. Results

3.1. Effect of BM Extract on NR2A and NR2B Expression. The combination of constituent NMDAR subunits is evident to impart unique neurophysiological role of this glutamate receptor. The NR2A dominating combination is known to mediate neuroprotection whereas those of NR2B induce neuronal death. According to Figures 1(a) and 1(b), as compared to the control rats, level of NR2A is found to be significantly reduced ($P < 0.001$) with a concomitant increase in NR2B level in the cerebellum of the CLF rats (Figures 1(c) and 1(d)), resulting in a significant decline in NR2A/2B ratio (Figure 1(e)). However, this pattern is observed to be recovered back with a significant enhancement of NR2A (Figures 1(a) and 1(b)) and a decline of NR2B (Figures 1(c) and 1(d)), resulting into attaining a control level NR2A/2B ratio (Figure 1(e)) in the cerebellum of the CLF rats treated with the BM extract.

3.2. Effect of BM Extract on nNOS and Nitric Oxide (NO) Production. Neuronal NOS (nNOS) has a direct molecular link with NMDAR and therefore, it is considered to be the main determinant of NMDAR activation based downstream signaling in the postsynaptic neurons. Accordingly, overactivation of nNOS is considered associated with the neuronal changes associated with NMDAR led excitotoxicity. As depicted in Figures 2(a) and 2(b), nNOS expression is observed to be enhanced significantly ($P < 0.001$) in the cerebellum of the CLF rats as compared to the control group rats. Moreover, due to the oral administration of BM extract, level of this enzyme is observed to be reduced up to the control value (Figures 2(a) and 2(b)). Such a pattern could coincide well with the similar changes in the NO level in cerebellum of the CLF and BM extract treated CLF rats (Figure 2(c)).

3.3. Effect of BM Extract on the Expression of Bcl2 and Bax. Bcl2 (antiapoptotic) and Bax (proapoptotic) ratio serves as a rheostat to determine cell susceptibility to apoptosis. According to Figures 3(a) and 3(c), as compared to the control group rats, level of Bcl2 is found to be declined significantly ($P < 0.05$) with a concomitant increase in the Bax level resulting in a significant decline ($P < 0.01$) in Bcl2/Bax ratio (Figure 3(e)) in cerebellum of the CLF rats. However, after treatment with BM extract, the pattern of Bcl2/Bax ratio is observed to regain its normal range in cerebellum of those CLF rats (Figure 3(e)).

4. Discussion

NMDA receptor overactivation is considered a common neurochemical event associated with a number of brain dysfunctions like epilepsy, ischemia, drug abuse, and HE [23, 26, 35–37]. However, NMDAR blockage is evident to be a poor therapeutic target for managing such excitotoxic conditions [25, 27]. In this background, while understanding neurochemical basis of CLF induced HE, we observed a clear

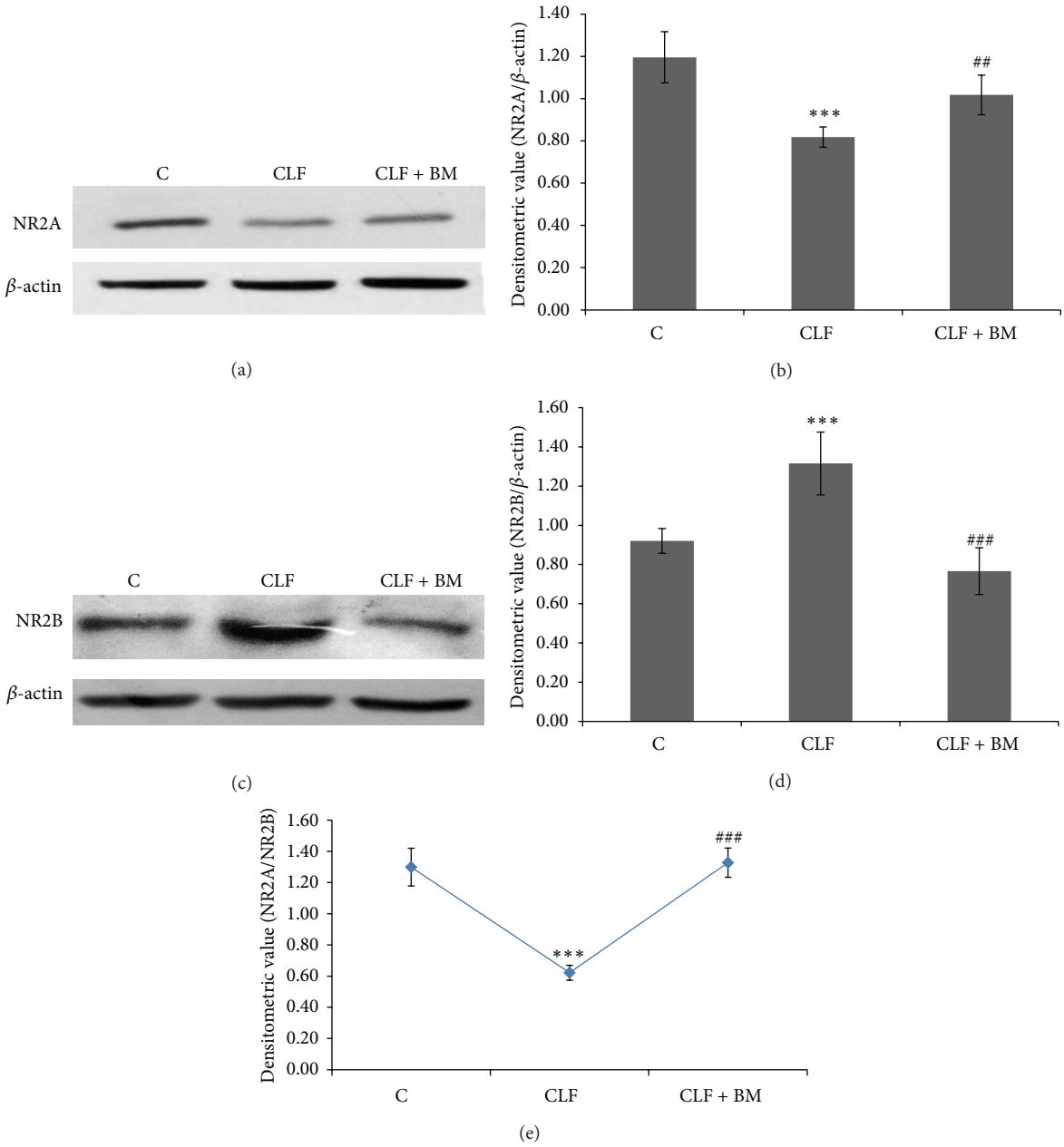

Figure 1: Expression profile of NR2A ((a) and (b)) and NR2B ((c) and (d)) in cerebellum of the CLF and BM extract treated CLF rats. Western blot analysis was performed as described in the method text. The level of β-actin was probed as the loading control. In panels (b), (d), and (e), normalized densitometric values of NR2A/β-actin, NR2B/β-actin, and NR2A/NR2B have been presented as mean ± SD from three western blot repeats. $^{***}P < 0.001$; (control versus CLF rats) $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; (CLF versus CLF + BM rats). C: control, CLF: chronic liver failure, CLF + BM: chronic liver failure + *Bacopa monnieri* extract.

shift in the expression of glutamate binding NMDAR subunit from a NR2A dominating combination to a NR2B rich combination in cerebellum of the CLF rats (Figures 1(a) and 1(c)). It is now becoming clearer that NR2A rich NMDAR imparts neuroprotection and that of NR2B dominating combination initiates neuronal damage and apoptosis [16, 38–40]. This is because NR2B has been reported to show delay in gating kinetics in comparison to that of NR1/NR2A combination resulting in increased Ca^{2+} influx and thus rapid activation of the downstream signaling [41]. In the present context, a significant decline in Bcl2 with a concomitant increase in Bax level in cerebellum of the CLF rats (Figure 3) also hints for a direct association between NR2B dominating NMDAR composition and altered neurochemistry of cerebellum of the CLF rats. Furthermore, alterations in oxidative and nitrosative factors in the postsynaptic neurons are considered to be the main downstream mediators of such unusual NMDAR activations [1] and it has been reported that cerebellum shows

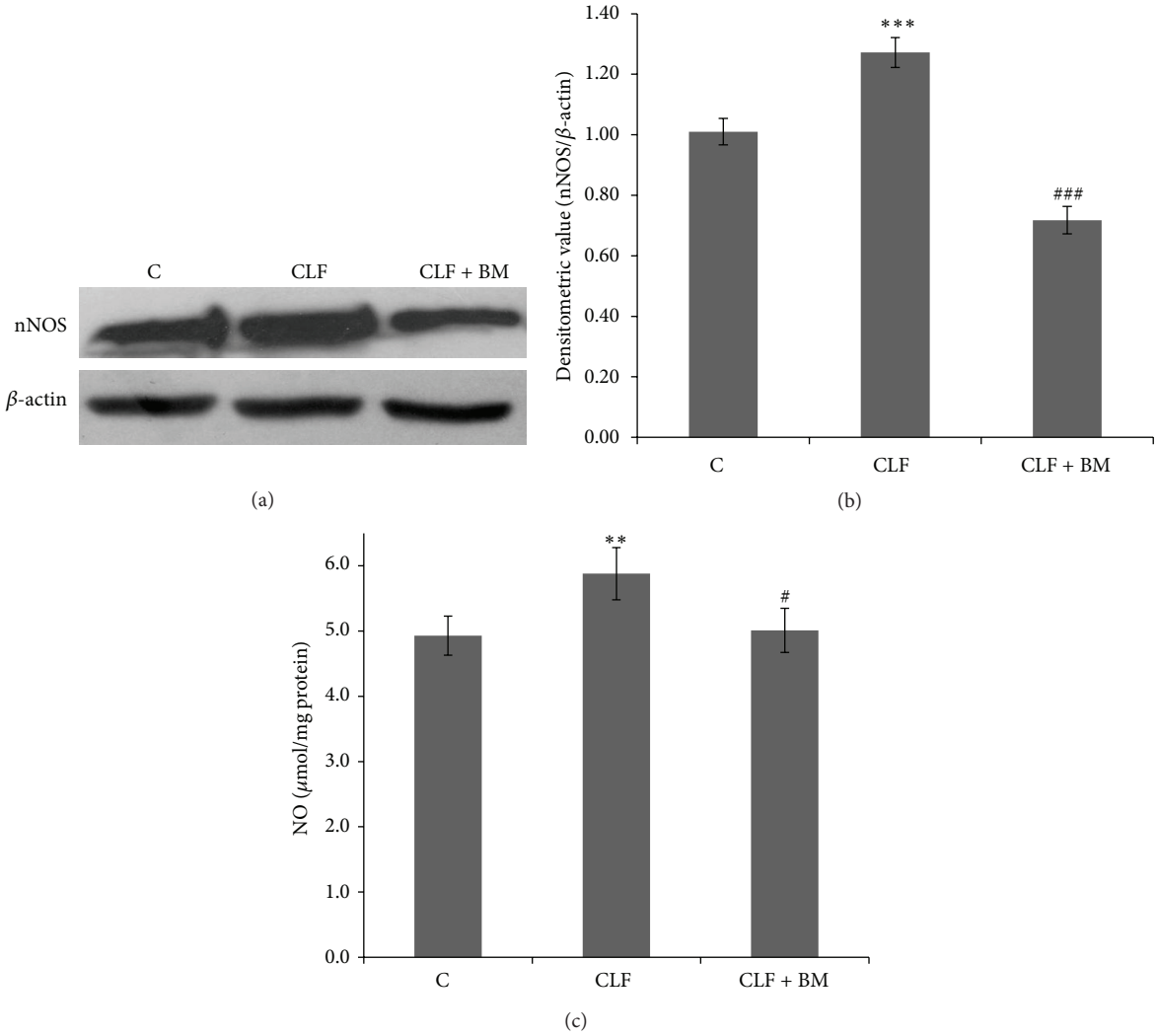

Figure 2: Expression profile of nNOS ((a) and (b)) and NO level (c) in cerebellum of the CLF and BM extract treated CLF rats. Western blot analysis of nNOS was performed as described in the text of methods. In panel (b), normalized densitometric values of nNOS/β-actin have been presented as mean ± SD from three western blot repeats. $^{**}P < 0.01$, $^{***}P < 0.001$; (control versus CLF rats) $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$; (CLF versus CLF + BM rats). C: control, CLF: chronic liver failure, CLF + BM: chronic liver failure + *Bacopa monnieri* extract.

greater susceptibility to undergo oxidative and nitrosative stress during HA and CLF led excitotoxicity [12, 13].

It has been described that lesion in cerebellum impairs acquisition of memory consolidation and deranges motor functions [10, 11]. The CLF rats used in this experiment have recently been reported to show cognitive impairment and deficit in motor functions as well [2]. Since such neurochemical alterations leading to neurobehavioural changes are known to emanate from abnormal NMDAR activity [1], it is argued that a shift from a NR2A combination to a NR2B combination of NMDAR in the cerebellum of the CLF rats might be accountable for developing HE associated symptoms observed in these CLF rats [2]. Moreover, keeping aside these explanations, the findings provided a basis to alter NMDAR constitution, instead of shutting this ion-channel off, as a therapeutic option to bring recovery from the HE symptoms.

To test this hypothesis, BM extract was chosen due to the two reasons. Firstly, amidst scarcity of the safer neuropharmacological agents, this plant extract is demonstrated to improve neuronal functions by modulating brain chemistry in many ways [28]. Secondly, BM is now evident to modulate activity of the neurotransmitter receptors like serotonergic receptors during epilepsy [29] and dopaminergic and cholinergic signaling in Parkinson's and Alzheimer's diseases [30, 31]. However, report is limited on modulation of NMDA receptor activity by BM. We observed a remarkable shift from a declined ratio of NR2A/NR2B (neurodegeneration supportive combination) in the cerebellum of CLF rats towards their normal level when these CLF rats were administered with BM extract (Figure 1(e)). This finding suggested that BM is able to alter constitution of the functional NMDAR tetramer by differential expression of the two glutamate binding subunits in cerebellum of the CLF rats. Indeed, in

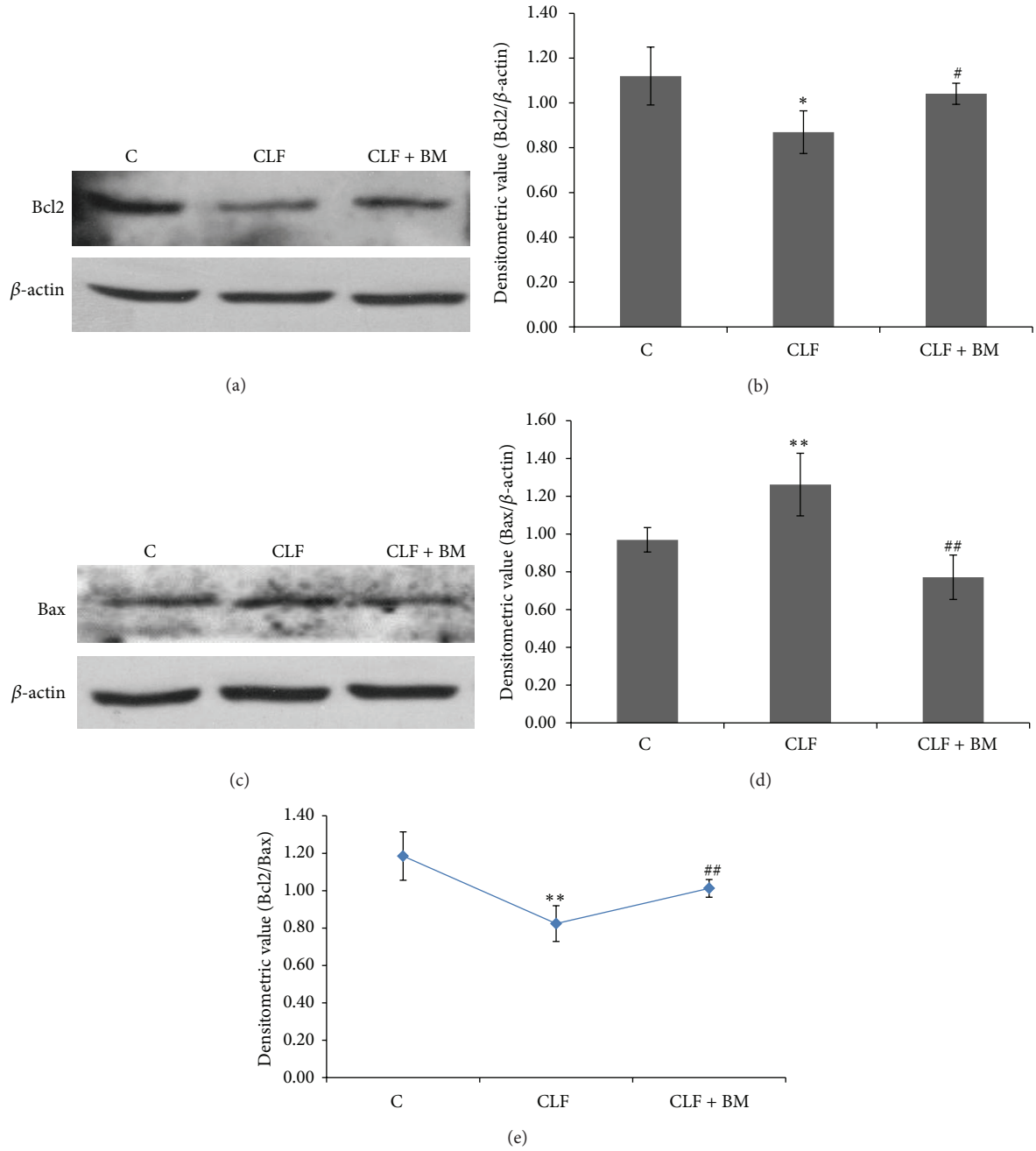

Figure 3: Expression profile of Bcl2 ((a) and (b)) and Bax ((c) and (d)) in cerebellum of the CLF and BM extract treated CLF rats. Western blot analysis of Bcl2 and Bax was performed as described in the text of methods. The level of β-actin was probed as the loading control. In panels (b), (d), and (e), normalized densitometric values of Bcl2/β-actin, Bax/β-actin, and Bcl2/Bax ratio, respectively, have been presented as mean ± SD from three western blot repeats. $^{*}P < 0.05$, $^{**}P < 0.01$; (control versus CLF rats) $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; (CLF versus CLF + BM rats). C: control, CLF: chronic liver failure, CLF + BM: chronic liver failure + *Bacopa monnieri* extract.

an epilepsy model, it has been demonstrated that BM could alter NR1 gene expression [32], thus suggesting BM as a modulator of NMDAR subunit expression. A similar finding on modulation of NR1 by BM was reported in a hypoxia model also [42]. Importantly, such a change in NR1 expression was found accountable for modulating glutamatergic signaling in cerebellum of those rats [32]. Thus, it is argued that shifting from a neurodegenerative NR2B overexpression towards the neuroprotective NR2A level due to the treatment with BME (Figure 1) could also account for preventing downstream undesirable neurochemical changes in cerebellum of the CLF rats.

In a number of excitotoxic models, including CLF led HE, nNOS activation is considered as the most common event after NMDAR activation [13, 43]. It is initiated by influx of Ca^{2+} which overactivates this enzyme to produce excess

NO in the postsynaptic neurons [44]. NO is a molecule of pleiotropic effects; however, when produced in excess in the brain cells, it uses more than one mechanism to induce neuronal dysfunction [20, 21]. A milder increase in NO level is likely to initiate mitochondrial dysfunction led neuronal apoptosis. Ratio of Bcl2 versus Bax is considered as the most effective regulators of mitochondrial dysfunction led apoptosis [45] and hence relative levels of both these proteins are considered as a reliable tool to assay whether a cell is likely to undergo internal apoptotic process [46]. In cerebellum of the CLF rats, a significantly increased level of nNOS coincides well with a similar increment in NO level (Figure 2). This is consistent with a significant decline in the Bcl2/Bax ratio (Figure 3). Moreover, all these factors were found to be reversed to regain their normal levels in the cerebellum when those CLF rats were treated with BM extract (Figures 1–3). Recent studies have also shown that BM extract downregulates Bax and upregulates Bcl2 and thus provides neuroprotection in several neurological disease models [47]. Thus, the findings of Figures 1–3 together advocate for concordant modulation of NMDA receptor constitution and nNOS led apoptotic activation by ethanolic extract of BM (CDRI-08) in cerebellum of the CLF rats.

5. Conclusion

Opposing roles of the two main glutamate binding subunits, NR2A and NR2B, of NMDA receptor advocate for modulation of NMDAR constitution, as an effective mechanism to normalize its excitotoxic effects without blocking its ion-channel activity. During recent past, studies on efficacy of *Bacopa monnieri* extract have been shown to prevent neurodegenerative diseases by modulating neurochemistry of the brain cells, but with little information about modulation of NMDAR overactivation led excitotoxicity. The present findings demonstrate that BM extract is able to modulate NMDA receptor signaling by bringing reciprocal changes in the expression of its two glutamate binding subunits, NR2A and NR2B, and thus provide a novel approach to normalize NMDAR overactivation effects without blocking this ion channel.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Review Article

Bacopa monnieri as an Antioxidant Therapy to Reduce Oxidative Stress in the Aging Brain

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The detrimental effect of neuronal cell death due to oxidative stress and mitochondrial dysfunction has been implicated in age-related cognitive decline and neurodegenerative disorders such as Alzheimer's disease. The Indian herb *Bacopa monnieri* is a dietary antioxidant, with animal and *in vitro* studies indicating several modes of action that may protect the brain against oxidative damage. In parallel, several studies using the CDRI08 extract have shown that extracts of *Bacopa monnieri* improve cognitive function in humans. The biological mechanisms of this cognitive enhancement are unknown. In this review we discuss the animal studies and *in vivo* evidence for *Bacopa monnieri* as a potential therapeutic antioxidant to reduce oxidative stress and improve cognitive function. We suggest that future studies incorporate neuroimaging particularly magnetic resonance spectroscopy into their randomized controlled trials to better understand whether changes in antioxidant status *in vivo* cause improvements in cognitive function.

1. Introduction

The world's population is aging rapidly [1]. One consequence of an aging population is an increased prevalence of chronic, age-related illnesses and disorders involving oxidative stress and low level chronic inflammation [2]. Increasing age is a major risk factor for dementia, including Alzheimer's disease (AD), and other prevalent neurodegenerative disorders [3]. The causes of brain aging and dementia are complex and incompletely understood.

Oxidative stress is one mechanism that detrimentally contributes to the aging process and is inextricably linked to neurodegenerative disorders [4]. Interventions that manipulate the oxidative stress mechanisms may decrease oxidative damage, slow the rate of aging, and lessen the risk of neurodegenerative disorders, increasing the lifespan of older adults. Research has begun to focus on developing effective health and lifestyle interventions so that older adults are able to remain both physically and cognitively healthy into older age, reducing the social and economic burden associated with an aging population [5].

The Indian herb, *Bacopa monnieri* (EBm) may serve as a dietary antioxidant, with several modes of action to protect the brain against oxidative damage and age-related cognitive decline. Several studies using the standardized CDRI08 extract have shown that EBm improves cognitive function particularly in the elderly [6–8]. Animal and *in vitro* studies using the standardized extract CDRI08 have revealed promising results to elucidate EBm's antioxidant properties (e.g., [9–12]). The aim of this review is to examine the evidence for EBm as a potential therapeutic antioxidant to reduce oxidative stress in the aging brain and as a mechanism by which it may improve cognition. We also discuss magnetic resonance spectroscopy (MRS) as a technique to elucidate the antioxidant mechanisms of action of EBm in human research *in vivo*.

2. The Aging Brain

Deterioration in memory performance is a signature of advancing age. Almost 50% of adults aged 64 years and over tend to report difficulties with their memory [13]. In addition

to memory, executive function, processing speed, attention, and spatial ability have also been shown to deteriorate with age [14–19]. While most cognitive functions decline with age, cognitive aging does not occur uniformly at the same rate and to the same extent for all people [20]. There may be several reasons for this including differences in lifestyle factors, particularly dietary intake.

Aging is a predominant risk factor for dementia, including AD [3]. An imbalance between the production and clearance of abnormal proteins called β-amyloid [21], the formation of neurofibrillary tangles, and neuroinflammation are hallmarks of advanced brain aging and dementia [22]. Neuroimaging studies have reliably identified that with increasing age, ventricular enlargement, white matter hyperintensities, reduction in gross brain volume, reductions in frontal and temporoparietal volume, and higher levels of cortical atrophy occur in the brain [23]. The shrinkage of cortical volume is believed to impact cognitive functioning negatively, reducing a person's cognitive ability [24]. Functional magnetic resonance imaging studies have revealed that age-related memory changes may be due to altered activation of the prefrontal cortex (PFC). For example, compared to their younger counterparts, older adults recruit a broader area of the PFC due to bilateral activation of this region [25]. Cabeza [26] conceptualized this phenomenon as a reduction in hemispheric asymmetry, a compensatory response to a loss in neural efficiency. Other researchers explain older adult's broad brain activation during working memory tasks with the compensation-related utilization of neural circuits hypothesis (CRUNCH) [27]. This hypothesis suggests that the compensatory response of the brain's bilateral over activation occurs due to older adults recruiting maximal neuronal resources earlier than younger adults when completing the same tasks, thereby leaving no neuronal resources left for a higher load of difficulty, resulting in poorer performance. Since these two theories, researchers believe the compensatory response is in fact a protective scaffolding effect to support or prop up underlying adverse factors associated with brain ageing like brain shrinkage, reduction in dopamine receptors, neural inefficiency, noise, or both (scaffolding theory of aging and cognition (STAC)) [28]. To enable researchers to predict cognitive status and change over time, a revised STAC model (STAC-r) incorporates aging and life experience factors like exercise and cognitive training that influence structure and function of the aging brain which in turn may enhance or deplete neural resources [29].

More recently, neuroimaging studies have investigated the correlation between brain neurometabolite levels, as an indication of underlying molecular or cellular changes that may be related to aging. The technique of MRS is a noninvasive method of obtaining biochemical information about body tissue [30]. MRS has been used to study age-related degenerative diseases like cognitive impairment and AD [31, 32] and neuropsychiatric disorders like depression [33] and schizophrenia [34]. MRS can be utilized for early detection of disease and for monitoring medical therapies or treatments [35]. Changes in metabolites are purported to reflect changes in different brain indices such as neuronal viability/function (N-acetyl-aspartate; NAA), cellular turnover (Choline; Cho), metabolic activity (glutamate, glutamine; GLX), inflammation in the brain (myo-inositol; Myo), and oxidative stress (glutathione; GSH) [36].

MRS studies have investigated the correlation between changing brain neurometabolite levels and cognitive performance in healthy aging populations [37, 38]. A study by Ross and colleagues [39] identified significant correlations between the integrity of frontal white matter NAA metabolite and cognitive function represented by processing speed, visual memory, and attention tasks with a healthy elderly cohort. A large study conducted by Ferguson and colleagues [40] investigated the relationships between NAA, Cho, and Cr and cognitive function in a group of healthy elderly men. Positive correlations were found between NAA/Cr and Cho/Cr ratios with test measures of logical and verbal memory. The authors postulated that high levels of Cr are the best predictor of poor cognitive performance. An increase in the Cr signal has been reliably identified in healthy elderly brains compared with their younger counterparts [41–43]. These studies support the premise that MRS is a valid technique to measure subclinical changes in cognition across the lifespan.

Alternatively, researchers investigating MRS metabolite markers in clinical cohorts with Alzheimer's disease (AD) have reliably found NAA to be lower and Myo to be higher when compared to cognitively healthy older adults (e.g. [31, 41]). However, inconsistent Cho levels have been identified with some studies reporting an increase in Cho of people with AD [44] while others have not [45]. Collectively, these physiological, pathophysiological, and structural changes that occur with increasing age highlight the complex nature of the aging brain.

Understanding the mechanisms and role of oxidative stress in the aging process is currently considered to be important to elucidate the key to longevity. An emerging theory in the literature postulates that the balance between oxidation/reduction reactions (redox state) within cells is important for healthy aging [46]. If there is a disruption to the mechanisms of redox state (impaired signalling and regulation), then age-associated functional losses will occur [46]. Oxidative stress, antioxidants, and the aging brain will be discussed below.

3. Oxidative Stress, Antioxidants, and the Aging Brain

3.1. Oxidative Stress Mechanisms. Although oxygen is needed for survival, the brain is sensitive to oxygen metabolic activity that produces ROS [47]. Approximately 95%–98% of ROS such as hydrogen peroxide (H_2O_2), hydroxyl free radical ($\bullet OH$), superoxide anion ($O_2^{-\bullet}$), and peroxynitrite (ONO_2^-) are formed in mitochondria as by-products of cellular respiration [48]. Studies of mitochondria isolated from the brain indicate that 2–5% of the total oxygen consumed produces ROS [49]. An imbalance between prooxidant and antioxidant reactions occurs when the equilibrium between the beneficial and harmful effects (redox homeostasis) is interrupted ([50];

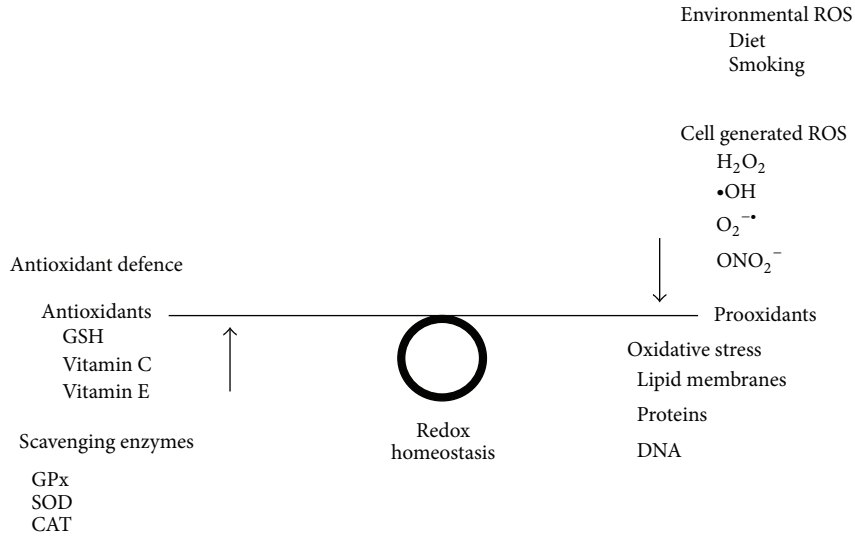

Figure 1: *Oxidative stress mechanisms*. GSH (glutathione) and vitamin C (ascorbic acid) and vitamin E (α-tocopherol) are nonenzymatic antioxidants that assist in antioxidant defence against reactive oxygen species (ROS), to inhibit or repair damage to cells. Scavenging enzymes GPx (glutathione peroxidase), SOD (superoxide dismutase), and CAT (catalase) work to prevent oxidative damage by detoxifying reactive oxygen species (ROS). Environmental ROS as well as cell generated ROS like H_2O_2(hydrogen peroxide), •OH (hydroxyl free radical), $O_2^{-\bullet}$ (superoxide anion), and ONO_2^- (peroxynitrite) are all prooxidants that when in abundance can lead to an imbalance in the redox homeostasis causing oxidative stress, which have detrimental effects to lipid membranes, proteins, and DNA.

refer to Figure 1). This imbalance produces a steady accumulation of oxidative damage in macromolecules that increase with aging, causing a progressive loss in cellular function and efficiency of processes [51]. Free radicals in the brain are responsible for significant harmful effects to cellular function and damage to DNA, proteins, membrane lipids, and components of mitochondria [47, 52]. The brain is particularly sensitive to free radical damage due to its high metabolic rate, concentration of unsaturated fatty acids, cytotoxic actions of glutamate and reduced antioxidant systems with a lower activity of glutathione peroxidase (GPx) and catalase (CAT) compared to other organs [9, 53].

Aging decreases the ability of the brain to scavenge free radicals, thus decreasing available antioxidants, particularly the most abundant endogenous antioxidant GSH [47, 50]. There is a delicate balance between the positive and negative effects of free radicals. In a normal physiological state, fluctuation in ROS production is balanced with ROS scavenging capacity [54]. Oxidative stress occurs when ROS production exceeds that of ROS scavenging capacity. Oxidative stress is a significant feature of aging, most likely due to a combination of reduced ROS scavenging capacity, impaired redox state, and increased ROS production [55]. This imbalance in the cellular redox mechanisms may contribute to the slow onset and progressive nature of neurodegenerative diseases, as well as age-related cognitive decline [46, 56]. Severe, extensive, or more prolonged oxidative damage is highly toxic and these toxic effects contribute significantly to the aging process [57, 58].

3.2. Antioxidant Mechanisms. The human body has an innate defence mechanism consisting of endogenous antioxidants to negate the detrimental effects of oxidants [59]. Antioxidants have the ability to reduce oxidative stress in the body by scavenging ROS to either inhibit or repair damage. Antioxidant enzymes superoxide dismutase (SOD), CAT, GPx, and glutathione reductase (GR) present the first line of defence against free radical damage under conditions of oxidative stress [60, 61]. Nonenzymatic antioxidants, glutathione (GSH), vitamin C (ascorbic acid), and vitamin E (α-tocopherol) are all phenolic compounds that offer protection by altering oxidants to either nonradical end products or transporting radicals to areas where their effects will be less damaging [56]. Vitamins A, C, and E, selenium, and coenzyme Q10 effect important antioxidant actions to protect neural tissue from "attack" by free radicals [62]. Eating a varied diet can provide a mixture of oxidants and antioxidants. Fruits and vegetables rich in vitamins A, C, and E provide a healthy defence against the formation of free radicals. Such fruits and vegetables also increase the number of cell receptors available for antioxidant enzyme action [47]. Dietary polyphenols with antioxidant properties have protective effects against many degenerative diseases including diabetes, cancer, and cardiovascular diseases aiding in the prevention of oxidative stress [63, 64].

The GSH redox cycle lowers H_2O_2 levels, thus lowering the formation of damaging hydroxyl radicals ([65]; refer to Figure 2). GSH is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) found everywhere within the cells of the body. It is involved in many physiological functions. GSH is responsible for detoxifying ROS into nontoxic substances (water and oxygen) [66] and is critical for the maintenance of normal function and neuronal survival [67]. It is involved in the synthesis of proteins and DNA, enzyme activity,

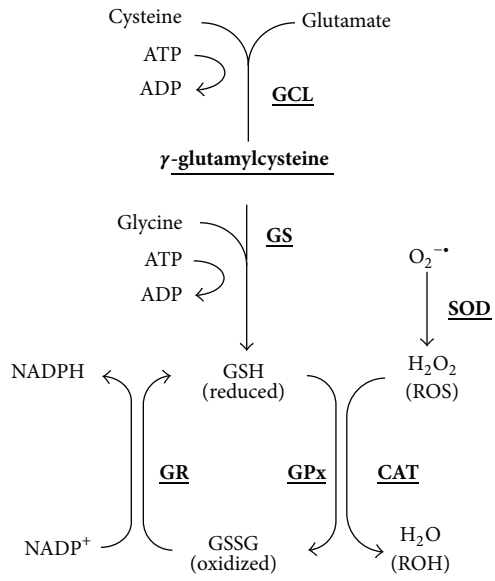

Figure 2: *Glutathione redox cycle*. Glutathione (GSH) is synthesized from the amino acids glutamate (Glu), cysteine (Cys), and glycine (Gly) in a two-step pathway requiring energy from ATP. Glu and Cys are combined via the action of glutamate cysteine ligase (GCL). This dipeptide then combines with Gly via a reaction from glutathione synthetase (GS). GSH undergoes a redox reaction using glutathione peroxidase GPx to detoxify reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2). The main source of H_2O_2 is from the conversion of superoxide anion ($O_2^{-\bullet}$) by the enzymatic action of superoxide dismutase (SOD). GSH is converted to an oxidized form (GSSG) and is recycled back to GSH by the enzymatic reaction of glutathione reductase (GR) which requires the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) to form a redox cycle. *NB:* bold and underlined text represents enzymes.

metabolism, transport, and cell protection [66]. GSH is oxidised to glutathione disulphide (GSSG) resulting in intracellular redox imbalance which is reflected in a decreased GSH to GSSG ratio, often referred to as oxidative stress [68]. GSH levels in tissue decrease with age [69]. Impaired GSH metabolism has been implicated in the pathogenesis of clinical mental disorders like schizophrenia and bipolar disorder [70] as well as neurodegenerative disorders including AD [31] and Parkinson's disease [71]. It is not surprising then that oxidative stress generated by ROS is consistently linked to these conditions.

A study conducted by Berger and colleagues [72] used MRS to investigate *in vivo* GSH levels before and after the administration of an omega-3 fatty acid (ethyl eicosapentaenoic acid; E-EPA) within patients who had experienced their first episode of psychosis. Supplementation with E-EPA was reported to increase GSH concentration by 38% in the temporal lobe of these patients. More importantly, the increase in GSH correlated with improvement in negative symptoms. This promising result provides support for further research to be conducted to elucidate the effect of other supplements on cerebral GSH levels in normal and clinical populations.

GSH levels, quantified using MRS, have been used to investigate oxidative stress in different brain regions (frontal cortex, parietal cortex, hippocampus, and cerebellum) of healthy adults and in the bilateral frontal cortices of patients with mild cognitive impairment and AD [32]. In healthy females compared to healthy males, mean GSH levels were higher (left frontal cortex, $P = 0.006$; right posterior cortex, $P = 0.01$) and that GSH distribution was different between the hemispheres for females and males. GSH levels were significantly depleted in the right frontal cortex of female AD patients compared to healthy female participants ($P = 0.003$), whereas, for males, the left frontal cortex was significantly depleted ($P = 0.05$) when comparing healthy males to AD patients. GSH was lower in mild cognitive impaired patients compared to healthy participants, but the difference was not statistically significant. GSH is therefore an important biomarker of redox state which can be monitored to investigate disease progression or normal age-related changes [32].

Despite the compelling research linking brain metabolite alterations to changes in cognitive function with age and changes in GSH levels in clinical populations, to date, no studies have incorporated the use of MRS as a technique to measure metabolite changes in response to EBm. Clinical trials have not investigated the antioxidant defence system, particularly targeting the ubiquitous antioxidant, GSH, in response to EBm using MRS. Finally, clinical trials have not examined the cognitive correlates of MRS after the chronic administration of EBm.

Altering the inefficiency of the antioxidant system by boosting the redox potential with thiols, particularly the ubiquitous antioxidant, GSH, may be a way to reduce age-associated decline in functional abilities. As discussed below, the administration of EBm may improve the antioxidant redox state, thus leading to improved functional outcomes such as enhanced cognitive performance.

4. What Is *Bacopa monnieri*?

Bacopa monnieri (Linn), commonly referred to as "Brahmi," from the plant family Scrophulariaceae is a creeping herb found in India and neighbouring tropical countries that grows in wet marshland up to 1500 m in altitude [73]. It has been traditionally used in Ayurvedic medicine to treat conditions such as fever, inflammation, pain, asthma, epilepsy, and memory decline [10]. It has been used in a standardized form in clinical research since 1996 [74]. Steroidal saponins and Bacosides A and B are the active chemical constituents responsible for improving both learning and memory [75, 76]. Other constituents include bacopasaponins D, E and F as well as alkaloids, flavonoids, and phytosterols [77, 78]. Some of the chemical constituents of EBm are lipophilic [79, 80]. This means that they can combine with or dissolve in lipids giving them the ability to cross the blood-brain barrier. Bacosides are believed to repair damaged neurons by enhancing kinase activity and neuronal synthesis linked with the restoration of synaptic activity, culminating in the improvement of nerve impulse transmission [81]. Antidepressant and anxiolytic effects have been reported in

animal studies [82, 83] although conflicting findings have been reported in human trials [6, 7, 84, 85]. However it is the memory enhancing effects of EBm that have generated the most attention [86]. Various mechanisms may be involved in the neuroprotective and memory enhancing effects of EBm, such as the binding and detoxification of metal ions [87], free radical scavenging [88], or increasing antioxidant activity [9]. Animal models have shown that EBm can exert vasorelaxant [89], adaptogenic [90], anti-inflammatory [91], metal ion chelating [92], and cholinergic modulatory effects [93]. Neuroprotective effects have been identified in animal models of epilepsy [94] and amnesia [95] as well as reducing ischemia-induced memory deficits in rats [96]. EBm also appears to inhibit numerous β-amyloid oxidative stress pathways involved in AD pathology [92] and antioxidant properties related to GSH redox state [97]. The role of oxidative stress and alterations in the antioxidant GSH redox state in response to EBm will be expanded upon below.

5. Antioxidant/Oxidative Stress Mechanisms of Bacopa

The antioxidant properties of EBm are widely recognised and have been discussed in various reviews [10, 98, 99]. Several histological (*in vitro*) and animal studies have established that EBm bacosides or extract improve the system's defences against oxidative stress by decreasing the formation of free radical accumulation in the brain. In an early study investigating the antioxidant activity of EBm, lipid peroxidation in the prefrontal cortex, striatum, and hippocampus of rats was inhibited. Bhattacharya and colleagues [9] found a dose related increase in enzyme activity responsible for scavenging reactive oxygen species, namely, SOD, CAT, and GPx in these brain regions of rats after 14 and 21 days of chronic administration of EBm. Interestingly, the same study compared the antioxidant effects of the drug deprenyl, which also improved antioxidant enzyme activity, but only in the prefrontal cortex and striatum of the rats and not the hippocampus. They suggested that this increase in the free radical scavenging activity of bacosides may be responsible for facilitating the cognitive action of EBm. Similarly, in a different study, the modulation of antioxidant activity in diabetic rats was again through a significant increase in SOD, CAT, GPx, and GSH levels showing a significant reversal of redox imbalance and peroxidative damage to enhance the defence system against ROS [11]. Other studies also support a free radical scavenging mechanism in response to EBm [75] by reducing the formation of free radicals [92, 100]. In addition, a more recent study found that a EBm ethanolic extract was able to adjust the level of endogenous oxidative markers in various brain regions of prepubertal mice [101].

Furthermore, an *in vitro* study by Russo and colleagues [88] investigated H_2O_2 induced cytotoxicity and DNA damage in human nonimmortalized fibroblast cells in response to an ethanol extract of EBm. They also investigated the free radical scavenging capacity and the effect on DNA cleavage induced by H_2O_2. EBm was able to inhibit superoxide anion formation in a dose dependent manner, indicative of free radical scavenging ability and a protective effect was observed against H_2O_2 cytotoxicity and DNA damage. A more recent *in vivo* and *in vitro* study conducted by Shinomol and colleagues [102] used 3-nitropropionic acid (NPA), a fungal toxin that causes neurotoxicity in both animals and humans, in comparison with the effects of an ethanolic extract of EBm in the mitochondria of the striatum of rats and dopaminergic (N27) cells. As predicted, the NPA caused oxidative stress in the mitochondria of the striatum, while pretreatment with EBm prevented NPA oxidative reactions and reduction of reduced GSH and thiol levels.

In experimental models of ischemia, diabetes and aluminium and cigarette induced toxicity, pretreatment with EBm (40 mg/kg/day to 250 mg/kg/day) and Bacoside A (10 mg/kg/day) has been identified to prevent lipid peroxidation and play a role in antioxidant activities by modulating the effects of enzymes (Hsp 70, cytochrome P450, and SOD in the rat brain) known to be involved in the production and scavenging of ROS, resulting in antistress activity in rats [103–105]. Again, these studies support the premise that EBm has the ability to restore antioxidant defence mechanisms and protect against the adverse effects of peroxidative damage. EBm has also been shown to either exert antioxidant effects through metal chelation at the initiation level of the free radical chain reaction by chelating ferrous ions, or be attributed to the detoxification of free radicals at the propagation level [87, 92]. In another study in rats, the effect of EBm detoxified ROS ONO_2^- in astrocytes [106].

Cumulatively, animal and *in vitro* studies provide support for antioxidant mechanisms of EBm. Animal and *in vitro* studies have identified that GSH is particularly useful to examine antioxidant capacity and changes in oxidative stress. Taking into account the findings of the studies described above, EBm may increase the cellular inefficiency of the antioxidant system by boosting the redox potential with GSH (e.g., [9, 11]). In turn, the administration of EBm as a therapeutic intervention may be a way to reduce age-associated decline in functional abilities. The therapeutic properties of plants like EBm have generated much scientific investigation due to their compelling antioxidant properties, little to no side effects, and economic sustainability [107].

6. Clinical and Research Implications

Oxidative stress plays a role in aging and neurodegenerative disorders. Based on the animal *in vitro* and *in vivo* studies discussed in this review, EBm can be utilized as a therapeutic strategy against oxidative damage and cognitive decline in the elderly. Supplementation with EBm is likely to support the antioxidant defence pathways altering the redox status, which are vital components for normal functioning, while improving cognitive ability. Given that with age it is believed that the antioxidant system is compromised and GSH levels are reduced, EBm has the potential as a therapeutic antioxidant to reduce oxidative stress and improve cognitive performance.

7. Future Directions

While the central role of oxidative stress in age-related cognitive decline and neurodegenerative diseases has driven studies to examine the potential antioxidant benefits of EBm, studies have not incorporated *in vivo* brain imaging techniques to systematically study brain aging and central oxidative stress. MRS may be a useful technique to understand the antioxidant mechanisms, particularly studying GSH ROS detoxification, *in vivo*, as a result of EBm supplementation. Applying neuroimaging research techniques is important to be able to understand the *in vivo* effects underpinning the cognitive changes due to EBm. Future studies should consider the application of brain imaging modalities, particularly MRS, to be able to extend results beyond the explanation of mood, general health, and cognitive behavioural outcomes in response to dietary supplementation in human randomized clinical trials.

8. Conclusion

Further exploration into the complex mechanisms of action of EBm in nutritional aging studies may reveal promising insights into antioxidant metabolic changes, supporting dietary nutritional supplementation for therapeutic means. This review has described how EBm has the potential as a therapeutic antioxidant to reduce oxidative stress, a mechanism that may be responsible for improving cognitive performance and offer neuroprotection. Employing the neuroimaging technique of MRS to investigate GSH antioxidant levels may be useful to elucidate the mechanisms of action underlying the cognitive enhancing effects of EBm. Such research may also assist in our understanding of how to improve cognition in the elderly.

Conflict of Interests

The authors declare that there is no conflict of interests in this paper.

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Review Article

Bacoside A: Role in Cigarette Smoking Induced Changes in Brain

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Cigarette smoking (CS) is a major health hazard that exerts diverse physiologic and biochemical effects mediated by the components present and generated during smoking. Recent experimental studies have shown predisposition to several biological consequences from both active and passive cigarette smoke exposure. In particular, passive smoking is linked to a number of adverse health effects which are equally harmful as active smoking. A pragmatic approach should be considered for designing a pharmacological intervention to combat the adverse effects of passive smoking. This review describes the results from a controlled experimental condition, testing the effect of bacoside A (BA) on the causal role of passive/secondhand smoke exposure that caused pathological and neurological changes in rat brain. Chronic exposure to cigarette smoke induced significant changes in rat brain histologically and at the neurotransmitter level, lipid peroxidation states, mitochondrial functions, membrane alterations, and apoptotic damage in rat brain. Bacoside A is a neuroactive agent isolated from *Bacopa monnieri*. As a neuroactive agent, BA was effective in combating these changes. Future research should examine the effects of BA at molecular level and assess its functional effects on neurobiological and behavioral processes associated with passive smoke.

1. Introduction

Cigarette smoking is an intractable and preventable public health problem. It is an important risk factor involved in the pathogenic pathways of a variety of disorders. The WHO has declared global tobacco epidemic and planned "Framework Convention for Tobacco Control" [1]. Tobacco smoke is a toxic air contaminant and secondhand tobacco smoke (SHS) is a formidable health hazard [2]. Epidemiological studies show increased risk for behavioral and cognitive problems and a greater incidence of mental disorders in children exposed to environmental tobacco smoke [3–5]. Prenatal maternal exposure to cigarette smoke has been well documented to induce neurological as well as many other lasting health effects [6, 7]. Etiological evidences support the involvement of neurotransmitter systems, oxidative and nitrogen stress, mitochondrial dysfunction, and neurogenetic and epigenetic changes in secondhand/passive smoking induced brain changes and the associated pathways have been extensively reviewed [8–11].

Despite the significant health impacts arising from passive smoking, little attention is paid to combat the neurological changes associated with it. This review critically examines and summarizes the study made on the neuroprotective role of BA in rats exposed to passive cigarette smoke and its sequelae with focus on the neurotransmitter systems, oxidative and lipid peroxidative, mitochondrial dysfunction, and apoptotic changes in rat brain. These results can be integrated with other theories in holistically combating passive smoking induced neurological changes.

2. Physical and Biochemical Properties of Cigarette Smoke

Cigarette smoke is divided into two phases: a tar phase and a gas phase. The tar or particulate phase is defined as the material that is trapped when the smoke stream is passed through the Cambridge glass-fiber filter that retains 99.9% of all particulate material with a size ~0.1 m [12]. The gas phase is the material that passes through the filter. The particulate

(tar) phase of cigarette smoke contains ~10^{17} free radicals/g, and the gas phase contains ~10^{15} free radicals/puff [12]. The radicals associated with the tar phase are long-lived (hours to months), whereas the radicals associated with the gas phase have a shorter life span (seconds) [12–14].

Cigarette smoke that is drawn through the tobacco into an active smoker's mouth is known as mainstream smoke (MS) and the smoke emitted from the burning ends of a cigarette is the sidestream smoke (SS). Mainstream cigarette smoke comprises 8% of tar and 92% of gaseous components [12]. Environmental tobacco smoke (ETS) results from the combination of sidestream smoke (85%) and a small fraction of exhaled mainstream smoke (15%) from smokers [13]. Importantly, the concentration of numerous toxins is dramatically (up to 100-fold) elevated in SS when compared with MS, and the complex mixture of toxins is attributed rather to a specific component of cigarette smoke to the potential adverse impact of passive smoke on health [15].

Aside from specific chemical constituents, certain physicochemical properties of smoke may participate in disease processes. The pH of the smoke affects the site and degree of nicotine absorption as well as the smoker's depth of inhalation. The oxidation-reduction state of the smoke is important because oxidants influence the maturation of cholesterol-laden plaques in the coronary arteries and other blood vessels. In short, cigarette smoke is far more than a triad of tar, nicotine, and carbon monoxide [16].

Although SS and MS smoke have qualitatively similar chemical compositions, the respective quantities of individual smoke constituents are different [17]. The exposure to SS smoke depends on the distance from the burning cigarette and conditions of ventilation; the higher concentrations of certain toxic and carcinogenic chemicals in SS smoke result in measurable levels of these chemicals in nonsmokers exposed to ETS [18].

3. Pharmacodynamics of Smoking

Although most of the toxicity of smoking is related to other components of cigarette smoke, it is primarily the pharmacologic effects of nicotine that produce the addiction to tobacco. An understanding of how nicotine produces addiction and influences smoking behavior provides a necessary basis for optimal smoking cessation intervention. Cigarette smoke contains 10–14 mg of nicotine [19], of which 1–1.5 mg is absorbed systemically in the lungs through inhalation [20]. Nicotine rapidly enters the pulmonary venous circulation, reaches the brain within 10–20 s, and readily diffuses into brain tissue [21] and binds to nicotine acetylcholine receptors (nAChRs) [22]. Acutely, cigarette smoking induces positive reinforcing effects, including mild euphoria, heightened arousal, reduced appetite, and reduced stress, anxiety, and pain [23].

Nicotine in cigarette smoke is alkaline and readily crosses the blood brain barrier. It mediates the stimulation of mesolimbic dopamine system. It is also involved in modulating other classical neurotransmitters in the brain including catecholamines, serotonin, GABA, and glutamate [24]. It induces addiction as it shares many properties of psychostimulant drugs such as cocaine and amphetamine [25]. With repeated exposure to nicotine, toxicity gives way to tolerance and addiction. Overtime tolerance is accompanied by increased intake of nicotine to attain the same effects initially taken which leads to physical dependence, compelling higher intake, lest it induces withdrawal symptom. This leads to reinforcement mechanisms, finally leading to addiction [26]. Substances other than nicotine present in cigarette smoke are also involved in mediating the harmful effects in nervous system. Other tobacco smoke constituents affect the structural and functional integrity of blood brain barrier [27, 28].

4. Neuropharmacological Effects of Cigarette Smoking

Cigarette smoking is an important environmental aging accelerator [29] partly because it induces oxidative stress in multiple organs including the brain and is presented in many diseases, including cognition-related or neurodegeneration-related pathological changes [30]. This should be presented to demonstrate a direct linkage between smoking and cognitive impairment.

The incidence of cerebrovascular diseases (CVDs) increases with cigarette smoking, as cessation of smoking decreases its incidence [31]. Smoking is a modifiable risk factor for stroke [32] primarily due to hypertension [33]. Other neurological diseases for which smoking is a risk factor include hypoxia, cerebral ischemia, cerebral hemorrhage, brain infarction, subarachnoid hemorrhage, and tardive dyskinesia [34]. Neuroleptic Parkinsonism, resembling Idiopathic Parkinson's disease, is associated with smoking, as higher doses of nicotine exert an inhibitory effect [35]. Cerebral symptoms like brain atrophy and ataxia are exaggerated with smoking [36]. Reports also suggest that cigarette smoking is protective in the development of Parkinson's disease [37] but is an important risk factor in Alzheimer's disease [38], as it accelerates cognitive decline and dementia [39].

5. Role of Passive Smoking

The evidence that active smoking is a risk factor for cardiovascular disease (CAD) and the leading cause of preventable death is overwhelming. However, exposure to passive cigarette smoke also exerts detrimental effects on vascular homoeostasis [40]. Importantly, most of these effects appear to be characterized by a rapid onset. For example, the relatively low doses of toxins inhaled by passive smoking are sufficient to elicit acute endothelial dysfunction, and these effects may be related, at least in part, to the inactivation of nitric oxide (NO). Moreover, passive smoking may directly impair the viability of endothelial cells and reduce the number and functional activity of circulating endothelial progenitor cells. In addition, platelets of nonsmokers appear to be susceptible to proaggregatory changes with every passive smoke exposure. Overall, passive smoke induces

oxidative stress and promotes vascular inflammation. Apart from vasoconstriction and thrombus formation, however, the myocardial oxygen balance is further impaired by adrenergic stimulation and autonomic dysfunction [41]. These data strongly suggest that passive smoking is capable of precipitating acute manifestations as it increases the odds of developing cognitive impairment [42] and 3-fold increase in the risk for dementia, causing neurofibrillary changes depictive of Alzheimer's disease [43].

Hence, one generally overlooked factor contributing to the escalation of tobacco abuse is passive smoking. Nicotine from secondhand smoke exposure results in an increase in plasma nicotine concentration of ~0.2 ng/mL and amounts to substantial brain $\alpha 4\beta 2$ nAChR occupancy (19%) in both smokers and nonsmokers compared with 0.87 ng/mL and 50% $\alpha 4\beta 2$ nAChR occupancy from actively smoking one cigarette [44, 45]. Secondhand smoking is clearly linked to serious illnesses among nonsmokers including asthma, heart disease, sudden infant death syndrome, and cancer [46].

6. Pharmacological Intervention in Cigarette Smoking

With the available understanding of the biological effects of cigarette smoking many treatment strategies are available. The primary target is nicotine; hence nicotine replacement therapy or nicotine substitution in the form of chewing gums, transdermal patch, and spray are employed in smoking cessation aid [47–49]. Other pharmacological therapies include receptor antagonists and nicotinic antagonists (mecamylamine and chlorisondamine) [50]; opiate antagonists and naloxone and naltrexone [51]; nonreceptor antagonists [52]; serotonin uptake inhibitors (zimelidine and citalopram) and monoamine oxidase inhibitor [53]; and antidepressants [54, 55]. Angiotensin converting enzymes (ACE) inhibitors and calcium antagonists are also effective in combating smoking induced toxicity [56]. These drugs are mainly indicated to reduce the severity of tobacco withdrawal, but not as an aid to stop smoking [57].

Currently, the first line therapy for smoking cessation includes bupropion (amfebutamone), an atypical antidepressant that inhibits norepinephrine uptake and dopamine uptake [58]. The metabolite of bupropion, (2S,3S) hydroxybupropion, is an antagonist on the $\alpha 4\beta 2$ (nAChR) [59]. Another agent is varenicline, which is a highly selective partial agonist of the $\alpha 4\beta 2$ (nAChR) that stimulates dopamine release in the nucleus accumbens (nAC) but to a much less extent than nicotine itself [60].

These interventions are improbable and ineffective in rendering protection against secondhand smoke. Due to poor pharmacological management of passive smoking, it was hypothesized that intervening with a neuroactive agent can prevent or minimize neurological changes. Studies on supplementation with vitamins E, C, and A [61, 62]; antioxidants: glutathione, N-acetyl cysteine, and superoxide dismutase [63]; and fish oil, curcumin, and green tea [64–66] have reported to offer protection against smoking induced damages.

In this context, this study evaluated the effect of BA, an active constituent isolated from *Bacopa monnieri* against smoking induced damages in rat brain. *Bacopa monnieri* exerts neuropharmacological effects [67] and is effective in the treatment of mental illness and epilepsy [68]. Its biological effects include free radical scavenging [69]; vasodilatory [70, 71]; and mast cell stabilizing [72] activities. The various biological activities of BA have been reviewed in detail [73, 74].

Bacoside A is 3-(a-L-arabinopyranosyl)-O-b-D-glucopyranoside-10, 20-dihydroxy-16-keto-dammar-24-ene [75] and is the major chemical entity responsible for neuropharmacological effects and the nootropic action or antiamnestic effect of *Bacopa monniera*. Bacoside A cooccurs with bacoside B, the latter differing only in optical rotation and is probably an artefact produced during the process of isolating BA [76]. On acid hydrolysis, bacosides yield a mixture of aglycones, bacogenins A1, A2, and A3 [77], which are artefacts, two genuine sapogenins, jujubogenin and pseudojujubogenin, and bacogenin A4, identified as ebelin lactone pseudojujubogenin [78].

7. Methods

7.1. Isolation of Bacoside A. The plant *Bacopa monniera* was collected in and around Chennai, India, and authenticated by Dr. P. Brindha, Central Research Institute (Siddha), Chennai, India. The dammarane type triterpenoid saponin BA was isolated from the plant by the standard procedure. The purity of the isolated BA was identified by thin layer chromatography (TLC) and infrared (IR) spectrum analysis using standard BA [79].

7.2. Experimental Setup. Adult male albino rats of Wistar strain (120–200 g) were used for the present study. The rats were provided with standard pelleted rat feed and water ad libitum. They were acclimatized to the laboratory conditions and maintained under 12 h light and dark cycles. The experiments were carried out in accordance with the guidelines provided by the Institutional Animal Ethical Committee [79].

The animals were divided into four groups of 6 animals each. Group I: control. Group II (CS): rats exposed to cigarette smoke. Group III (BA): rats administered with BA (10 mg/kg bw/day, p.o.). Group IV (CS + BA): rats exposed to cigarette smoke and simultaneously administered with BA. Group II and Group IV rats were exposed to cigarette smoke, following a standard method as described [79] for a period of 12 weeks.

The rats were exposed to side stream cigarette smoke in whole body smoke exposure chamber. The rats were exposed twice daily as described [80, 81]. The experimental period lasted for 12 weeks. Drug control animals received aqueous suspension of BA in 1% gum acacia orally at a dosage of 10 mg/kg bw/day for 12 weeks, whereas experimental animals exposed to cigarette smoke (Scissors Standard Cigarette) were simultaneously administered with BA at the same dose. Control animals received a corresponding volume of the vehicle suspended in normal saline. The same brand of locally

Table 1: Constituents of the cigarette smoke.

Smoke constituents	Concentration/cigarette
Nicotine	1.8 mg
Carbon monoxide	20 mg
Total particulate matter	32 mg
Acetaldehyde	0.9 mg
Hydrogen cyanide	225 mg
Benzene	38 mg
N'nitrosonorcotine	240 mg

available cigarette was used throughout the experiment (Scissors Standard, W.D & H.O.Wills, Hyderabad Deccan Cigarette Factory). Control animals were subjected to the same handling and time in the smoke exposure chamber with air replacing smoke/air mixture. The composition of cigarette smoke was analyzed at Tamil Nadu Pollution Control Board, Chennai, and the constituents present are listed in Table 1.

8. Results and Discussion

8.1. Structural Brain Changes and Clinical Correlates. Cigarette smoking is associated with diverse structural changes in brain, probably as a consequence of toxicity or as an adaptive response, causing a reduction in integrity of cerebral white matter microstructure [82] and gray matter volumes [83, 84] and these changes appear correlated with the magnitude of cigarette exposure. Smoking induced structural changes in brain are associated with cognitive deficits [85] as well, with the integrity of white matter and glial proliferation [86]. In gross, the microstructural changes in key brain regions and white matter tracts have a negative impact in cigarette smokers.

In the present study, histological changes were prevalent in brain of rats exposed to cigarette smoke that were inflammatory and edematous in the cerebrum (Figure 1). Smoking induced inflammatory changes were also marked by increased activity of CK-MB isoenzyme in serum [79], an early marker for pathological changes like cerebral damage [87]. 4-N-Methyl-N-nitrosamino-1-(3-pyridyl)-1-butanone (NNK), is a major nitrosamine present in substantial concentration in MS and SS that causes oxidative stress and triggers neuroinflammation in brain [88, 89]. Inflammation plays a pivotal role in extremely wide array of disease conditions ranging from viral diseases of CNS to neurodegenerative disorders. NKK mediated microglial activation leads to profound increase in inflammatory mediators. The inflamed milieu may cause neuronal damage [90]. A decrease in the inflammatory changes was noted in BA treated rats exposed to cigarette smoke, which could be due to the anti-inflammatory effect of BA [91] and the reduction in cerebral inflammatory changes in treated rats were also reflected in lowered levels of CK-MB as against untreated rats [79].

Electroencephalography (EEG) of rat brain monitored frontal and parietal regional changes in brain as electrical changes as α, β, δ, and θ waves. Cigarette smoke exposed rats presented depressed δ and increased α waves (Figure 2). A desynchronized and electrically active EEG pattern is noted in smokers [92]. Acute smoking accelerates dominant frequency fast waves α and β with a reduction in slow wave δ and θ waves illustrate a stimulant action [93], whereas chronic smoking induces less α wave and more β wave [94]. In rats treated with BA and exposed to cigarette smoke, the EEG pattern was devoid of desynchronization and lacked stimulatory wave, an effect also noted among cholinergic agonists: mecamylamine and scopolamine. This shows the anticholinergic effect of BA and effective against smoking induced stimulation of brain.

8.2. Neurotransmitter Systems. Neurotransmitters mediate diverse pharmacological effects on central and peripheral nervous system and participate in reinforcing, mood elevation, and cognitive functions [95]. A balance in their rate of synthesis and utilization constitutes the regulatory mechanism in neurotransmission. Smokers have positive effects like pleasure, arousal, and relaxation, as well as negative effects like depression and anxiety. The functional antagonism presented in cigarette smoking is related to desensitization of nAChR. Nicotine in cigarette smoke upregulates nAChR (pre- and postsynaptic), which in turn interacts with the noradrenergic, cannabinoid, dopaminergic, cholinergic, and serotonergic systems [96] and increases the levels of norepinephrine, dopamine, acetylcholine, and serotonin [97].

Cigarette smoking upregulates nAChR in the brain, including the common $\alpha4\beta2^*$ nAChR subtype [23]. In the present study, an upregulation of $\alpha4$ subunit was evident in rats exposed to cigarette smoke (Figure 3). Chronic administration of nicotine also upregulates nAChRs [98, 99] causing an increased receptor function and sensitivity to nicotine. This results in increased trafficking of nAChRs to the cell surface, increased receptor assembly and/or maturation, or other mechanisms [100]. In smokers, abstinence from smoking normalizes the nAChR upregulation to the levels of nonsmokers [101, 102]. Similarly, commonly used treatments for smoking cessation also decrease $\alpha4\beta2^*$ nAChR to near normal levels as in nonsmokers. In the exploratory analyses, decreases in $\alpha4\beta2^*$ nAChR levels are associated with decrease in the perceived rewarding properties of nicotine [103, 104]. Hence a downregulation of $\alpha4$ nAChR in BA treated smoke exposed rats could be associated with diminished reward from cigarettes (presumably mediated at least in part through dopamine release). Taken together, these findings indicate that the role of BA on nAChR regulation could be vital in modulating nicotine response and reward pathway in chronic cigarette smoking. However, the mechanism on how BA influences the upregulation remains to be understood.

Nicotine is cholinergic by increasing the release of acetylcholine (ACh) from axonal stores and inhibits its clearance by inhibiting acetylcholine esterase (AChE) [105–107]. Increased accumulation of ACh increases the electrical activity in rat brain [107]. This accounts for the increase in most of the neurotransmitters in rats exposed to cigarette smoke (Figures 4–6). In BA treated rats, the activities of AChE were increased

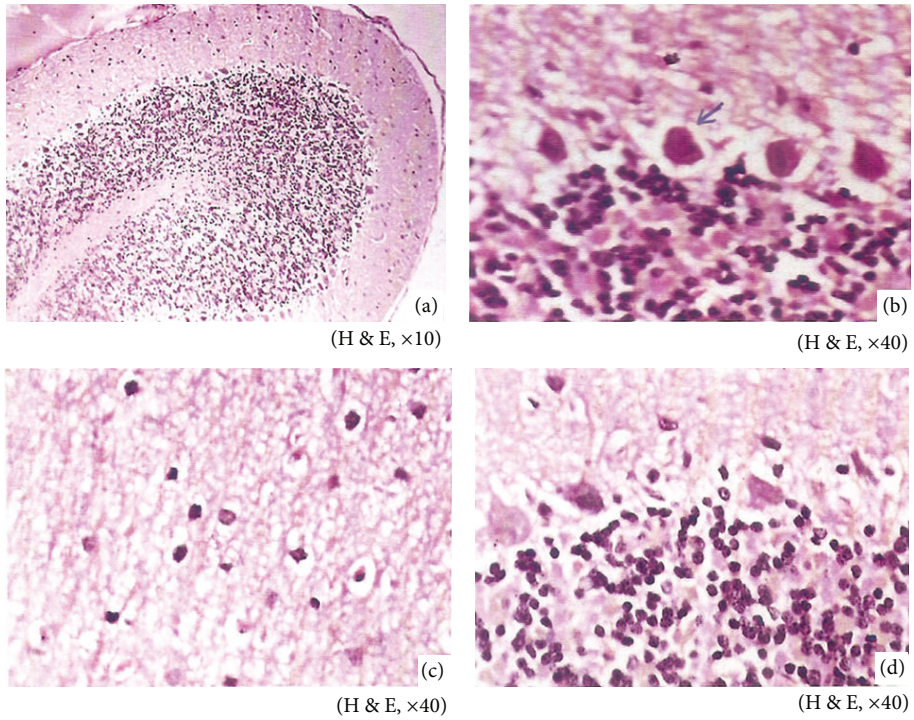

Figure 1: Sections of rat brain cerebellum. (a) Control rats showing normal architecture. (b) CS rats showing mild gliosis, edema, necrosis, and Purkinje cell damage. (c) BA rats showing normal architecture with no significant changes. (d) CS + BA rats showing normal morphology of Purkinje cells.

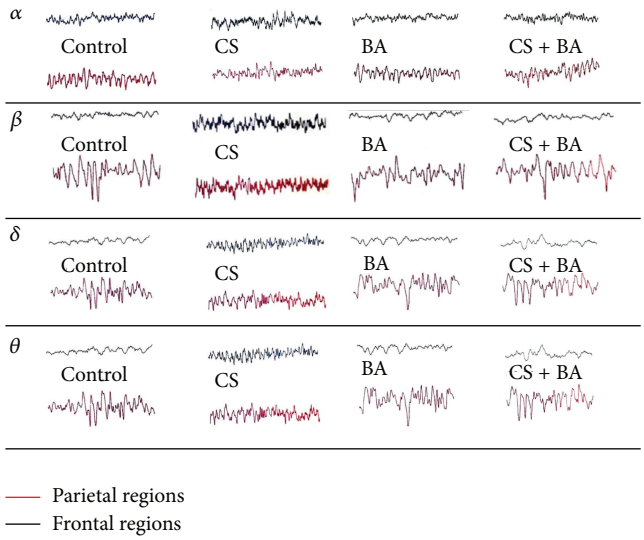

Figure 2: Electroencephalographic pattern of frontal and parietal regions of rat brain.

(Figure 7), which could have decreased the lowered levels of ACh. This confirms the anticholinergic effect of BA [108].

Increases in plasma catecholamines are known to occur with smoking [109]. Upregulation of nAChR increases the release of catecholamines: epinephrine and norepinephrine, an effect mediated through the tyrosine hydroxylase activity [110]. Vasoconstrictor effects observed in smoking are related to increases in norepinephrine [111]. In the present study, smoking induced an increase in the levels of epinephrine and norepinephrine in rat brain (Figure 8). However, BA administration maintained the levels of norepinephrine in treated rats. The observed lowering could be due to the downregulation of nAChR by BA. Apart from its ability to induce downregulation of nAChR expression, BA could have interacted with tyrosine hydroxylase [112] and modulated the release of catecholamines.

Nicotine also influences the release of serotonin, and it has been reported to have a dual role as it induces both an increase and decrease [113, 114]. In the present study, cigarette smoking increased the serotonin level in rats. Serotonergic dysfunction has also been in smokers [115]. Serotonergic dysfunction is associated with clinical depression and depression is far more prevalent among smokers [116] suggesting a possible link. Further, compounds that increase dopamine and its metabolites concentration have abuse potential like opiates and cocaine, whereas those which lower dopamine induce cognitive, behavioral, and motor coordination defects [117]. The role of BA on serotonin [118] could have maintained the levels in treated animals (Figure 8). Physiologically, high level of neuronal dopamine induces greater oxidative stress derived from dopamine [119]. These results confirm the effect of *Bacopa monnieri* extract in normalizing norepinephrine, serotonin, and dopamine in cortex and hippocampus of rats, in both acute and chronic unpredictable stress [120]. In the cigarette smoke exposed rats, an increase in dopamine levels was observed, but in BA administered rats the levels

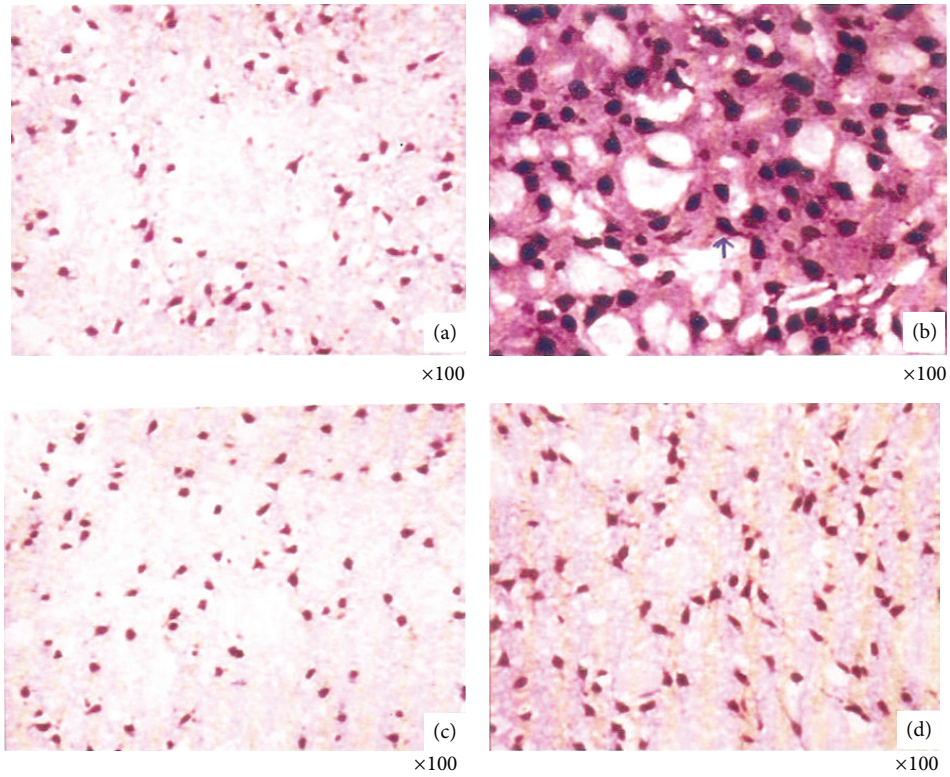

Figure 3: Immunohistochemical analysis of nAChR ($\alpha 4$) expression in rat brain cerebellum. (a) Control rats showing normal expression of nAChR. (b) CS rats showing increased expression of nAChR. (c) BA rats showing normal expression of nAChR. (d) CS + BA rats showing decreased expression of nAChR.

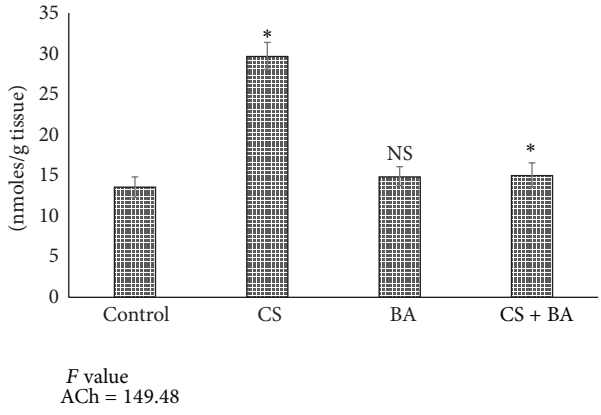

Figure 4: Levels of acetylcholine in brain of control and experimental animals. Values are expressed as Mean ± S.D. Significance is indicated for comparisons between control and CS and BA; Group CS versus CS + BA with Dunnett's T3 post hoc multiple comparison test; $^{*}P < 0.001$; NS: nonsignificant.

were maintained at near normal. This reflects the safety and subsequent tolerability of BA in preclinical models as it did not induce any untoward and toxic effect.

Most of the nicotine-mediated release of neurotransmitters occurs via modulation by presynaptic nAChRs, although direct release of neurotransmitters also occurs [121]. Dopamine release is facilitated by nicotine-mediated augmentation of glutamate release and with long term treatment, by the inhibition of GABA release [122]. In addition to direct and indirect stimulation of neurotransmitter release, chronic cigarette smoking (but not nicotine administration) reduces brain monoamine oxidases A and B (MAO-A and MAO-B) activity, which would be expected to increase monoaminergic neurotransmitter levels such as dopamine and norepinephrine in synapses, thus augmenting the effects of nicotine and contributing to addiction [123]. Inhibition of MAO facilitates acquisition of nicotine self-administration in rats, supporting the idea that MAO inhibition interacts with nicotine to reinforce tobacco dependence [124]. Decreased activity of MAO in cigarette smoking exposed rats (Figure 8) confirms reports that have shown downregulation of MAO expression, including MAO-A and MAO-B in the brain, [125, 126] as well as influencing methylation of MAO promoter genes [127]. This lowering could have resulted in an increase in dopamine content in cigarette smoke exposed rats. Increases in MAO activities in BA treated rats (Figure 8) confirm the reports of recent studies which have shown the influences of *Bacopa monnieri* on the activities of MAO [128].

Polyamines play a key role in brain cell replication, differentiation, and regulation of nAChRs and they influence synaptic transmission [129, 130]. Alterations in polyamine gating of cholinergic synaptic signaling contribute to adverse neurobehavioral effects of numerous neuroteratogens [130].

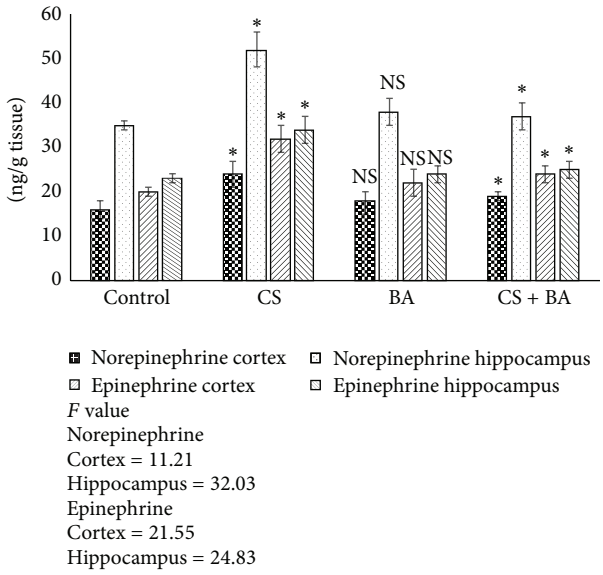

Figure 5: Levels of norepinephrine and epinephrine in brain of control and experimental animals. Values are expressed as Mean ± S.D. Significance is indicated for comparisons between control and CS and BA; Group CS versus CS + BA with Dunnett's T3 post hoc multiple comparison test; $^{*}P < 0.001$; NS: nonsignificant.

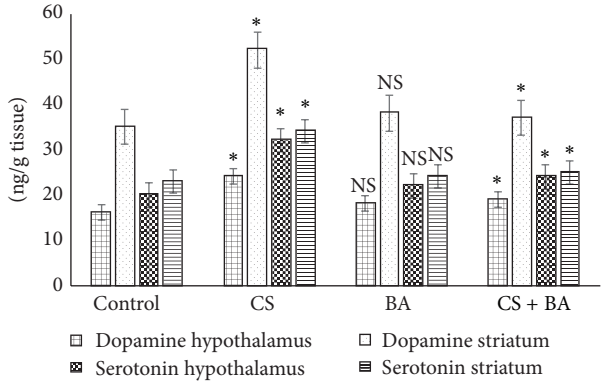

Figure 6: Levels of dopamine and serotonin in brain of control and experimental animals. Values are expressed as Mean ± S.D. Significance is indicated for comparisons between control and CS and BA; CS versus CS + BA with Dunnett's T3 post hoc multiple comparison test; $^{*}P < 0.001$; NS: nonsignificant.

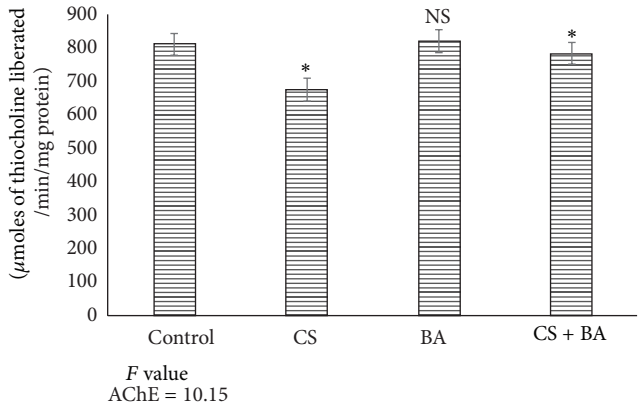

Figure 7: Activities of acetylcholine esterase (AChE) in brain of control and experimental animals. Significance is indicated for comparisons between control and CS and BA; CS versus CS + BA with Dunnett's T3 post hoc multiple comparison test; $^{*}P < 0.001$; NS: nonsignificant.

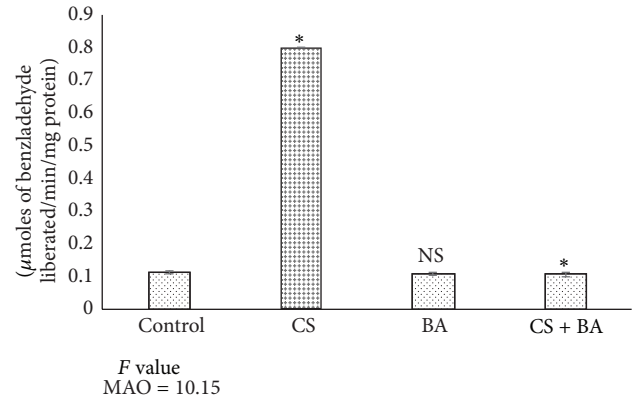

Figure 8: Activities of monoamine oxidase (MAO) in brain of control and experimental animals. Significance is indicated for comparisons between control and CS and BA; Group CS versus CS + BA with Dunnett's T3 post hoc multiple comparison test; $^{*}P < 0.001$; NS: nonsignificant.

Ornithine decarboxylase (ODC) is the rate limiting enzyme in the maintenance of polyamine levels. Inhibition of ODC inhibits growth and induces gross dysmorphology, upregulating the $\alpha 7$ and $\alpha 4\beta 2^{*}$ nAChR. This is accompanied by abnormalities in macromolecular indices of cell packing density and cell membrane surface area. In chronic cigarette smoking exposed rats, ODC activity increased significantly (Table 2).

Excitotoxic challenge induces neuronal proliferation and induces ODC [131]. Induction of ODC is neuroprotective in cerebral ischemia [132], and, however, is also a common response in various pathological stimuli in brain such as physical, chemical, thermal, and metabolic injuries [133]. A relatively long lasting increase in ODC and consequently its product putrescine are causally related to neurodegeneration [134]. In the present study, cigarette smoking increased the activities of ODC. BA treated rats recorded a decrease in ODC activity confirming its role in inhibiting neurodegenerative process following cigarette smoking induced excitotoxicity in brain.

8.3. Nicotine and Cotinine Levels. Cigarette smoking increases the levels of nicotine and its metabolite cotinine to pharmacologically active concentrations that are responsible for mediating the aspects of nicotine dependence. In rats exposed to cigarette smoke, accumulation of cotinine in brain was noted (Table 3), and the levels were lowered in BA treated rats. The decrease in the levels could have probably resulted from the increased clearance of cotinine by the

Table 2: Levels of nicotine and cotinine in brain of control and experimental animals. Values are expressed as Mean ± S.D.

Parameter	Control	Cigarette smoke (CS)	Bacoside A (BA)	Cigarette smoke + bacoside A (CS + BA)	F value
Nicotine (ng/g tissue)	n.d	180 ± 12	n.d	89 ± 5*	870.08
Cotinine (ng/g tissue)	n.d	210 ± 15	n.d	120 ± 8*	718.14

Significance is indicated for comparisons between control and CS and BA; CS versus CS + BA with Dunnett's T3 post hoc multiple comparison test; * $P < 0.001$. n.d: not detected.

CYP system. Although *Bacopa monnieri* extract reportedly inhibits CYP enzymes [135], increased clearance of cotinine, as noted from a decrease in cotinine levels in BA treated rats, confirms that purified bacosides do not inhibit CYP; instead, the constituents in crude extract exert an inhibitory effect [136, 137].

Cigarette smoking accelerates the metabolism of drugs, especially the ones primarily metabolized by CYP1A2 [138]. It delays the clearance of nicotine [139]. In smokers, nicotine clearance is increased by 14% in 4-day smoking abstinence and by 36% higher in 7-day smoking abstinence compared to overnight abstinence. Apart from nicotine, substance(s) in cigarette smoke, as yet unidentified, also affect the metabolism of nicotine. For instance, cotinine slows the metabolism of nicotine since both are metabolized by the same enzyme [140]. However, carbon monoxide in cigarette smoke has no effect on nicotine and cotinine clearance [141], but β-nicotyrine, a minor alkaloid in cigarette smoke, effectively inhibits CYP2A6 in vitro [142]. Thus, reduced nicotine clearance may also result from downregulation of CYP expression and not inhibition [143].

Cigarette smoking also induces glucuronidation of some drugs, such as propranolol and oxazepam, and UGT1A9 is the inducible component of 3′-hydroxycotinine O-glucuronidation [143]. Excretion of 3′-hydroxycotinine O-glucuronide is induced by smoking, but the extent of nicotine and cotinine N-glucuronidation is not significantly affected. In rats exposed to cigarette smoke increase in UDP-GT was noted and the activities remained unaltered in BA treated rats [144]. The adaptogenic role of *Bacopa monnieri* is evident from increased cotinine clearance [145].

8.4. Oxidative and Peroxidative Changes. Free radicals mediated oxidative stress has been implicated in the pathogenesis of smoking-related diseases and antioxidant nutrients are reported to prevent the oxidative damage induced by smoking. Cigarette smoking modulates antioxidant status in various organs by increasing lipid peroxidation and prooxidative state [146]. Increased basal and induced lipid peroxidation were observed in cigarette smoke exposed rat brain [147]. Acute exposure to cigarette smoke enhances the production of antioxidant enzymes as a result of adaptive response that mitigates the damage [148], but chronic exposure decreases the inherent antioxidant defense in brain [149, 150].

The constituents of cigarette smoke affect the individual cellular antioxidants differently. The quinone/semiquinone radicals from the tar phase of cigarette smoke inactivate superoxide dismutase [151] and inhibit catalase in brain [152]. Acetaldehyde, a major aldehyde from the smoke, depletes cell of cellular glutathione [153]. Other cellular antioxidants, tocopherols, carotenoids, and retinol, are destructed by cigarette smoke [154].

Further the cigarette tar contains large amounts of metals, complexed to some components of tar such as odiphenols [155], which can mobilize reactive iron from ferritin and copper from copper binding protein inducing damage to brain [156]. The heavy metal cadmium, in cigarette smoke, decreases the bioavailability of selenium (Se) and zinc (Zn) and thus depletes the antioxidant status [157]. The role of BA as chelator of transition metal, inhibition of free radicals, and termination of lipid peroxidation at the initiation level itself [69] accounts for its protection in cigarette smoke induced lipid peroxidative damage and combative against oxidative damage.

8.5. Mitochondrial Functions. Mitochondria are the site of cellular oxidation and provide ATP for various metabolic processes and hence are vulnerable to free radical attack. Mitochondrial damage is prevalent in both heart and brain following cigarette smoke exposure [158, 159]. Exposure to cigarettes can lead to mitochondrial dysfunction as demonstrated by increased levels of cholesterol, lipid peroxides, and increased cholesterol/phospholipid ratio, in conjunction with decreased mitochondrial enzymes in rats exposed to cigarette smoke [160]. Chronic cigarette smoking prevented exercise-induced improvement in brain mitochondrial function and neurotransmission [161]. Perturbed mitochondrial energetics is critical in normal brain development [6, 162]. Cerebellar perturbation can broadly impact regulation of behavioral and cognitive domains [163].

Aerobic demands increase postnatally with heighted synaptic development, requiring more ATP to maintain membrane polarity. Exposure to cigarette smoke perturbed the mitochondria and associated aerobic pathways. The effect of BA in regulating the key aerobic ATP production, probably by preventing the peroxidative changes in mitochondria, could be crucial in mitochondrial mediated neurotransmission pathways. Brain energetics is highly regulated process and further studies in the mechanistics can provide an insight into the role of BA.

8.6. Membrane Integrity and Electrolyte Balance. Derangement of membrane bound enzymes and modifications of lipid bilayer alterations following cigarette smoke exposure

Table 3: Activities of ornithine decarboxylase in brain of control and experimental animals. Values are expressed as Mean ± S.D.

Parameter	Control	Cigarette smoke (CS)	Bacoside A (BA)	Cigarette smoke + bacoside A (CS + BA)	F value
Ornithine decarboxylase nM of $^{14}CO_2$ released/hr/g tissue	2.0 ± 0.12	5.65 ± 0.52	2.23 ± 0.22	2.45 ± 0.023*	3489

Significance is indicated for comparisons between control and CS and BA; Group CS versus CS + BA with Dunnett's T3 post hoc multiple comparison test; * $P < 0.001$.
n.d: not detected.

resulted in significant decrease in the activities of ATPases [164]. Free radicals in cigarette smoke deplete cell protein sulfhydryl groups and increase in protein carbonyl formation [165] and so does acetaldehyde in cigarette smoke [166]. Membrane bound ATPases are thiol-dependent enzymes, and modification of thiol groups within the active sites of these enzymes lowers their activities in cigarette smoke rats. The antioxidant role of BA prevented the membrane damage and restored the activities of ATPases. Also the restitution of ATP levels by altering the mitochondrial dysfunction maintained the activities of ATPases.

Inhibition of Na^+/K^+-ATPase and elevation of Na^+ in chronic exposure to cigarette smoke are attributed to the increased cholesterol/phospholipid ratio [167] followed by neuronal apoptotic death mediated by intracellular depletion of K^+ and accumulation of Na^+ and Ca^{2+} [168]. Plasma membrane Ca^{2+}-ATPase (PMCA) is a regulator of intracellular calcium which undergoes early developmental changes in rat brain as a function of its maturity [169]. PMCA is very sensitive to the inhibitory effect of reactive oxygen species (ROS) due to the age dependent oxidative modification of PMCA and the related chronic oxidative stress [170].

In addition to generation of free radicals, cellular degeneration that is involved in cigarette smoking is related to the accumulation of advanced glycosylation end-products (AGE). Activities of several enzymes are inhibited due to enzyme protein glycation [171, 172]. The changes in the Ca^{2+} ATPase can be related to the increased glycation found in cigarette smoke exposed rats that in turn may lead to the enzyme protein glycation [173]. Alterations in the capacity to maintain normal calcium homeostasis underlies the reduced cellular function bound with the aging process. In the brain, multiple methionines within the calmodulin molecule become oxidized to methionine sulfoxides, resulting in an inability to activate a range of target proteins, including plasma membrane Ca^{2+}-ATPase [174].

Mg^{2+}-ATPase is not uniformly distributed and differs in respect to affinity for ATP in rat brain regions [175] and is activated by millimolar concentrations of Mg^{2+}. Comparison of Na^+, K^+-ATPase, and Mg^{2+}-ATPase activities in the synaptic plasma membrane from various regions of rat brain reveals that moderate hypoxia increases the activity of synaptosomal Mg^{2+}-ATPase whereas activities of both Ca^{2+}- ATPase and Na^+, K^+-ATPase are decreased [176].

Increased concentrations of Ca^{2+} by stimulating Na^+/Ca^{2+} exchanger produce cellular Mg^{2+} depletion since excessive calcium displaces magnesium from its binding sites [177]. Decrease in Mg^{2+} in turn inhibits Na^+/K^+-ATPase further, as ATP-Mg complex is the actual substrate for the enzyme [178]. Rats exposed to cigarette smoke showed a decrease in the activity of brain Mg^{2+}-ATPase. The restoration of membrane bound ATPases maintained the electrolyte homeostasis in brain, impairing electrolyte balance in cigarette smoking.

8.7. Apoptotic and Neurogenic Changes. Dysregulation of apoptosis is an important factor in the pathogenesis of cigarette smoking [179]. Nicotine is involved in both stimulation and inhibition of neuronal apoptosis [180–182]. Apoptosis is suggested as a possible contributing factor in the pathogenesis of smoking-induced toxicity. Exposure to cigarette smoke induced apoptosis as characterized by DNA laddering, increased TUNEL-positive cells, and apoptotic features evident ultrasctructurally in the brain. Administration of BA prevented expression of hsp70 and neuronal apoptosis during cigarette smoking [183]. Extract of BM reduced oxidative stress by improving Nrf2 expression and results in improvement in antiapoptotic (Bcl2) expression and decreased proapoptotic (Bax and caspase-3 activity) indicating neuroprotection [184].

8.8. Therapeutic Implications of BA in Passive Smoking. An insight into these observations supports the role of BA as a supplement for secondhand smoking. Its role on nAChR expression may underpin its effect on cigarettes induced neurochemical alteration. Generally antidepressants are noncompetitive inhibitors of nAChRs [185] and so it is possible that the role of BA as a noncompetitive inhibitor to nAChRs could potentially help in controlling the nAChR mediated upregulation of neurotransmitters and nicotine dependence [186], apart from its role on nAChR expression at the transcriptional level.

Other potential sites of action for BA worthy for consideration include its ability to control inflammation and oxidative stress. Antioxidants and anti-inflammatory drugs potentially negate the anxiolytic behaviors [187, 188], a feature also prevalent in passive smokers. Exploitation of the antioxidant property of BA could aid in overcoming oxidative anxiety disorders.

9. Conclusion

A number of admonitions exist in the data presented. The interpretations are drawn from a study involving chronic

exposure of rats to cigarette smoke and not acute cigarette smoke. The cross-sectional nature of this work is hampered from conclusions not drawn from molecular pathways. Future research efforts in this area should attempt to address these shortcomings. It would be useful to ascertain the effects of BA on individual components of cigarette smoke constituents involving multiple pathways. Given that passive smoking affects multiple pathways and may increase risk of developing anxiety, triangulation of potential effects involving a combination of animal and human models will likely be required. As the role of BA appears to be multifaceted, it may represent a future therapeutic means for secondary smoke. In addition, to its neuroactive role, BA as an anti-inflammatory and antioxidant agent may assist in improving the symptoms, as they may do in other conditions pertaining to oxidative stress. Further studies addressing this area may elicit insights into new therapeutic opportunities.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Validation of Quantitative HPLC Method for Bacosides in KeenMind

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Brahmi (*Bacopa monnieri*) has been used by Ayurvedic medical practitioners in India for almost 3000 years. The pharmacological properties of *Bacopa monnieri* were studied extensively and the activities were attributed mainly due to the presence of characteristic saponins called "bacosides." Bacosides are complex mixture of structurally closely related compounds, glycosides of either jujubogenin or pseudojujubogenin. The popularity of herbal medicines and increasing clinical evidence to support associated health claims require standardisation of the phytochemical actives contained in these products. However, unlike allopathic medicines which typically contain a single active compound, herbal medicines are typically complex mixtures of various phytochemicals. The assay for bacosides in the British Pharmacopoeia monograph for *Bacopa monnieri* exemplifies that only a subset of bacosides present are included in the calculation of total bacosides. These results in calculated bacoside values are significantly lower than those attained for the same material using more inclusive techniques such as UV spectroscopy. This study illustrates some of the problems encountered when applying chemical analysis for standardisation of herbal medicines, particularly in relation to the new method development and validation of bacosides from KeenMind.

1. Introduction

The human brain is a complex organ that neuroscientists are still attempting to understand. As people live longer, dysfunction of the brain is becoming a predominant issue for the healthcare system. Cognitive decline, particularly in elderly people, often derives from the interaction between age-related changes and age-related diseases and covers a wide spectrum of clinical manifestations, from intact cognition through mild cognitive impairment and dementia. Neural dysfunction of the brain is becoming a predominant issue for the healthcare system as a result of human longevity.

In recent years, the interest in the use of herbal products has grown exponentially, particularly in the western world as well as in developed countries [1]. It is now becoming exceedingly apparent that available psychotherapeutics does not properly meet therapeutic demands of a vast majority of patients with mental health problems and that herbal remedies remain to be the alternative therapeutic hope for many such patients. In the folklore of Indian medicine, several herbs have been used traditionally as brain or nerve tonics. One of the most popular of these herbs is *Bacopa monniera* Wettst. (syn. *Herpestis monniera*), which belongs to the family Scrophulariaceae.

2. Materials and Methods

2.1. Brief Description of the Plant. Brahmi [2–6] has been used by Ayurvedic medical practitioners in India for almost 3000 years. The earliest chronicled mention is in the Ayurvedic treatise, the Charaka Samhita (100 A.D.), in which Brahmi is recommended in formulations for the management of a range of mental conditions including anxiety, poor cognition, and lack of concentration. According to the Charaka, Brahmi acts as an effective brain tonic that boosts one's capabilities to think and reason. The Sushruta Samhita [7] (200 A.D.) attributes the plant with efficacy in maintaining acuity of intellect and memory.

The herb is from a family Scrophulariaceae and is a small creeping herb with numerous branches, small oblong

leaves, and light purple or small and white flowers, with four or five petals. It is found in wetlands throughout the Indian subcontinent in damp and marshy or sandy areas near streams in tropical regions. The genus *Bacopa* includes over 100 species of aquatic herbs distributed throughout the warmer regions of the world, apart from India, Nepal, Sri Lanka, China, Taiwan, and Vietnam and is also found in Florida and other southern states of the USA [8].

2.2. Active Constituents. Compounds responsible for the pharmacological effects of Bacopa include alkaloids, saponins, and sterols. Detailed investigations first reported the isolation of the alkaloid "brahmine" from Bacopa [9, 10]. Later, other alkaloids like nicotine and herpestine and isolation of D-mannitol and saponin, hersaponin, and potassium salts have also been reported [9, 10]. The major chemical entity shown to be responsible for neuropharmacological effects and the nootropic action or antiamnestic effect of Bacopa is bacoside.

2.3. Bacosides Description. The term bacosides refers to dammarane-type triterpenoid glycosides found in extracts of *Bacopa monnieri*. There are over 30 bacosides reported, with most being either jujubogenin or pseudojujubogenin glycosides.

Triterpenoid glycosides fall into the broader category of "saponins," as their amphoteric nature allows them to form emulsions in water. Triterpenoids are widely reported actives in plant based medicines and synthetic analogues have been developed for specific pharmacological functions.

Bacosides were first reported by Chatterji et al. in 1963 [11] and described as Bacoside A and Bacoside B. They were isolated by crystallisation and separated by silica column chromatography and therefore categorised as only two distinct molecules. Later research demonstrated that Bacosides A and B were in fact groupings of coeluting compounds known as Bacosides A and Bacosides B, consisting of at least 4 different but closely related jujubogenin and pseudojujubogenin glycosides. Beyond those major bacosides regarded as Bacosides A and B, there are more highly glycosylated bacosides, various minor jujubogenin/pseudojujubogenin glycosides as well as cucurbitacin glycosides, and aglycone forms of both pseudojujubogenin and jujubogenin (Figure 1).

2.4. Analytical Techniques for Measuring Bacosides. Early methods for quantification of Bacopa saponins involved conversion to ebelin lactones by acidic hydrolysis and then measuring these by UV-spectrophotometry Pal and Sarin (1992) [12]. Bacosides, like most triterpenoids, are largely saturated and therefore have only a small UV absorbance coefficient. Ebelin lactones which can be formed by acidic hydrolysis of various triterpenoids including bacosides have a strong chromophore and are readily detected by UV-spectroscopy at 278 nm.

It was not until 2004 that Ganzera et al. [13] published the first analytical procedure to separate and quantify bacosides by HPLC. The following year, Deepak et al. [14] published a method for quantitative determination of the major saponin

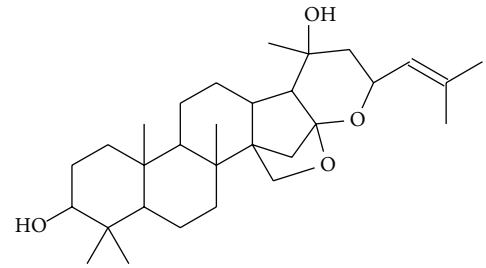

Figure 1: Jujubogenin MW:472.707.

mixture Bacoside A in *Bacopa monnieri* by HPLC. Murthy et al. [15] followed with a similar approach to Deepak but more comprehensive in its inclusion of 12 bacosides calculated. In 2011 both the British Pharmacopoeia (BP) and the United States Pharmacopoeia (USP) for the first time included monographs for *Bacopa monnieri*. Each included an assay for bacosides by HPLC, which appear to be based upon methods described by Murthy et al. [15] and Deepak [14] respectively.

It is generally expected that compendia methods are validated and can be applied directly without requirement for further validation. While this may be workable for uncomplex pharmaceuticals, it is less realistic when applying methods to complex herbal formulas such as those made from *Bacopa monnieri*. We challenged the current BP method for quantification of a bacosides to routine method validation to assess the suitability of this method for stability evaluation of the potency of KeenMind (http://www.keenmind.info/).

Validation of analytical methods involves examining the uncertainty associated with each component of a methodological procedure as a means to assessing the suitability of a method for its desired purpose.

General procedures and parameters for validation of analytical methods for the measurement of pharmacologically active substances are guided by regulatory guidelines established by the WHO and PIC/S as well as National Pharmaceutical Compendia such as the BP and the USP.

The method separates bacosides by HPLC using an isocratic mobile phase with detection by UV-Vis detector at 205 nm (Figure 2). Bacopaside II is used as the calibrating standard and selected bacoside peaks are identified by their relative retention time to Bacopaside II.

We used an Agilent 1100 HPLC with a UV-Vis detector, and a reverse phase Phenomenex Synergi 250 mm × 4.6 mm HPLC column with 5 μm, C18 (octadecyl) packing. The isocratic mobile phase was prepared by mixing 315 volumes of acetonitrile and 685 volumes of 0.72% w/v anhydrous sodium sulphate, previously adjusted to pH 2.3 with sulphuric acid. This was run isocratically over 75 minutes with a flow rate of 1.0 mL/min and an injection volume of 20 μL.

A stock solution of the calibrating reference standard Bacopaside II was prepared diluting 5 mg into 5 mL with methanol (1 mg/mL). This was further serial diluted to create 5-point standard curve across a concentration range of approximately 1.0 to 0.01 mg/mL. The contents of 20 capsules of KeenMind were combined to provide a representative sample. Approximately one gram of the powdered extract

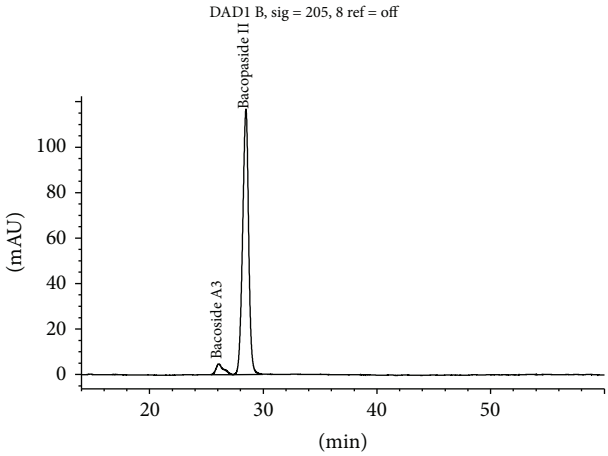

Figure 2: Bacopaside II HPLC by BP method at 205 nm.

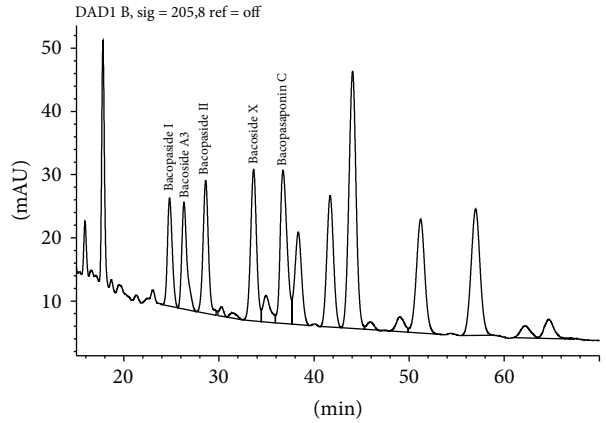

Figure 3: Bacoside peaks (labelled) included in total Bacosides by BP.

Table 1: Peak purity values for BP bacoside analytes. Purity is indexed from 0 to 1000.

Compound	Peak purity
Bacoside A3	973.8
Bacopaside II	951.1
Bacoside A	827.9
Bacopasaponin C	977.8
Bacopaside I	787.5
Other peaks	>800

was diluted in 70% methanol in 50 mLs and sonicated for 30 minutes, followed by centrifugation. The solution was sampled for injection onto HPLC (Tables 1–4).

According to the BP, when the chromatograms are recorded using, the prescribed conditions the retention time of Bacopaside II is about 36 minutes. According to the BP, the retention times relative to Bacopaside II are as follows: luteolin, about 0.3; Bacoside A3, about 0.9; Bacoside A, about 1.2; Bacopasaponin C, about 1.3; Bacopaside I, about 1.4 (Figure 3, Table 5). According to the BP method the test is not valid unless, in the chromatogram obtained with the test solution, the resolution factor between the peaks due to Bacoside A3 and Bacopaside II is at least 1.5 and the resolution factor between the peaks due to Bacoside A and Bacopasaponin C is at least 2.4. The total content of Bacopa saponins, expressed as Bacopaside II, is calculated from the chromatograms obtained and using the declared content of Bacopaside II in the certified reference standard.

Table 2: % RSD of standard replicate injections across the calibration range.

	Concentration (mg/mL)	Mean	Standard deviation	% RSD
LOD	$4.45E-03$	62	6.81	12.53
LOQ	$8.90E-03$	94.84	9.47	9.98
	$4.45E-02$	454.86	11.42	2.51
Working level	$8.90E-02$	934.95	6.78	0.73
	$4.45E-01$	4409.71	16.03	0.36
	$8.90E-01$	9346.45	42.18	0.45

Table 3: Accuracy results.

Placebo (%)	Placebo (mg)	Bacopaside II (mL)	Bacopaside II (mg)	(%w/w)
0	0	1	0.223	100.00
80	16	1	0.223	104.97
100	20	1	0.223	102.76
120	24	1	0.223	100.51

Table 4: Extraction efficiency results.

Extraction number	%w/w bacosides	% recovered
1	9.235	97.7
2	0.213	2.30
3	0.000 (nd)	0.00

3. Results

3.1. Validation of the BP Bacosides Assay. We applied validation procedures to the current BP assay for bacosides in KeenMind *Bacopa monnieri* extract.

Validation parameters examined included specificity, linearity, limit of detection, limit of quantitation, system precision, method precision, extraction efficiency, intermediate precision, and robustness (Tables 1–6).

"Specificity" determines that the analyte/s are correctly identified and suitably distinct to allow for accurate measurement. Specificity was assessed initially using the peak purity function on HP Chemstation and by examination of peak profiles and symmetry. Because of the structural similarity of bacosides with respect to their chromophore, the peak purity function was unable to differentiate between different overlapping bacoside peaks. It did however indicate that bacoside analytes were not coeluted with compounds of a different structural class. Table 1 shows peak purity values for the bacoside analytes calculated by BP bacosides assay.

Table 5: Peak areas and retention times of replicate injections of standard solution at 0.089 mg/mL.

Replicate injection number	Peak area	Retention time (min)
1.00	936.26	27.882
2.00	929.30	27.809
3.00	936.67	27.886
4.00	945.46	27.875
5.00	925.90	28.009
6.00	936.10	27.935
Mean	**934.947**	**27.889**
STDEV	**6.783**	**0.067**
%RSD	**0.73**	**0.24**

Table 6: Results of method precision (1st operator) and intermediate precision (2nd operator).

Sample replicate	Injection replicate	Bacosides (%w/w)	
		1st operator	2nd operator
1	1	1.02609	0.9489
	2	1.0181	0.9501
	3	1.0053	0.9494
2	1	1.03001	1.047
	2	1.05028	0.9872
	3	1.03453	0.97095
3	1	1.00957	0.97228
	2	1.02717	0.97656
	3	1.05503	0.9754
4	1	0.99785	0.938
	2	0.9765	0.97555
	3	0.9665	0.9839
5	1	1.09557	0.9748
	2	1.03263	1.0428
	3	1.04265	1.01844
6	1	0.9804	1.0184
	2	0.97473	0.9899
	3	0.99676	0.99117
%w/w (mean)		**1.018**	**0.984**
STDEV		0.031	0.024
% RSD		**3.00**	**2.46**

Close examination of peaks included as bacosides by the BP shows minor peaks occurring on the front tails indicating nonspecificity. Given their equivalent UV-Vis response it is likely that these represent minor bacosides.

We purchased available reference standards for the major bacosides to confirm the correctness of the BP peak identification guide which ascribes relative retention times for peaks to be calculated as bacosides relative to the retention time of Bacopaside II. According to this guide Bacopaside I is the large peak eluting at 38.5 min whilst the reference standard purchased from Sigma-Aldrich coelutes with the peak at 22.5 min (Figure 3). Further, the BP method refers to Bacoside A as being a single peak at 1.2 times the retention time of Bacopaside II. The identification of Bacoside A as a single compound is an historical error. The peak at 1.2 times the retention time of Bacopaside II is Bacopaside X, as confirmed by comparison to the purchased reference standard.

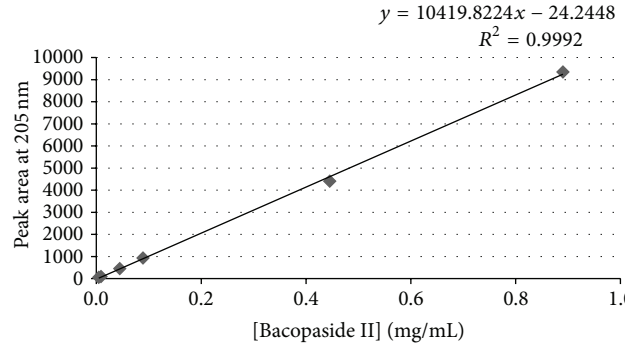

Figure 4: Bacopaside II calibration curve.

Interestingly, this method does not include bacosides eluting after 29.5 min, which are clearly evident in the trace. This effectively diminishes the value for calculated bacosides in KeenMind from about 30% to about 10%. However, clinical trials which have provided supporting evidence for claims for *Bacopa* efficacy and thereby effective dosage ranges have been standardised for total bacoside by more inclusive methods based on UV-spectroscopy. Such methods calculate total bacoside values of about 50%. This creates problems for sponsors when displaying appropriate dosage recommendations on packaging.

To assess linearity, a 6-point set of Bacopaside II, standard solutions prepared in the range of LOQ 150% of the nominal concentration were injected onto HPLC in triplicate. The linearity curve was plotted and the R^2 and Y-intercept were calculated. Across this range an R^2 of 0.9992 was attained where the acceptance criteria are ≥0.990 (Figure 4).

The range of concentration over which the assay is valid is determined by confirming the linear correlation of analyte concentration to instrument response; the limit of detection (LOD); limit of quantitation (LOQ); system and method precision; and accuracy/recovery. We found that the range over which precision, accuracy, and linearity met their defined criteria was from 0.0089–0.89 mg/mL.

LOD (limit of detection) is the concentration at which the analyte is detectable, but where interference from background noise occupies at least 30% (signal : noise ratio = 3) of the peak height, rendering measurement too inaccurate to be recorded. LOQ is the concentration at which the background noise occupies up to 10% (signal : noise ratio = 10) of the peak height, allowing for a reasonable estimate of peak area to be measured.

A series of standard (Bacopaside II) solutions with concentrations ranging from 0.01% of the nominal concentration of the active peak to 100% working strength of the impurities was accurately prepared. Triplicate injections of each were performed and the signal-to-noise ratios determined for all samples starting with the least concentrated. The acceptance criteria for LOD is typically S/N of all 3 injections per

solution ≥3.0, which for Bacopaside II is a concentration of 0.00445 mg/mL.

For LOQ, a series of standard solutions was accurately prepared from the LOD concentration to 100% of the standard working concentration. Triplicate injections of each of the above solutions were performed and signal-to-noise ratios of all the solutions determined. The acceptance criteria for LOQ is typically S/N of all 3 injections per solution ≥10.0, which for Bacopaside II is 0.0089 mg/mL.

Range can also be limited by recovery of the analyte from the matrix in which it is bound, prior to extraction for analysis. "Recovery," also termed "accuracy," is typically assessed by spiking known amounts of the analyte substance into a formulation or carrier matrix and compared to the amount measured in the samples upon analysis. In our validation of the BP method we applied accuracy by both spiked addition and extraction efficiency studies.

For accuracy by spiked addition, Bacopaside II was added at known concentrations to the product placebo. 2.2 mg Bacopaside II reference standard was accurately weighed into a 10 mL volumetric flask and dissolved and diluted to volume with the solvent mix. 1 mL of this solution was accurately transferred to HPLC sample vials containing 16, 20, and 24 mg placebo and sonicated for 15 minutes. The spiked samples were analysed by HPLC and the amount of Bacopaside II calculated. The % recovery of Bacopaside II from theoretical amounts added was determined and used to indicate the impact on matrix binding of the analyte.

The acceptance criteria for accuracy are that the recovery of Bacopaside II is 90.0% to 110.0%, from concentrations ranging from 80 to 120% of nominal stated content of the analyte. Table 3 shows accuracy data within the acceptance criteria at all three spiked addition concentrations.

In our validation of the BP bacoside assay we also applied a technique coined "extraction efficiency" wherein the test sample is extracted in series up to 5 times and each serial extract is analysed quantitatively. The total analyte from all serial extracts is determined and the respective % yield of each progressive extract calculated. This approach is used by our laboratory for herbal substances where the plant extract matrix cannot be readily replicated. It measures the efficiency of the extraction process of the analytical method.

To assess extraction efficiency, about 1 g of the sample powder was weighed into a 40 mL vial. 30 mL of methanol/water (70/30) was added and sonicated for 15 minutes. The solution was centrifuged and the supernatant transferred to a 50 mL volumetric flask and made up to mark. Another 15 mL of methanol/water (70/30) was added to the 40 mL vial containing the residual pellet, sonicated for 15 minutes and the centrifugation step repeated. The process was repeated once more and aliquots were sampled from each of the three 50 mL volumetric flasks for injection onto HPLC.

A nominal acceptance criteria of >95% were set for recovery from first extract, which is prepared according to the BP bacoside method. Table 4 shows extraction efficiency results. Because a small volume of solvent remains in the undissolved tablet material after the supernatant has been decanted from the first extraction, the remaining analyte in this volume is then dissolved in the second extraction. As such, a small amount, typically less than 5%, will be present in the second extract. If analyte is still present in the third extract this is a clear indication that not all analyte was recovered in the first extraction.

System precision is a measure of the uncertainty associated with the instrument operation and is commonly an outcome of sample injection error. For HPLC systems, precision is assessed by measuring the % RSD of 3–6 repeat injections of the same sample, typically a reference standard dilution. For a HPLC in good operating condition acceptance criteria for the retention time are ≤1.0%, and the % RSD of the peak area is ≤10.0%. The average peak area of bacoside peaks is about 1000 mAU which equates to Bacopaside II at 0.089 mg/mL. At this concentration the instrument attained system precision for injection of Bacopaside II of 0.24% RSD for retention time of 27.889 min and 0.73% RSD for an average peak area of 934.95 mAU.

Method precision, also called repeatability, is a measure of the inherent error in sample preparation. A test sample is prepared 6 times and each preparation injected 3 times. The means of repeat injections of each sample are compared and the % RSD measured.

Table 6 shows method precision results for KeenMind when prepared according to BP bacosides. The acceptance criteria were defined such that the mean result at method working strength is within a specified range ≤5.0% RSD.

Intermediate precision is the same task performed by a second operator. A comparison of method precision with intermediate precision is an indication of the human error associated with the sample preparation methodology. This is important as a high level of skill may mask a cumbersome or problematic method.

We attained an intermediate precision result of 2.45% RSD which is comparable to that attained by the first operator. In preparation of herbal specimen for analysis, error can occur with the use of the measuring apparatus, through insufficient extraction of analytes from the product matrix as well as variable peak area calculation affected by poor resolution from other peaks absorbing in the same region at the similar retention time.

Robustness assesses the effect of minor changes to HPLC conditions on the analyte measurement. The robustness of the BP bacoside assay was examined at increased (1.1 mL/min) and decreased (0.9 mL/min) HPLC flow rates, at modified mobile phase buffer concentration (0.7, 0.71, and 0.72% Na_2SO_4), and also at column temperature of 29°C and 31°C compared with a control at flow rate of 1.0 mL/min and 30°C.

The chromatographic separation of bacosides was negatively affected by all system changes applied.

Figures 5–7 demonstrate the effect of adjusting the concentration of sodium sulphate in the mobile phase. These minor variations in mobile phase concentration illustrate how peaks can shift and how merged peaks can result in erroneous identification of individual bacosides.

3.2. Updated Clinical Efficacy of KeenMind. After twelve years of research at Swinburne University, Melbourne, KeenMind suggests that this clinically proven *Bacopa monnieri* product is a safe and efficacious cognitive enhancer [16, 17]. Robust

Table 7: Summary of validation results.

Test	Limits	Conclusions/results	
Specificity	No interfering peaks with that of the target.	Complies, however, bacoside peaks are coeluting	
Linearity (calibration coefficient)	The Y-intercept should not be more than ±2%.	0.38%	
	Linearity $R^2 \geq 0.990$	$R^2 = 0.9992$	
Instrument precision	The % RSD of the retention time is ≤1.0%	0.24% RSD	
	The % RSD of the peak area is ≤10.0%	0.073% RSD	
Detection limit	S/N ≥ 3	0.00445 mg/mL	
Quantitation limit	S/N ≥ 10	0.0089 mg/mL	
Method precision	The mean result at method working strength is within the specification	Pass (1.02 %w/w)	
	The % RSD is ≤10.0%	3.0% RSD	
Intermediate precision	The mean result at method working strength is within the specification	Pass (1.02 %w/w)	
	The % RSD is ≤10.0%	2.5% RSD	
Extraction efficiency	>95% recovery from first extract	97.7% of total recovered	
Accuracy/recovery	At concentrations ranging from 80 to 120% of nominal stated content, the recovery Bacopaside II is 90.0% to 110.0%	Sample	%
		80	100.51
		100	102.76
		120	104.97
Range	Precision, accuracy, and linearity must meet their criteria from LOQ% to 150% of the label claim	0.00445–0.89 mg/mL	

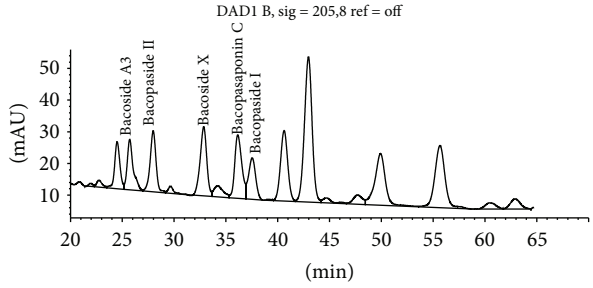

Figure 5: $[Na_2SO_4] = 0.70\%$.

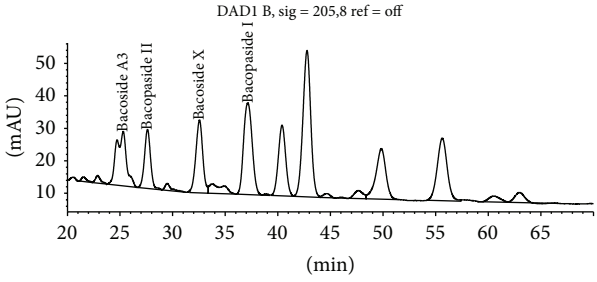

Figure 6: $[Na_2SO_4] = 0.71\%$ showing Bacopasaponin C merged with Bacopaside I.

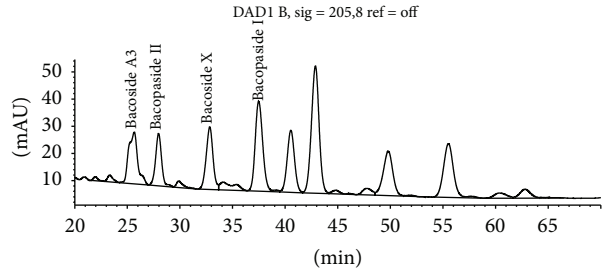

Figure 7: $[Na_2SO_4] = 0.72\%$ showing Bacoside A3 merged with peak 1.

4. Discussion and Conclusion

We found that the BP Bacopa assay is valid for the analysis of Bacopaside II as the validation results have met the acceptance criteria for this molecule (Table 7). However, the assay was not valid for accurate quantification of total bacosides due to issues with specificity as well as poor robustness and reproducibility.

Even very minor changes to HPLC conditions resulted in large shifts in peak shape and resolution. We found that the quality of separation attained was dependent upon the column condition at the time of use. This creates significant problems in stability trial evaluation where testing time-points are often months apart in which time changes to HPLC and HPLC column condition is inevitable. In order to provide reproducible stability results we run a characterised specimen of KeenMind which is stored frozen and adjust

evidence for its chronic enhancing effect is strongest, with recent studies also suggesting an acute cognitive enhancing effect [18, 19]. Additional trials with longer administration durations [20] are ongoing at Swinburne University.

the buffer concentration of the HPLC mobile phase until optimal separation of bacosides is achieved. It is necessary to equilibrate the HPLC column in buffer solution for up to 2 hours before use and condition the column after use with blank injections of butanol. Two blank runs use a butanol injection of 20 μL at the beginning of the run to confirm the absence of contaminants/carryover. To ensure the suitability of the column condition for adequate separation of bacosides a secondary reference standard is run to confirm that the peak order of elution and resolution is consistent with that prescribed by the method.

Standardisation of herbal medicines is fraught with challenges. While claiming pharmacological efficacy and having clinical evidence to support such claims, the actual mechanisms of activity are often not well understood. While we are confident that bacosides are the active constituents of *Bacopa monnieri*, we do not yet understand how these are metabolised and in what form they are functionally active. There are suggestions that Bacosides A are active while Bacosides B are not. While this may be true, differentiating between the two classes creates significant analytical problems.

Bacosides are saponins and as such have "detergent" like properties. They are therefore more susceptible to subtle change in the solid phase condition of HPLC columns. Without a buffered HPLC system, bacosides do not separate well. But this does not mean they cannot be effectively measured. Ganzera et al. [13] described a method for basic separation of bacosides from the flavonoids and phenolic acids also present in Bacopa extracts. While they did not achieve baseline separation the method is very inclusive of bacosides and produces results which are similar to those attained by the UV-spectrophotometry methods used to standardise products upon which current clinical evidence is based.

Ultimately the most accurate measure of bacosides will be achieved by gravimetric isolation using liquid/liquid partitioning and preparative column chromatography. This approach is more of research activity than being routine analytical so it is not suitable for quality control laboratories. The original ebelin lactone methods by UV-spectrophotometry are simple to apply and could be standardised by comparison to gravimetric results.

Conflict of Interests

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Research Article

A Special Extract of *Bacopa monnieri* (CDRI-08)-Restored Memory in $CoCl_2$-Hypoxia Mimetic Mice Is Associated with Upregulation of *Fmr-1* Gene Expression in Hippocampus

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Fragile X mental retardation protein (FMRP) is a neuronal translational repressor and has been implicated in learning, memory, and cognition. However, the role of *Bacopa monnieri* extract (CDRI-08) in enhancing cognitive abilities in hypoxia-induced memory impairment via *Fmr-1* gene expression is not known. Here, we have studied effects of CDRI-08 on the expression of *Fmr-1* gene in the hippocampus of well validated cobalt chloride ($CoCl_2$)-induced hypoxia mimetic mice and analyzed the data with alterations in spatial memory. Results obtained from Morris water maze test suggest that $CoCl_2$ treatment causes severe loss of spatial memory and CDRI-08 is capable of reversing it towards that in the normal control mice. Our semiquantitative RT-PCR, Western blot, and immunofluorescence microscopic data reveal that $CoCl_2$-induced hypoxia significantly upregulates the expression of Hif-1α and downregulates the *Fmr-1* expression in the hippocampus, respectively. Further, CDRI-08 administration reverses the memory loss and this is correlated with significant downregulation of Hif-1α and upregulation of *Fmr-1* expression. Our data are novel and may provide mechanisms of hypoxia-induced impairments in the spatial memory and action of CDRI-08 in the recovery of hypoxia led memory impairment involving *Fmr-1* gene encoded protein called FMRP.

1. Introduction

Brain requires a continuous supply of oxygen to perform its normal function. Being the largest consumer of oxygen, it is especially sensitive to hypoxia, a condition in which brain receives reduced oxygen. Several studies have shown that injury to the brain due to loss of oxygen triggers memory loss and causes learning and memory deficits [1, 2]. Although the whole brain is susceptible to hypoxia, hippocampus in particular has been reported to be severely affected by hypoxia [3, 4] as it plays crucial roles in encoding and consolidating memory [5, 6].

Memory formation, maintenance, and retrieval are dynamic processes involving transcription, translation, and expression of several proteins [7]. Fragile X mental retardation protein (FMRP), an mRNA-binding protein [8–11], is prevalently present in dendritic spines [12] and regulates protein synthesis relevant to synaptic plasticity [10]. FMRP was first characterized in context of the fragile X syndrome (FXS) which results from loss of function mutations in *Fmr-1* gene, which in turn results in mental retardation, loss of memory, and abnormal cognitive behavior in fragile X mental retardation syndromes (FXS). FMRP is a 70–80 KDa protein abundantly expressed in brain and testis [13, 14]. FMRP-mediated translational regulation plays important roles in proper synaptic connectivity [15] and plasticity [16, 17]. Since the dendritic protein synthesis required for achieving synaptic plasticity is under the strict control [18, 19], any change in the level of FMRP may lead to alterations in the synaptic plasticity, thus learning and memory. Whether hypoxia leads to any alterations in the expression of *Fmr-1* gene is not known. Therefore, we have examined the effects of hypoxia on the expression of the *Fmr-1* gene at transcript and protein levels in relation to learning and memory in cobalt chloride-induced hypoxia mimetic mouse model.

Bacopa monnieri, also known as Brahmi, is a traditional Ayurvedic medicinal plant and it has been extensively used in India as a nerve tonic for centuries [20]. In the Indian Ayurvedic system of medicine, *Bacopa monnieri* belongs to a group of medicine called "Medhya Rasayana" which is known to act on nervous system and improve mental abilities by enhancing memory and tunes cognition. *Bacopa monnieri* extract contains mixture of saponins, for example, bacoside A, bacopasides I and II, bacopasaponin C, and flavonoids [21–23], as active constituents. Its extract has been reported to facilitate cognitive functions as well as to augment mental retention capacity. There is evidence that the mechanism of action of *Bacopa monnieri* could be attributed to a combination of cholinergic modulation [24–27] and antioxidant effects [28–31]. Although many reports suggests the nootropic capabilities of *Bacopa monnieri* extract, its effect on *Fmr-1* gene expression in relation to learning and memory has not been studied to date. In the light of the crucial role played by the *Fmr-1* gene encoded FMRP in the formation and maintenance of synaptic connectivity, it is possible that *Fmr-1* gene could be one of the targets of bacoside's action during memory enhancement. Therefore, in the present study, we have investigated whether hypoxic condition leads to any alteration in spatial memory and this alteration is associated with change in the expression of FMRP in the hippocampus of cobalt chloride-induced hypoxic mouse model [32], and further we studied whether a selected dose of CDRI-08 (obtained from a pilot study) recovers the alteration in spatial memory and reverses alterations in the *Fmr-1* gene expression in the hippocampus due to hypoxia.

2. Materials and Methods

2.1. Materials. A standardized extract of *Bacopa monnieri* (CDRI-08) containing 58.18% of bacosides was received as a kind gift from Dr. H. K. Singh, Director, Lumen Research Foundation, Chennai, India. Cobalt chloride was purchased from Sisco research laboratory, India (SRL). All other chemicals and reagents were of analytical grade and purchased from Merck, India, and Sigma Aldrich, USA.

2.2. Animals and Drug Treatment. Male Swiss albino mice of age 20 ± 5 weeks, weighing 25 ± 5 g were used in the present study. Mice were housed in the animal house maintained at 25 ± 2°C with alternating 12 h light and dark cycles, access to standard mice feed and water *ad libitum*. All experimental procedures were approved by the ethical committee of Banaras Hindu University. Prior to exposure to hypoxia mimetic condition mice were trained in Morris water maze for 8 days. After training, mice were randomly divided into six groups ($n = 7$ mice per group) for different treatments as (1) control group (C) administered with 5% Tween 80, (2) Brahmi group orally treated with standardized dose of *Bacopa monnieri* extract (CDRI-08) (200 mg/Kg BW) dissolved in 5% Tween-80 for 8 days, (3) hypoxia group 1 (HA) in which mice were administered with standardized dose of cobalt chloride (40 mg/kg BW) for 15 days [32] and then were kept for 8 days without any treatment to check if hypoxic condition reverts back to normal in this time period, (4) hypoxia group 2 (HB) in which mice were administered with standardized dose of cobalt chloride (40 mg/kg BW) for 15 days, (5) mice who were orally administered with CDRI-08 (200 mg/Kg BW in 5% Tween-80) for 8 days as mentioned above followed by induction of hypoxic condition (B + H), (6) and mice were first orally treated with cobalt chloride followed by treatment with CDRI-08 (200 mg/Kg BW in 5% Tween-80) for 8 days (H + B). After completion of respective treatments, mice of all the groups were subjected to Morris water maze test. The animals were sacrificed and the brain was dissected out on ice. The hippocampus was removed for RNA isolation and protein lysate preparation for gene expression studies. For preparation of cryostat brain sections, the mice were anesthetized using 50 mg/Kg BW sodium pentobarbital and perfused with 4% paraformaldehyde in PBS before sacrificing.

2.3. Morris Water Maze Test. Morris water maze test, a well established behavioral test for evaluation of spatial navigation memory in rodents, was performed on the experimental mice following the procedure of Morris et al. [5]. The Morris water maze consisted of a black circular tank (106 cm diameter, 76.2 cm height) filled with water up to 1/3 height maintained at a temperature of 24 ± 2°C. A Plexiglas escape platform (9.5 cm × 35 cm) was submerged at a fixed position 1 cm below the water surface. Distinct geometric visual cues were fixed in each quadrant at specific locations which were visible to mice while under training and test. Performance of mice in the maze was recorded by video camera suspended above the maze and interfaced with a video tracking system (ANY-maze software, Microsoft version 4.84, USA). Mice were given an acclimatization session of 60 s in the water maze 2 days before the start of training. The training consisted of 3 trials each of 90 s/day with an intertrial interval of 5 min for 8 days. Each trial consisted of gently placing the mice by hand into the water, facing the wall of the pool and being allowed to swim freely for 90 s and find the hidden platform. Mice which failed to locate the platform within 90 s were guided to the platform and allowed to remain on the platform for 15 s. After the completion of training period of 8 days, mice were divided randomly into six groups as described earlier and after the completion of all treatments, Morris water maze test was performed to investigate hypoxia induced loss of memory and evaluation of its recovery by CDRI-08. Alteration in spatial learning and memory was assessed in terms of latency (sec) and path length (m). Latency is defined as the time taken by mice to locate the hidden platform, expressed in sec whereas the path length is defined as distance travelled by mice to reach the hidden platform, expressed in m. In the probe-trial experiment in which the hidden platform was removed alteration in memory was studied in terms of time spent in target quadrants and number of platform crossings to infer the strength of the memory of the mouse for locating the platform.

2.4. Cryosectioning and Immunofluorescence Detection of FMRP. To study the *in situ* expression of FMRP, first 15–20 mL of normal saline was passed transcardially to flush

Table 1: Details of gene specific primer sequences, temperature conditions, cycle numbers and amplicon sizes.

Genes	Primers	PCR condition		Amplicon size
Hif-1a	F 5′-AGACAGACAAAGCTCATCCAAGG-3′ R 5′GCGAAGCTATTGTCTTTGGGTTTAA-3′	94°C - 3′ 94°C - 45″ 59°C - 30″ 72°C - 45″	30 cycles	100 bp
Fmr-1	F 5′-TTACAGAAATAGGGGGCACG-3′ R 5′-TACGCTGTCTGGCTTTTCCT-3′	94°C - 3′ 94°C - 45″ 59°C - 30″ 72°C - 45″	34 cycles	388 bp
Actb	F 5′-ATCGTGGGCCGCTCTAGGCACC-3′ R 5′CTCTTTGATGTCACGCACGATTTC-3′	94°C - 3′ 94°C - 45″ 57°C - 30″ 72°C - 45″	28 cycles	543 bp

out the blood. Thereafter, intra-arterial perfusion of 4% paraformaldehyde solution was given. The brain was dissected out and was kept in paraformaldehyde medium at 4°C overnight. Then the brain tissues were cryopreserved in different grades of sucrose, that is, 10%, 20%, and 30% sucrose. Finally, cryosectioning was carried out using HM525 Microcryotome and sections of 15 μm thickness were obtained. Cryosections were washed in PBS and were then permeabilized by soaking in 0.3% triton X-100 in PBS medium for 10 min, washed in 1X PBS for 5 min, and were kept in blocking solution containing 5% goat serum, 0.2% Tween-20, and 0.2% NP-40 in PBS for 3 hrs at RT. Thereafter, the sections were incubated in anti-FMRP primary antibody (1 : 200 dilution; Sigma Aldrich), overnight at 4°C followed by washing in 1X PBS for 15 min. The sections were then incubated with FITC conjugated goat anti-rabbit IgG (1 : 500 dilution; Bangalore Genei) for 4 hrs at RT in dark. Sections were then mounted in fluoroshield mounting medium containing DAPI and photographs were taken at 540 nm for FITC and 460 nm for DAPI at 20x magnification using Nikon 90i Motorized Research Microscopy, equipped with NIS Elements 4.0 AR software. The immunofluorescence intensity was analyzed as integrated densitometric value (IDV) using Image J software.

2.5. Total RNA Isolation. Total RNA from the hippocampal samples was isolated using TRI reagent (Sigma, USA) following the suppliers manual. The aqueous phase was collected and mixed with equal volume (v/v) of isopropanol and precipitated at –70°C. The RNA pellet was collected, washed with ice-chilled 70% ethanol, and dissolved in DEPC-treated water. Extracted RNA was treated with DNase-I (DNAfree, Ambion) according to the manufacturer's guidelines to remove any DNA contamination. RNA content was determined by measuring the absorbance at 260 nm using UV-Visible Spectrophotometer and its integrity was checked by 1% formaldehyde agarose gel electrophoresis following the procedure described earlier [33], and quality of its preparation was found suitable for RT-PCR experiment (results not shown).

2.6. Semiquantitative RT-PCR. To carry out semiquantitative RT-PCR, cDNA strands were synthesized in each case by mixing 2 μg of the DNA free total RNA and 200 ng random hexamer primers (MBI Fermentas, USA) in 11 μL reaction volume and incubating the whole mix at 70°C for 5 min. Thereafter, 2 μL of 5X reaction buffer, 2 μL of 10 mM dNTP mix, and 20U of RNase inhibitor (Ribolock, MBI Fermentas, USA) were added, and the volume was made up to 19 μL. The tube was incubated for 5 min at 25°C, and 200U of M-MuLv reverse transcriptase (New England Biolabs) was added. Further, the tube was incubated for 10 min at 25°C initially and then at 42°C for 1 h in the thermal cycler (G-Storm, UK). The reaction was terminated by heating the reaction mix at 70°C for 10 min followed by its incubation at 4°C.

The resulting cDNA was used as template to carry out polymerase chain reaction using thermal cycler (G-Strom, UK). PCR reactions were carried out in a 25 μL reaction mixture containing 2 μL cDNA, 1X Taq polymerase buffer with $MgCl_2$, 0.2 mM of each dNTP (MBI Fermentas, USA), 1.0 unit of Taq DNA polymerase (Banglore Genei, India), and 10 pmol of appropriate primers (as shown in Table 1). Reactions were carried out using thermal cycler (G-Strom, UK) with the reaction conditions as described in Table 1. The amplified products were resolved by 2% agarose gel electrophoresis and detected by ethidium bromide staining. The ethidium bromide stained gels were photographed and intensity of the bands as described above was scanned and quantified using Alpha Imager 2200 software separately to obtain integrated density values (IDV) and were normalized with that of β-actin to obtain the relative density values (RDV) for individual amplicons.

2.7. Total and Nuclear Lysate Preparation. For western blot analysis, the cytosolic and nuclear proteins lysate were prepared following the procedure as described earlier [32]. Briefly, the protein lysate from hippocampus was prepared in the buffer containing 20 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.2% NP40, 10% glycerol, 1 mmol/L PMSF, and 1 μg/mL protease inhibitor cocktail. After 5 minutes of incubation on ice, the samples

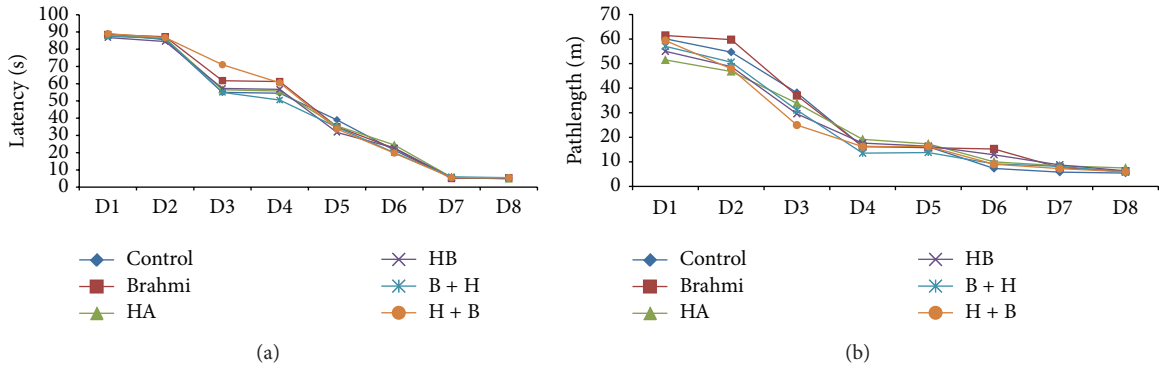

Figure 1: Latency of mice of various groups during training in Morris water maze (a). Path length of mice during training in Morris water maze (b).

were centrifuged at 13,000 ×g for 10 minutes. The supernatants (cytosolic extracts) thereafter were used for the western blot analysis of FMRP. The resulting pellets were suspended in 50 μL buffer containing 350 mmol/L NaCl, 20% glycerol, 20 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 20% SDS, 10% Sodium deoxycholate, and 1 μg/mL protease inhibitors cocktail and the suspension was vigorously mixed with finger tips and incubated on ice for 30 minutes. Thereafter, samples were centrifuged at 13,000 ×g for 10 minutes at 4°C and the resulting supernatants (nuclear extracts) were used for detection of hypoxia marker protein HIF-1α. The total protein contents in both the preparations were estimated by Bradford method using bovine serum albumin as standard [34].

2.8. Western Blot Analysis. In order to examine the levels of expression of HIF-1α and FMRP a uniform 50–80 μg of the protein lysate were resolved by SDS-polyacrylamide gel electrophoresis [35] and transferred onto PVDF membrane by wet transfer method. The membrane was blocked with 5% nonfat milk in PBS (35 mM NaCl, 8 mM Na_2HPO_4, 5 mM KCl, 7 mM KH_2PO_4, pH 7.4) medium for 4 h at room temperature. The blot was then incubated using rabbit polyclonal antibodies for HIF-1α (1 : 1000; Cayman, USA) and FMRP (1 : 2500; Sigma Aldrich) in 5% nonfat milk in PBS (pH 7.4) overnight at 4°C. The blot was further treated with secondary antibody against mouse IgG conjugated with horse radish peroxidase (1 : 2,500) in blocking buffer for 6 h at room temperature. Horse radish peroxidase (HRP) conjugated antibody for β-actin (1 : 25,000; Sigma) was used for the detection of β-actin as internal control. HIF1α, FMRP and β-actin (internal control) signals were detected by enhanced chemiluminescence (ECL) method and the intensity of resulting signals on the X-ray film were scanned and quantified using Alpha Imager 2200 software separately. Scan data of proteins as mentioned above was normalized with that of the β-actin to obtain relative densitometric value (RDV).

2.9. Statistical Analysis. All the experiments were repeated thrice. Data were expressed as mean ± standard error means (S.E.M.). Results obtained from Morris water maze test were analyzed by One way ANOVA followed by post hoc least significance difference test (LSD). For analysis of the molecular data, Tukey's post hoc test was used after one way ANOVA. P value < 0.05 was considered statistically significant.

3. Results

3.1. CDRI-08 Attenuates Hypoxia Induced Alteration in the Spatial Learning and Memory. As shown in Figure 1(a), training of mice for 8 days in Morris water maze leads to progressive improvement of acquisition, the ability of mice to explore the hidden platform in the target quadrant. The decline in latency time indicates that mice got trained with task given on the maze. This is further evident by decrease in path length (Figure 1(b)). Exposure to hypoxic condition resulted in significant increase ($P < 0.05$) in the latency and path length as compared to the control group. The above finding can well be seen in Figures 2(a), 2(b), and 2(c). Hypoxic conditions brought about by cobalt chloride treatment for 15 days (HA) and withdrawal of the treatment for next 8 days (HB) show similar effects. During these conditions, the hypoxia significantly decreased the acquisition of information and poor consolidation as evident by significant increase in the latency period and the path length. The control mice administered with CDRI-08 showed significant decrease in the latency time as well as path length as compared to control mice. Both pre- and posthypoxic treatment of mice with the CDRI-08 caused significant decline ($P < 0.05$) in latency (Figure 2(a)) and path length (Figure 2(b)) as compared to hypoxic groups. In the probe trial test, in which the hidden platform was removed, mice in the hypoxic conditions (HA and HB) showed significant decrease ($P < 0.05$) in number of platform crossings (Figure 2(c)) and time spent in the target quadrant (Figure 2(d)) as compared to the control group. Conversely, the hypoxic mice which were given pre- and posttreatment of CDEI-08 showed a significant increase ($P < 0.05$) in the number of platform crossings (Figure 2(c)) and time spent in the target quadrant (Figure 2(d)) as compared to hypoxic groups (HA and HB). Also, we observe that the CDRI-08, when administered to normal control mice, the number of

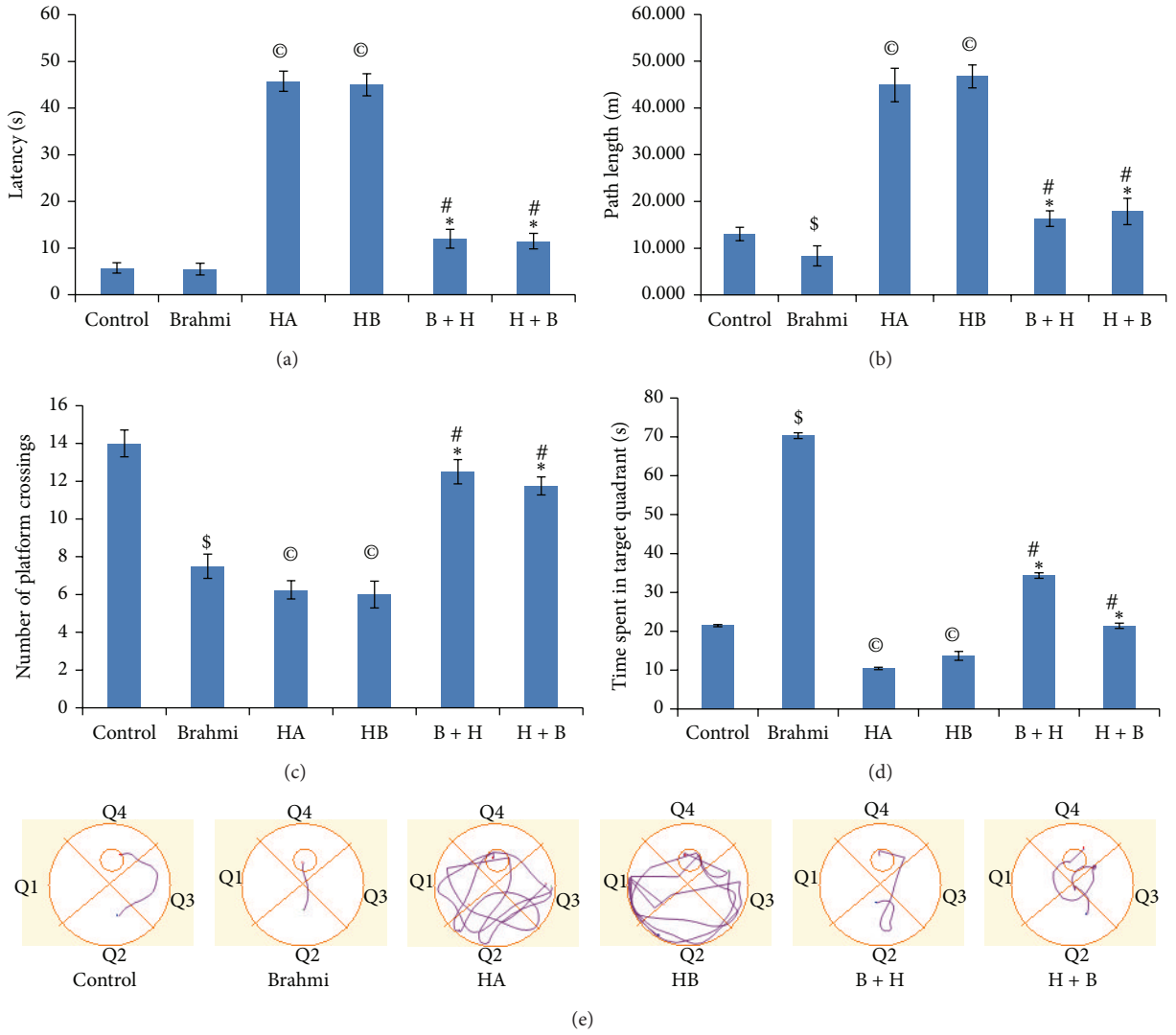

Figure 2: Effects of CDRI-08 on spatial memory of hypoxic mice. (a) Pattern of latency; (b) path length; (c) number of platform crossing, and (d) time spent in target quadrant. Values are expressed as mean ± S.E.M. $^{\$}P < 0.05$ versus control; $^{©}P < 0.05$ versus control; $^{*}P < 0.05$ versus HA; $^{\#}P < 0.05$ versus HB. (e) Track record for probe trail of mice following exposure to hypoxia mimetic condition and CDRI-08 treatment. Q1: Quadrant 1; Q2: Quadrant 2; Q3: Quadrant 3; Q4: Quadrant 4 (target quadrant). Mice administered with BME: Brahmi; HA, treatment of $CoCl_2$ for 15 days to induce hypoxia; HB: hypoxic mice left for 8 days without any treatment after $CoCl_2$ treatment for 15 days; B + H: mice pretreated with CDRI-08 followed by $CoCl_2$ treatment; H + B: CDRI-08 treated hypoxic mice.

platform crossing is significantly reduced and the time spent in the target quadrant is significantly increased. This indicates that the CDRI-08 possesses the ability of enhancing spatial learning and memory.

3.2. CDRI-08 Ameliorates Hypoxia Induced Expression of HIF-1α in the Hippocampus. Our RT-PCR analysis data indicate that expression of *Hif-1α* mRNA is significantly upregulated ($P < 0.05$) due to hypoxia in both the conditions (HA and HB) as compared to control group (Figure 3(a)). CDRI-08 when administered to mice before hypoxia was generated (prehypoxic treatment) and after the hypoxia (posthypoxi treatment) as described above, significantly downregulated the level of *HIF-1α* mRNA ($P < 0.05$) towards the normal as compared to both hypoxic conditions. Our Western blot data reveals that the level of HIF-1α protein is significantly upregulated ($P < 0.05$) in the hippocampus of hypoxic mice of both HA and HB conditions; however, its level is prominently higher in HB conditions as compared to normal control, which confirms the establishment of hypoxic condition (Figure 3(b)). CDRI-08 treatment to hypoxic mice (prehypoxic and posthypoxic) was found to significantly downregulate the level of HIF-1α protein ($P < 0.05$) towards that in the normal control mice.

3.3. Effect of Hypoxia and Bacopa monnieri Extract on Fmr-1 mRNA Expression in the Hippocampus. As shown in Figures 4(a) and 4(b), our RT-PCR data shows that hypoxia does not affect the level of *Fmr-1* mRNA in the hippocampus in the initial phase of hypoxia (HA); however, its level is significantly

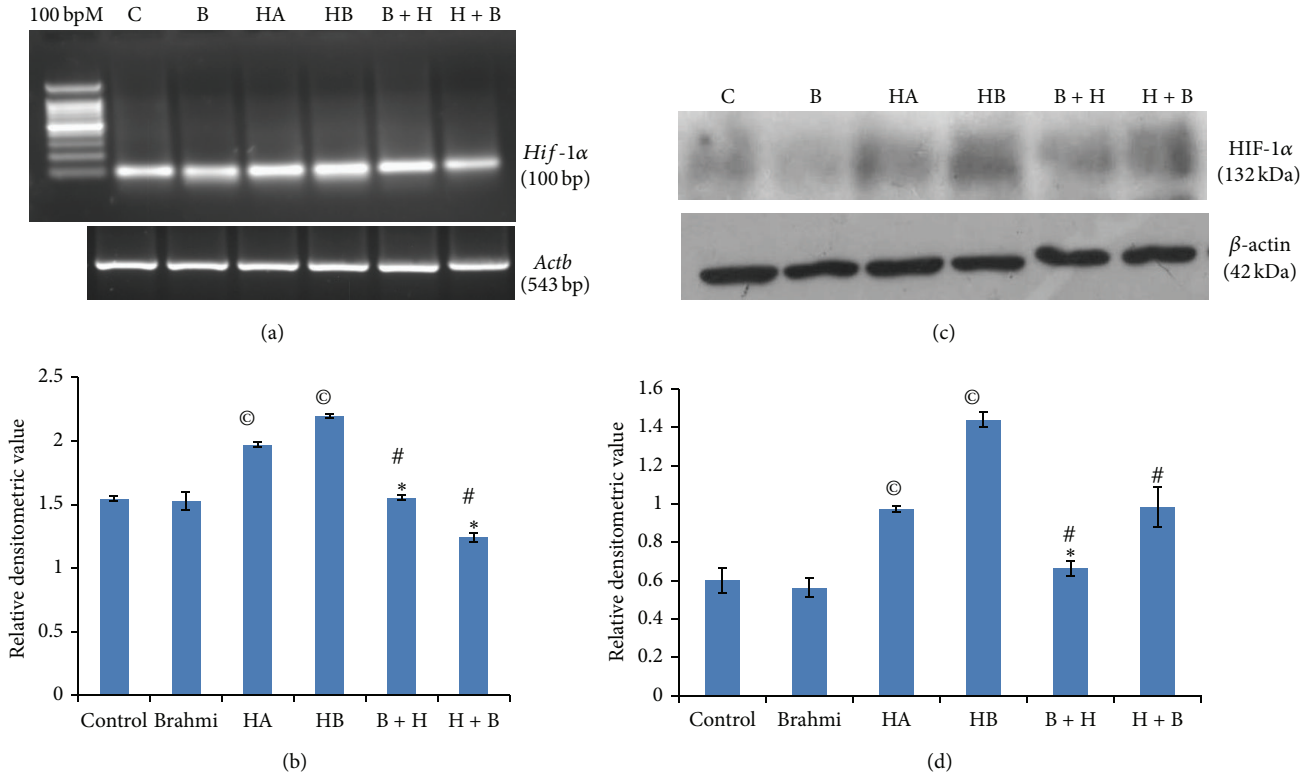

FIGURE 3: Effects of hypoxia and CDRI-08 on *Hif-1α* gene expression. Semiquantitative RT-PCR analysis of *Hif-1α* (a) and Western blot analysis of HIF-1α (c). Bar shows the relative density value developed by integrated densitometric values (IDV) of HIF-1α by IDV of β-actin. Each bar represents the mean ± SEM. $^{©}P < 0.05$ versus control; $^{*}P < 0.05$ versus HA; $^{\#}$, indicates $P < 0.05$ versus HB.

downregulated during the period when hypoxia continued without cobalt chloride treatment (HB) as compared to normal control ($P < 0.05$). Pretreatment with CDRI-08 or posthypoxic CDRI-08 treatment did not show any significant change in the level of *Fmr-1* transcript.

3.4. CDRI-08 Causes Recovery of FMRP Expression in the Hippocampus of Hypoxic Mice. Western blot data reveal that hypoxic conditions (HA and HB) cause significant downregulation of the FMRP level; however, the decline was more prominent in the withdrawal period (HB) ($P < 0.05$). On the other hand, administration of CDRI-08 to the normal control mice caused significant upregulation of FMRP expression ($P < 0.05$) as compared to that in the control. Treatment of mice with CDRI-08 before hypoxic condition (B + H) and after hypoxic condition (H + B) both causes significant upregulation in the level of FMRP when compared to hypoxic condition. Mice *pretreated with CDRI-08 followed by $CoCl_2$ and the $CoCl_2$-(hypoxic) mice treated with CDRI-08 resulted in significant upregulation in the expression of FMRP in the hippocampus as compared to mice of hypoxic groups (HA and HB)* (Figures 4(c) and 4(d)). These results were further confirmed by immunofluorescence microscopic based studies on the *in situ* detection of FMRP expression in brain sections in CA3 (Figures 5(a) and 5(b)) and CA1 (Figures 6(a) and 6(b)) regions of the hippocampus showed the patterns similar to that in Western blot results.

4. Discussion

Use of herbal preparations in the treatment of nervous disorders and many other diseases has tremendously increased especially in the last decade. These preparations are rich in multiple active components and have emerged as preferred prophylactic agents owing to their wide spectrum therapeutic benefits and minimum risks due to significantly less side effects as compared to their synthetic variants. *Bacopa monnieri* is one of the plants that have been widely used in Indian medicinal system of Ayurveda for the treatment of various nervous disorders in general and memory related diseases in particular [36]. In the present study, we have used alcoholic extract of *Bacopa monnieri* named CDRI-08 which is well characterized to be rich in Bacoside A and Bacoside B and studied its effects on the cobalt chloride-induced hypoxia led loss of spatial learning and memory and its effects on the expression of fragile X mental retardation protein (FMRP), one of the proteins that regulate synaptic plasticity, a neurophysiological mechanism underlying learning, memory, and cognition. In order to assert the learning and memory loss in mice due to hypoxia and the possible action of CDRI-08 in restoring the learning and memory loss, we chose to use the Morris water maze paradigm as this test has been often used to assess the alterations in hippocampal spatial learning and memory in rodents [37].

Our data suggests that hypoxia, during which the normal oxygen supply is reduced to organs including brain, causes

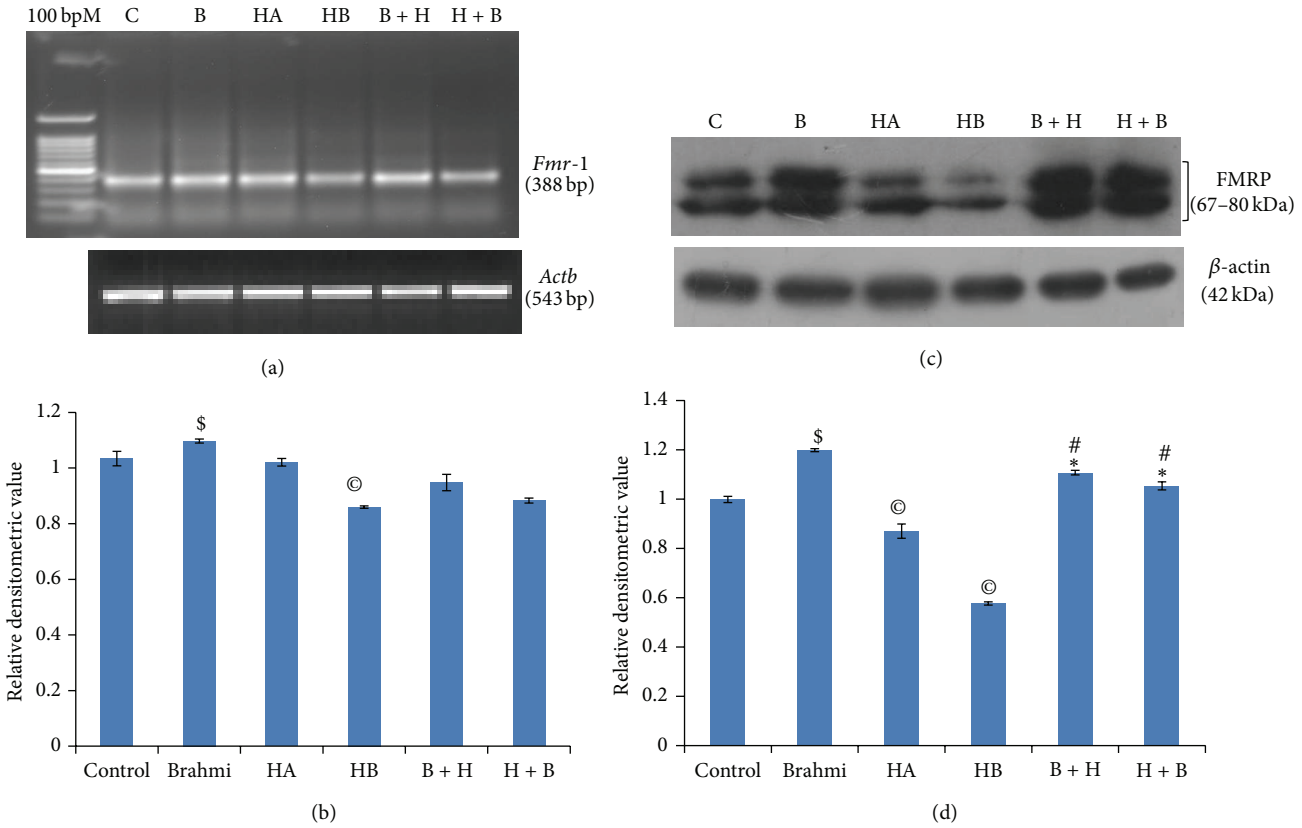

FIGURE 4: Effects of hypoxia and CDRI-08 on *Fmr-1* gene expression. Semiquantitative RT-PCR analysis of *Fmr-1* (a) and Western blot analysis of FMRP (c). Bar shows the relative density value developed by integrated densitometric values (IDV) of HIF-1α by IDV of β-actin. Each bar represents the mean ± SEM. $^{©}P < 0.05$ versus Control; $^{*}P < 0.05$ versus HA; $^{\#}$, indicates $P < 0.05$ versus HB.

impairments in the learning and memory consolidation process. Also, it reveals that when hypoxic condition is prolonged further without any further treatment of cobalt chloride (hypoxia withdrawal effects), the cognitive impairment effects are similar to hypoxic conditions with continuous cobalt chloride treatment for the experimental period. This indicated that the period of withdrawal had no separate effects on the level of memory impairment (Figures 1 and 2). This impairment in the spatial memory due to hypoxia could be attributed to rise in the level of the hypoxia marker protein Hif-1α, a transcription factor which regulates the early hypoxia responsive genes including glutamate transporter type-1 (GLUT-1), erythropoietin (Ep), and late responsive genes like superoxide dismutase (SOD) and catalase (CAT) and many proteins related to synaptic plasticity [38]. In order to confirm whether Hif-1α expression is altered and associated with decline in learning and memory, we examined alteration in its expression in the hippocampus of the normal control and experimental mice. It was observed that the hypoxia-induced memory impairment in mice is related with enhanced expression of Hif-1α, which could have affected the levels of the antioxidative stress enzymes such as SOD and CAT. This data corroborates with our earlier findings on the relation between increased Hif-1α level due to hypoxia and decline in the activities and expression of these enzymes [38]. Our data suggest that effects of hypoxia might not differ much once that hypoxia led neurological derangement has occurred. Memory impairment due to hypoxia, as evident from rise in the level of Hif-1α, may be due to possible alterations in expression of synaptic plasticity related proteins such as AMPA, NMDA, and metabotropic glutamate receptors (AMPAR, NMDAR, and mGluR) which control long term potentiation (LTP) or long term depression (LTD), the cellular basis of learning and memory [39–42]. Our data demonstrated that cobalt chloride induced hypoxic condition resulted in alteration in spatial memory which is found to be in accordance with several other studies which report that chronic exposure to hypobaric hypoxia leads to memory impairment in rats [43, 44]. We also observed that CDRI-08 treatment to hypoxic mice improves their impaired spatial memory which can be understood by significant decrease in the latency and path length along with significant increase in number of platform crossings and time spent in the target platform. This can be correlated with the neuroprotective role of the CDRI-08 in restoration of the altered spatial memory towards the normal condition. Similar role of CDRI-08 has been shown in earlier studies where CDRI-08 plays positive roles in animals affected with altered spatial memory due to hypobaric hypoxia [45], Alzheimer's disease [46], and scopolamine-induced amnesia [47].

As HIF-1α is hallmark of hypoxic condition, it is possible that CDRI-08 alters its expression or its stability. Our results,

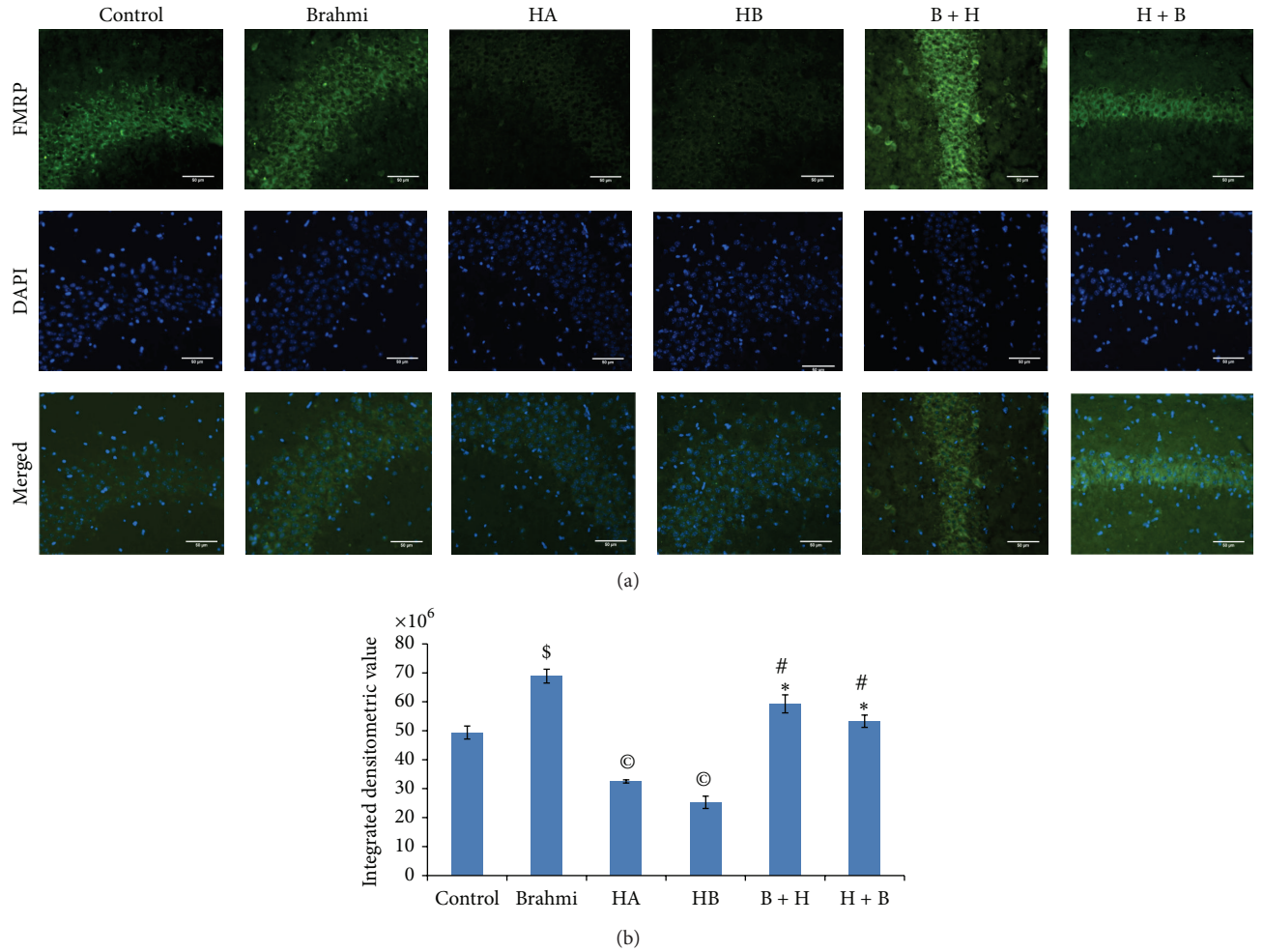

Figure 5: Effects of hypoxia and CDRI-08 on the expression of FMRP in hippocampus. Photomicrophotographs shows immunofluorescence (20x magnification) illustrating FITC-labeled signals of FMRP in CA3 region of hippocampus (a). Data were calculated in terms of integrated densitometric value (b). Bar represents the mean ± SEM. $^{\$}P < 0.05$ versus control, $^{©}P < 0.05$ versus control, $^{*}P < 0.05$ versus HA, $^{\#}P < 0.05$ versus HB, Scale bar = 1 μ.

to our surprise, show upregulation of *Hif-1α* at both transcript and protein levels in the hippocampus of $CoCl_2$-induced hypoxic mice and it was found that CDRI-08 treatment to hypoxic mice reversed the level of Hif-1α towards that in the normal mice. Therefore, it is suggestive that CDRI-08 treatment based restoration of learning and memory is correlated with the levels of HIF-1α. Also, from our study, it can be concluded that CDRI-08 has similar effects whether it is given before hypoxia is developed or after the hypoxia was developed. However, the precise mechanism by which CDRI-08 modulates the expression of HIF-1α and which thereby protects or restores memory cannot be assertively explained by our results and it is needed to be thoroughly studied. Nonetheless, CDRI-08's positive role in impaired spatial learning and memory is evident from our studies. The CDRI-08 treatment-dependent restoration of memory in hypoxia caused decline in learning and memory might be attributed to its free radical scavenging function [28–31] and cholinergic modulation [24–27] which are being investigated in our group.

As indicated earlier in the discussion, cobalt chloride-dependent hypoxic condition that decreases the level of learning and memory may also be correlated with alterations in the synaptic plasticity related proteins. Since FMRP is one of the proteins that regulate LTP and LTD via regulation of various glutamate receptors like AMPAR, NMDAR, and mGluR, it is likely that hypoxia may cause decline in memory, and CDRI-08 treatment reverses the impaired memory towards that in the normal control, which may be due to alterations in the level of FMRP which might in turn affect synaptic plasticity. Therefore, we thought to examine alterations in the expression of *Fmr-1* gene at transcript as well as protein levels. We observed that impairment in spatial memory was significantly correlated with the expression of FMRP, an important protein associated with synaptic plasticity. We report here for the first time that hypoxic condition leads to a remarkable decrease in hippocampal *Fmr-1* expression at both mRNA and protein levels as analyzed by RT-PCR and Western blotting. Consistent with the Western blotting results, the immunofluorescence studies also revealed

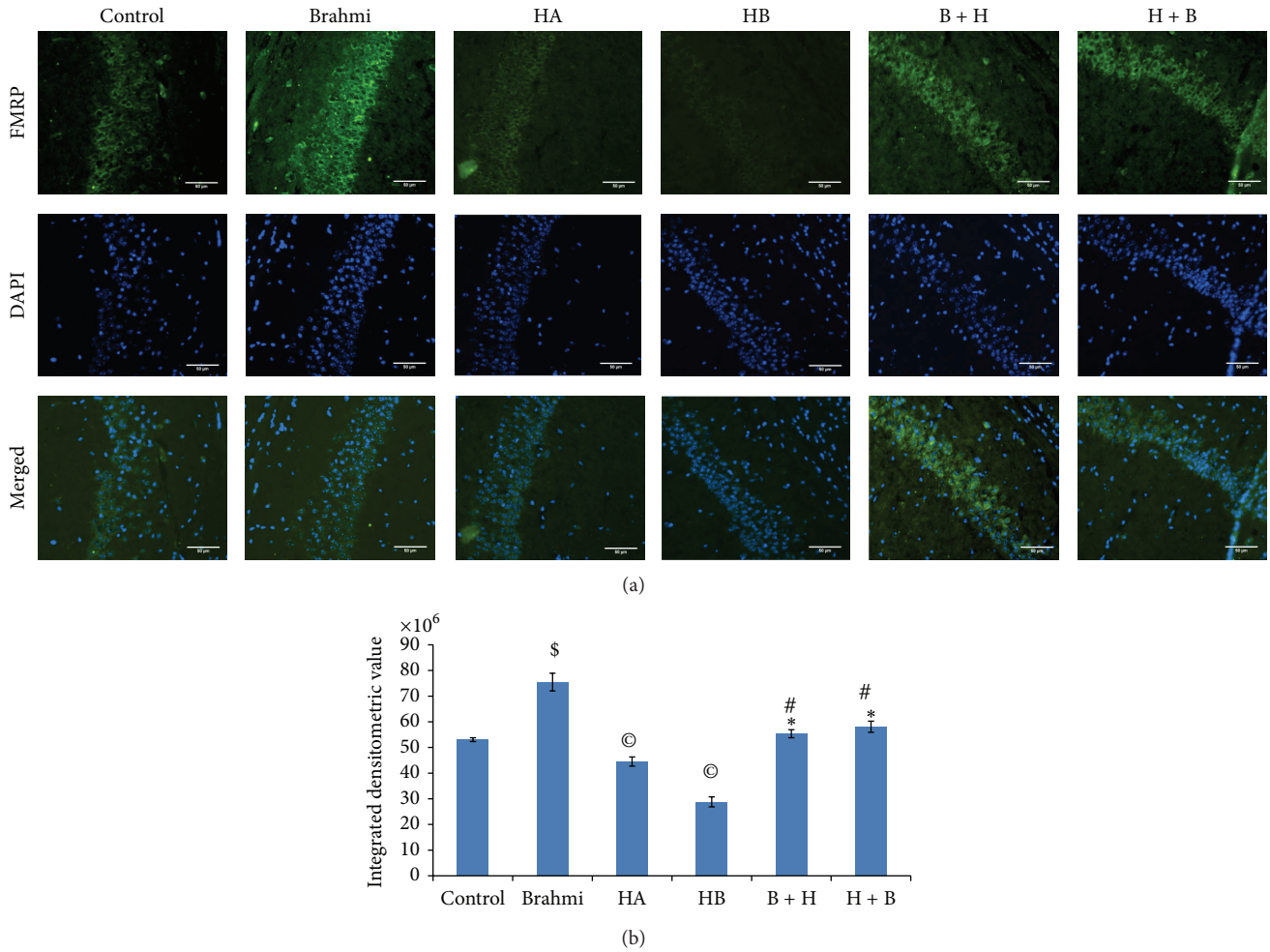

Figure 6: Effect of hypoxia and CDRI-08 on the expression of FMRP in hippocampus. Photomicrophotographs shows immunofluorescence (20x magnification) illustrating FITC-labeled signals of FMRP in CA1 region of hippocampus (a). Data were calculated in terms of integrated densitometric value (b). Bar represents the mean ± SEM. $^{\$}$indicates $P < 0.05$ versus Control, $^{©}P < 0.05$ versus Control, $^{*}P < 0.05$ versus HA, $^{\#}P < 0.05$ versus HB, Scale bar = 1 μ.

remarkable downregulation of FMRP in the hippocampus of mice of both the groups of hypoxic mice, and it upregulates its expression in the CDRI-08-treated prehypoxic or hypoxic mice towards its level in the normal control mice. Our results are consistent with the findings which show impairment in spatial memory in *Fmr-1* knockout mice [48, 49] suggesting a crucial role of FMRP in the hypoxia led memory impairments and CDRI-08-dependent memory restoration processes. The expression of FMRP is reported be high in the hippocampus [50] and since, the hippocampus has been shown to be necessary for memory in humans and rodents, specifically for the formation of spatial memory in rodents, FMRP seems to play a critical role in the function of hippocampus. FMRP is found in dendritic spines [51], the important postsynaptic sites of plasticity induction and maintenance, it plays role in the regulation of dendritic mRNA translation [11, 52] which is required for multiple forms of plasticity [53] and it is dynamically regulated by activity-dependent synaptic activation can trigger its local translation and rapid degradation [54], it is established that FMRP is a candidate protein involved in regulating synaptic plasticity. Other studies have revealed that translation of proteins regulated by FMRP includes microtubule-associated protein 1B (MAP1B) and activity-regulated cytoskeleton-associated protein (ARC) [55, 56]. Studies have shown that *Fmr-1* promoter possesses the CRE site that binds CREB in the regulation of its own transcription in neural cells [57, 58]. In a recent study, it has been shown that CREB may specifically contribute to the upregulation of FMRP by stimulating Group I mGluRs [59], suggesting the CREB-dependent regulation of FMRP level. Therefore, it can be speculated that the hypoxia-induced decline in the learning and memory may be due to alterations in above to which FMRP is intricately associated which in turn might cause defects in synaptic plasticity. A recent report suggests that the chronic administration of *B. monniera* extract improves cognitive behavior by upregulation of PKA, MAPK and pCREB. Our study also reveals that CDRI-08 upregulates FMRP expression and it is likely to possess the neuroprotective or restorative effects, respectively, by way of FMRP-dependent regulation of pCREB and its binding

with CRE site on the *Fmr-1* gene promoter leading to transcriptional regulation of *Fmr-1* and several other genes which in turn may facilitate the role of synaptic proteins and synaptic plasticity, hence learning and memory.

Although various reports on Bacosides have suggested its antioxidant properties [60] and cholinergic property [26] which contributes in restoration of altered memory by *Bacopa monnieri*, based on the strength of available publications, we can claim that our report is novel on the effects of *Bacopa monnieri* on the expression of *Fmr-1* gene and its association with spatial memory. Thus our study suggests a possible mechanism for the hypoxia-induced memory loss involving FMRP and the mode of action of CDRI-08 during recovery of memory impaired due to hypoxia, which needs to be addressed in more details.

Conflict of Interests

Authors hereby declare that there is no conflict of interests among them in respect to publication of this paper.

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