

Spatial memory impairment is associated with oxidative stress, neurodegeneration and abnormalities neuromorphology in the hippocampus of rotenone-induced Parkinson's mouse model: neuroprotective role of a specialized *Bacopa monnieri* extract (CDRI-08)

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Abstract

Parkinson's disease (PD), a neurodegenerative disorder linked with ageing, is associated with both motor and non-motor problems. Memory loss is a common non-motor symptom of PD, particularly as the disease progresses, and it can lead to cognitive impairment. The objective of this study was to investigate the underlying biochemical, molecular, and neuroanatomical pathways that contribute to learning and memory impairment in PD. The study investigates the potential neuroprotective and neurotherapeutic effects of the *Bacopa monnieri* extract (CDRI-08), a natural nootropic medication, on memory deterioration associated with PD. To comprehend the information provided, we developed a PD mouse model by administering rotenone subcutaneously at a dose of 2mg/kg BW. Spatial working memory and the expression of α -Synuclein in the hippocampus was examined. Additionally, evaluation of the oxidative stress parameters, neuronal cell density, apoptosis in subregions of the hippocampus, and dendritic arborization and dendritic spine density in CA1 pyramidal neurons of the hippocampus. Results showed that PD exhibited a decline in their ability to remember spatial information, which can be correlated with high oxidative stress characterized by increased reactive oxygen species and lipid peroxidation levels whereas decreased activity of SOD and catalase. This oxidative stress resulted in a drop in the density of neurons due to increased cell death characterized by increased caspase-3 expression and TUNEL-positive cells, leading to a loss of dendritic length, branching, and dendritic spine density. Administering BME (CDRI-08) orally effectively alleviates the illness and additionally improves neuroprotection by strengthening and nourishing neurons by increasing the activity of antioxidant enzymes and decreasing the expression of pro-apoptotic factor caspase-3. Collectively, these findings indicate that *Bacopa monnieri* extract can be utilized as a herbal remedy to treat memory impairment induced by PD.

Introduction

Parkinson's disease (PD) is the second most common neurological disorder after Alzheimer's. It is characterised by dopaminergic neuronal degeneration in the nigrostriatal area and the aggregation of α -synuclein-rich clumps called Lewy bodies and Lewy neurites. PD is characterized by a gradual decline in motor function, which manifests as tremors, slow movement, difficulty initiating movement, muscle stiffness, problems with balance, and difficulty walking. Additional symptoms, such as cognitive impairment, melancholy, loss of smell, anxiety, and sleep disruption are commonly observed in patients with PD [1–3]. The cognitive impairments associated with PD mostly affect executive function, encompassing tasks such as multitasking, problem-solving, planning, organization and learning and memory formation [4]. The occurrence of cognitive dysfunction in PD is likely attributed to a confluence of chemical and anatomical alterations. PD impacts the neurotransmitters involved in memory and cognitive function, as well as causing damage to brain cells in the corresponding regions.

The role of oxidative stress in the development of several late-onset neurodegenerative disorders has been widely documented. The prevailing ideas about aging-related neurological disorders, such as Alzheimer's disease (AD) and PD, propose that the gradual accumulation of oxidative stress results in mitochondrial dysfunction, and oxidative damage [5–6]. Oxidative stress is a significant factor in the

degeneration of dopaminergic neurons in PD. The occurrence of dopaminergic neuron degeneration has repeatedly been linked to mitochondrial dysfunction and oxidative stress [7–8]. Rotenone specifically targets complex I in mitochondria, leading to the overproduction of reactive oxygen species (ROS). ROS consists of hydrogen peroxide (H₂O₂), nitric oxide (NO), superoxide anions (O₂⁻), as well as the extremely reactive hydroxyl and monoxide radicals (OH⁻, NO⁻) [9–10]. In PD, there is a state of excitotoxicity that occurs in neurons. This condition is caused by an increase in intracellular calcium-dependent nitric oxide synthase (NOS), which causes nitric oxide (NO) production [11–13]. Nitric oxide exhibits a strong attraction to the superoxide anion, resulting in the formation of peroxynitrite (ONOO⁻). This compound then proceeds to damage membrane lipids, leading to lipid peroxidation. Lipid peroxidation seriously damages lipoproteins and the membranes of cells and organelles. Acroléin, isoprostanes, 4-hydroxynonenal (HNE), malondialdehyde (MDA), and conjugated dienes are byproducts of this process that harm neuronal cells [14]. As oxidative stress advances, it causes chromatin condensation, DNA breakage, and the activation of cysteine-proteases and caspases. This, in turn, impacts the antioxidant defence system, which includes antioxidant enzymes like as SOD, and catalase. Imbalances in the equilibrium between tissue antioxidants and oxidants may play a significant role in the development of both macro and microangiopathic problems in patients with PD. Alpha-synuclein induces oxidative stress by decreasing the activity of complex I, leading to mitochondrial dysfunction [15].

The hippocampus plays a well-established role in modulating cognitive functions, such as learning and memory. The hippocampus formation is deeply linked to the acquisition and consolidation of information [16]. A significant number of synapses possess flexible qualities, which have been suggested to play a crucial part in the processes of learning and memory formation. The process of memory formation and consolidation in the hippocampus involves the transmission of information between synapses in a circuit known as the trisynaptic circuit. The relay of synaptic transmission takes place through the transfer of information from the entorhinal cortex to the granular neuron in the dentate gyrus (DG) via the perforant pathway. From there, the information is transmitted from the DG to the CA3 pyramidal neurons through mossy fibres, and finally from the CA3 neurons to the CA1 pyramidal neurons via the Schaffer collaterals [17]. CA1 pyramidal cells serve as the principal neurons that transmit information from the hippocampus. They receive excitatory signals from specific routes that target different parts of their dendritic branches, both near and far from the cell body [18–19]. Neuronal branches facilitate effective neurotransmission and promote synchronized functioning among brain networks. Any interruption in this process may result in changes in communication, including the breakdown of synaptic plasticity.

Although there is currently no known cure for Parkinson's disease, many herbal medicines and supplements have been investigated for their ability to enhance cognitive function and memory in patients with PD. *Bacopa monnieri* is a renowned nootropic Ayurvedic medication used to treat cognitive dysfunction in several neurological diseases. *Bacopa monnieri*, also referred to as Brahmi, is a herb with a long history of usage in traditional Ayurvedic medicine. It has gained attention for its potential neuroprotective and neurotherapeutic properties [20–23].

This study aims to investigate the therapeutic potential of *Bacopa monnieri* extract (CDRI-08) in treating memory impairment caused by hippocampal neuroanatomical disturbance in Parkinson's disease. Furthermore, we will examine the neuroprotective properties of BME for strengthening and nourishing neurons.

Materials and methods

Animals

The Swiss albino male strain mice were housed in the animal facility of the Department of Zoology at Banaras Hindu University, Varanasi. The mice were maintained at a 12-hour light-dark cycle and provided with a standard mouse diet and drinking water ad libitum. The animals were maintained following the rules set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Additionally, all experimental procedures conducted in this study received approval from the Institutional Animal Ethical Committee (IAEC) at Banaras Hindu University in Varanasi. (BHU/DOZ/IAEC/2021–2022/007, Dated 15/02/2022)

Chemicals

The chemicals and reagents utilized in the experiments were procured from reputable companies such as Sigma Aldrich (USA), E-Merck India Ltd. (Mumbai, India), Milipore (USA), MBI Fermentas (USA), and Qualigens Fine Chemicals (Mumbai, India), all of which provide analytical and molecular grade products. α -Synuclein and active caspase-3 primary antibody was purchased from DSHB, USA and Bioss, USA respectively. The HRP-conjugated secondary antibody from GeNei, Bangalore, India. The One-step TUNEL In-Situ Apoptosis Kit (Green, Fluor 488) (Cat. No. E-CK-A321) was purchased from Elabscience, USA. The *Bacopa monnieri* extract (CDRI-08) was generously provided by Prof. H.K. Singh, the former Director of the Central Drug Research Institute (CDRI) in Lucknow.

Drug administration

The mice were divided into four groups, each consisting of six mice. The groups were as follows: (1) Control group, which received DMSO subcutaneously for 14 days and after that 5% Tween 80 was given orally; (2) Rotenone Treated group, which received 2mg/ kg BW Rotenone dissolved in DMSO subcutaneously for 14 days; (3) Rotenone pre-treated with *Bacopa monnieri* extract (BME) group, which received 200mg/ kg BW of BME dissolved in 5% tween 80 orally for 21 days before administration of Rotenone; and (4) Rotenone post-treated with *Bacopa monnieri* extract (BME) group, which received 200mg/kg BW of BME dissolved in 5% tween 80 orally for 21 days after administration of Rotenone. After the completion of treatments, Y-maze test was conducted and then all mice were sacrificed and brains were isolated for Nissl's, TUNEL, and Golgi-cox staining separately and the hippocampus were collected for biochemical assay and western blotting.

Y-maze test

The experimental setup consists of a Y-shaped maze including three identical opaque arms, positioned at a 120° angle relative to each other. The test is employed to assess cognitive deficits in mice.

The three arms of the Y-maze were designated as A, B, and C.

In the training session, the recording was initiated, and the mouse was positioned in arm-C, with its orientation directed towards the central region of the maze. The animal was allowed a period of 5 minutes without disturbance to thoroughly investigate each of the appendages. After a duration of 5 minutes, the mouse was returned to its designated enclosure and the process of recording was terminated. The Y-maze apparatus was subjected to a cleaning procedure before the commencement of testing with each subsequent animal. The aforementioned technique was replicated with the remaining mice [24]. The videos that were recorded underwent analysis using the ANYmaze version 7.2 software to calculate the total count of arm entries and alternations. The calculation of percent (%) alternation was performed using the following formula:

$$\% \text{ Alternation} = [\text{Number of alternations} / (\text{Total number of arm entries} - 2)] \times 100$$

An entrance is said to occur when all four limbs of the mouse are positioned within an arm. An alternation is operationally defined as the sequential participation in all three arms of a study or experiment.

Total reactive oxygen species (ROS)

The total ROS production was fluorometrically quantified by the multimode reader. Following the protocol of our lab, 10% tissue homogenate was prepared in 20mM Tris-Cl buffer pH 7.5 followed by centrifugation at 12000xg at 4°C, and the supernatant was collected for estimation of protein by Bradford method. 50ug protein from each group was incubated with DCFDA (100uM, 20µl) at 37°C temperature for 30 min in dark. The total ROS was estimated by measuring the conversion of non-fluorescent 2 ,7 - dichlorofluorescein-diacetate (H2DCFDA) into very high fluorescent compound 2 ,7 -dichlorofluorescein (DCF) by available reactive oxygen species at an excitation wavelength of 485nm and an emission wavelength of 530nm. The amount of total ROS was calculated in relative fluorescent intensity per milligram of protein (RFI/mg protein) [25].

Lipid peroxidation or MDA level

The MDA level was spectrophotometrically quantified. Following the protocol of our lab, 10% tissue homogenate was prepared in 20mM Tris-Cl buffer pH 7.5 followed by centrifugation at 12000xg at 4°C, and the supernatant was collected for estimation of protein by the Bradford method. 0.2M Tris-malate buffer (pH 5.9) was mixed with 10% homogenate and incubated for 30 min at 37°C then the same volume of 0.8% thiobarbituric acid (TBA) of the initial volume and incubated in boiling water for 10 minutes. 3:1 ratio of pyridine: butanol mixture was added with 100ul of NaOH and mixed well. Absorbance was taken at 548nm [26].

Superoxide dismutase (SOD) assay

After centrifugation of 10% tissue homogenate, supernatant (100µl) was mixed with 1.4 ml of the cocktail mixture-which included 1% Triton X-100, 50 mM phosphate buffer pH 7.4, 20 mM L-methionine, 10 mM hydroxylamine hydrochloride, and 50 µM EDTA-it was incubated for five minutes at 37°C. Each sample was treated with 80µl of 100µM riboflavin and left in an LED light box for ten minutes. Each tube received 1 ml of Griess reagent (0.1%) naphthylethylenediamine and 1% sulphanilamide to end the assay. A microplate reader detected absorbance at 543nm to calculate enzyme activity in units/mg protein [27].

Catalase assay

0.8M (500µL) hydrogen peroxide, 625µL phosphate buffer, and 125µL brain homogenate were added to the test tube. After a 1-minute room-temperature incubation, 2 ml of colour reagent (5% aq. $K_2Cr_2O_7$ and glacial acetic acid) was added. The tubes with the mixture were kept in a 100°C water bath for 10 min. Microplate readers measured absorbance at 570nm and enzyme activity in micromole/min/ml [28].

Nissl's staining

The sections were immersed in 95% ethyl alcohol at room temperature for 5 hours. Subsequently, they were rinsed in 75% ethyl alcohol for 5 minutes, followed by a rinse in distilled water for an additional 5 minutes. The sections were subjected to incubation in a solution of 0.1% cresyl violet acetate, which had been heated to 60°C, for 30 minutes. Following this incubation period, the specimens were washed in water for 5 minutes and subsequently rinsed in distilled water. To facilitate dehydration, the specimens were briefly exposed to 75% alcohol, followed by immersion in 90% ethanol for 2 min, and finally in absolute alcohol for 2 min. Subsequently, the samples were subjected to xylene clearing process for 2 minutes, followed by mounting with DPX [29]. The slide was examined using a bright-field microscope (Leica, Germany). Cell density was determined using ImageJ software (NIH, USA).

TUNEL assay

The TUNEL staining method was executed following the protocol specified in the One-step TUNEL In-Situ Apoptosis Kit (Green, Fluor 488) (Cat. No. E-CK-A321) Elabscience, USA, following careful adherence. Briefly, the sections underwent a triple wash in 1×PBS, followed by protein digestion through treatment with proteinase-k for 20 minutes. Following the washing step with 1×PBS, the sections were subjected to incubation with the labeling solution at a temperature of 37°C for 2 hours. Subsequent slices were once again washed with a 1× PBS solution. The nuclei were exposed to DAPI staining at ambient temperature for 10 minutes and afterwards placed in an anti-fade mounting solution that included DABCO. The detection of the fluorescent signal was accomplished via a Confocal Microscope system (Model: SP8 STED, Manufacturer: Leica Microsystems).

Golgi-cox

The Golgi-Cox staining technique is used to analyze the structure of neurons, with a particular emphasis on features like dendritic arborization and spine density. The mouse brain was removed by decapitating it and dislocating its cervical vertebrae. We submerged the brains in freshly made Golgi-Cox solution. Vibratome-cut coronal slices of 100 µm thickness were produced after a 24-hour incubation period at

37°C. Ammonia and sodium thiosulfate solution was applied to the section for ten minutes each. Following this, every segment was cleaned, dried, and then mounted using DPX on glass slides coated with Poly-L-Lysine [30–31]. Sholl's analysis [32] was carried out using Neuroexplorer software (MBF Bioscience, Williston, Vermont, USA) after the 3D tracing procedure. The 50 µm-diameter concentric rings were positioned on the tracing and extended outward from the soma's core. The length of the dendrites and the number of branching sites in each succeeding segment were estimated using the program.

Statistical analysis

Each experiment is conducted three times. The data acquired from the behavioural analysis, western blotting, oxidative stress parameter, Nissl's staining, TUNEL assay and Golgi cox staining were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The values were reported as the mean $\pm$ standard error of the mean (SEM). Statistical analysis was conducted using SPSS 16.0, with a significance level of $p < 0.05$ indicating statistical significance.

Results

Effect of CDRI-08 on altered spatial working memory in PD mice

Rotenone-induced-PD mice exhibited a significant reduction ($p < 0.05$) in the percentage of spontaneous alteration compared to the control mice. However, the mice that were treated before and after with CDRI-08 showed a significantly ($p < 0.05$) improved percentage of spontaneous alteration compared to the PD mice (Fig. 1A-B). However, the mice that received pre-treatment exhibited significantly better improvement compared to the mice that received post-treatment.

Effect of CDRI-08 on α -Synuclein expression alteration due to PD

The level of α -Synuclein expression is significantly elevated ($P < 0.05$) in mice affected with Parkinson's disease in comparison to mice in the control group. Nevertheless, the PD mice that received pre- and post-BME treatment exhibited a significant reduction in the expression of altered α -Synuclein compared to the normal control mice (Fig. 1C-D).

Effects of CDRI-08 on the neuronal density of various hippocampal regions in PD mice

The neuronal cell density was quantified by enumerating the number of viable neurons/ 100µm² area of the cresyl violet-stained thin sections of the hippocampus in the brain. The results from cresyl violet staining (Nissl's staining) indicate a significant reduction ($p < 0.05$) in the density of viable neuronal cells in the DG area of the hippocampus in the PD mice, as compared to the control mice (Fig. 2). Pre-treated BME resulted in a significant ($p < 0.05$) rise in density. Additionally, post-treatment of PD mice with BME

showed a substantial increase in density. A p-value of less than 0.05 indicates that BME therapy restores the altered density of live neurons towards that of normal control animals. Nevertheless, the pre-BME treatment has a greater impact on sustaining the density of neuronal cells when compared to the post-BME treatment. Surviving neurons were significantly decreased ($p < 0.05$) in the CA3 region of hippocampus in the PD group compared to the control group (Fig. 2). Pre- and post-treatment of the PD mice with BME results in a significant elevation in the density of neuronal cells ($p < 0.05$) in comparison to PD mice. Additionally, a similar pattern was acquired from the CA1 area of the hippocampus. In addition, the PD mouse exhibits a significant decrease in neuronal density ($p < 0.05$) compared to the control. The data from the pre- and post-treated BME mice demonstrates a remarkable rise in density ($p < 0.05$). However, the increase in neuronal density is greater in the pre-treated BME mice compared to the post-treated BME mice.

Effects of CDRI-08 on the dendritic arborizations of hippocampal CA1 pyramidal neurons of Parkinson's mice

Sholl analysis was used in the CA1 regions of hippocampal pyramidal neurons to evaluate the effect of CDRI-08 on the PD-induced alteration in dendritic branching (Fig. 3A). The neuron was positioned in multiple concentric rings with increasing radii to measure total dendritic length, total number of intersections and total number of branching points. More complex processes create more "Sholl" spheres as the distance rises.

After rotenone and BME treatment, we examined the dendritic branching pattern in hippocampus CA1 pyramidal neurons. The Golgi-Cox stained CA1 pyramidal neurons and their corresponding tracing from the control, rotenone, pre-treated BME, and post-treated BME groups are shown in Fig. 3A. While there is a noticeable recovery in the length of CA1 pyramidal neurons on both the BME treatment, it is clear that the overall length of CA1 pyramidal neurons in PD mice is much shorter than in the control group (Fig. 3C-D). The findings indicate that the overall length of PD mice's CA1 pyramidal neurons was considerably ($p < 0.05$) shorter than that of the control group, whereas the condition was greatly improved both before and after BME treatment (Fig. 3C). When the total branching points were evaluated, it was found that the PD led to significant ($p < 0.05$) decrease in total branching points as compared to the control (Fig. 3D), and both the pre- and post-BME treated mice showed a significant rise in total branching point.

Effects of CDRI-08 on the apical dendritic spine density of hippocampal CA1 pyramidal neurons of Parkinson's mice

Our findings demonstrate that Parkinson's disease (PD) causes a significant decrease ($p < 0.05$) in the density of dendritic spines (number of spines per $10\mu\text{m}$) in the CA1 region apical dendrites in PD mice, compared to the control mice (Fig. 3E-F). While BME treatment reinstates the effect. A significant increase ($p < 0.05$) was seen in the apical dendrites of the CA1 region in pre-treated BME PD mice. Similarly, a significant increase ($p < 0.05$) was also observed in post-treated PD mice. However, the recovery was more pronounced in the pre-treated mice compared to the post-treated mice (Fig. 3E-F).

Effects of CDRI-08 on the levels of total ROS and lipid peroxidation in the hippocampus of Parkinson's mice

To determine the impact of oxidative stress on rotenone-induced PD and its potential recovery with CDRI-08 treatment, we assess the levels of pro-oxidant markers associated with oxidative stress. The study's findings revealed a significant elevation ($p < 0.05$) in the total reactive oxygen species (ROS) in hippocampus of the group of mice with PD, in comparison to the level observed in the control mice (Fig. 4A). The hippocampus of PD mice that were treated with CDRI-08 before and after treatment exhibited a significant recovery in the total level of reactive oxygen species (ROS). The p-value is less than 0.05.

The results of the lipid peroxidation assay indicate a significant rise ($p < 0.05$) in the level of MDA in the hippocampus of mice with Parkinson's disease compared to the control group (Fig. 4B). Pre-treating PD animals with BME resulted in a significant ($p < 0.05$) reduction in the level of MDA compared to PD mice. This reduction brought the MDA level closer to the same level as the control mice (Fig. 4B). Nevertheless, following the administration of BME to mice with Parkinson's disease (post-treated group) there was a significant yet modest reduction in the level of MDA ($p < 0.05$) compared to PD mice.

Effects of CDRI-08 the SOD, and catalase (CAT) activities in the hippocampus of PD mice

The spectrometric assessment of SOD activity reveals that PD significantly reduces SOD activity ($p < 0.05$) (Fig. 4C). The administration of BME both before and after treatment resulted in a significant increase in the reduced activity of SOD ($p < 0.05$) compared to the PD mice, and this increase brought it back to normal levels. Nevertheless, animals treated before BME administration exhibited more favorable outcomes.

The data from the CAT activity analysis indicates a notable decrease in the catalase activity in the hippocampus of individuals with PD ($p < 0.05$) when compared to the control group (Fig. 4D). Pre-treatment of BME in PD mice resulted in a significant increase ($p < 0.05$) in CAT activity compared to PD mice, where CAT activity was reduced. Nevertheless, the administration of BME after the treatment of PD mice also significantly elevates the CAT level, although to a lesser extent compared to the BME group that was pre-treated.

Effect of CDRI-08 on the expression of active caspase-3 in the hippocampus of PD mice

To assess the extent of apoptosis, the cleaved caspase-3 expression was checked. The immunoblot data revealed a considerable increase in the level of activated caspase-3 in the hippocampus of PD animals compared to the control mice ($p < 0.05$) (Fig. 4E-F). Treatment of BME in PD mice, both before and after treatment, resulted in a significant decline in the expression of active caspase-3 compared to its level in

PD mice. However, the neuroprotective impact of the BME is more when compared to its therapeutic effect.

Role of CDRI-08 in the recovery of apoptotic neuronal cell death in various regions of the hippocampus of PD mice

The study focused on investigating apoptotic cell death in the different hippocampal regions i.e. DG, CA3, and CA1 regions in PD mice. This was done by assessing the level of DNA fragmentation, which is a good signal of apoptosis, using the TUNEL assay on cryosections. When the TUNEL assay was conducted on DG neurons (Fig. 5A-B), it showed a statistically significant rise ($P < 0.05$) in the TUNEL-positive cells in the PD mice group compared to the control mice. Additionally, the results indicated a significant decrease ($p < 0.05$) in apoptosis in the DG region of the hippocampus in both pre- and post-treated PD mice that were administered CDRI-08, in comparison to the PD mice group.

A considerable increase in the frequency of TUNEL-positive cells was also noted in the CA3 region of PD (Fig. 5C-D) in comparison to the normal control ($p < 0.05$). The data we obtained demonstrated a notable reduction in DNA fragmentation in PD mice treated with both dosages of CDRI-08. However, the degree of DNA fragmentation was reduced in PD mice when they were treated before the administration of rotenone.

Further, our data revealed a significant increase ($p < 0.05$) in TUNEL-positive cells in the CA1 region of the hippocampus in PD mice (Fig. 5E-F) as the control ($p < 0.05$). However, both the pre-and post-CDRI-08 treated PD groups showed a significant decrease in TUNEL-positive cells compared to the rotenone-induced PD group. The pre-treatment dose exhibited a higher level of recovery, compared to the normal in all three regions of the hippocampus.

Discussion

Parkinson's disease is typically seen as a movement disorder resulting from the degeneration of dopaminergic neurons in the substantia nigra. However, there is increasing evidence suggesting that the pathology of Parkinson's disease may have impacts beyond the substantia nigra and the dopamine system. Cognitive impairment is a non-motor symptom that can occur at any stage of Parkinson's disease and can progress to a more severe form known as dementia. It impacts various cognitive functions, including executive function, attention, information processing, learning, and memory. While managing all the symptoms of Parkinson's disease might be challenging, there are medications and exercises available that can help mitigate its impact.

The current investigation centered on cognitive decline in Parkinson's disease (PD) and the potential use of *Bacopa monnieri* extract (CDRI-08) as a means of both protecting and treating the neurological impairments associated with it.

In order to develop the PD model, we employed rotenone, a pesticide widely recognized for its ability to induce Parkinson's disease in rodents. We primarily focused on memory and examined the expression of the PD biomarker protein, α -synuclein, in the hippocampus. Our data indicates that, in addition to the substantia nigra, Lewy bodies aggregate in the hippocampus of mice treated with rotenone. This strongly suggests the successful development of a PD model and its correlation with memory decline. Nevertheless, therapy with BME significantly reduces its expression in both a neuroprotective and therapeutic manner.

Further, the mice were behaviourally analysed by the Y-maze test. The Y Maze Spontaneous Alternation test is a behavioral test used to assess the short-term memory of rodents by monitoring their tendency to explore novel situations. Rodents generally exhibit a preference for exploring a novel arm of the maze rather than revisiting a previously explored one. Several brain regions, including the prefrontal cortex, hippocampus, septum, and basal forebrain are engaged in this cognitive process [33].

Our data indicates that PD mice exhibited a considerably reduced percentage of spontaneous alteration compared to the control group. Furthermore, we noticed that administering BME to PD mice before and after PD enhances their defective spatial memory, as evidenced by a significant increase in the percentage of spontaneous alterations. Nevertheless, the neuroprotective effect of BME had a greater influence on enhancing memory compared to the post-treatment.

Oxidative stress is thought to have a substantial impact on the emergence and advancement of PD. Several factors, including mitochondrial dysfunction, DNA damage, neuroinflammation, α -Synuclein aggregation, and defective antioxidant defence system, have all led to an elevation in oxidative stress and the onset of neurodegeneration. According to these findings, the current study discovered that elevated levels of malondialdehyde (MDA) and reactive oxygen species (ROS) are linked to Parkinson's disease. This shows an increase in lipid peroxidation and free radicals. The membrane lipid is directly attacked by free radicals such as hydroxyl radicals ($\text{OH}^\cdot$) and H_2O_2 [34]. This incursion may be connected to diminished or abnormal neural activity, which could then be connected to the decline in memory and learning shown in the present Parkinson's disease investigation. BME treatment both prior to and following therapy led to a decrease in total reactive oxygen species (ROS) and malondialdehyde (MDA), suggesting a reduction in oxidative stress in the PD mice. The restoration of Parkinson's-related memory loss has been associated with this decrease in oxidative stress. The antioxidative qualities of bacosides A and B, the main components of *Bacopa monnieri* extract, may be the cause of the significant reduction in oxidative stress, as shown by the decrease in ROS and lipid peroxidation in the PD mice treated with BME [35–36]. The antioxidant enzyme SOD provides the first line of defense for cells against reactive oxygen species (ROS) produced by Parkinson's disease (PD). This enzyme facilitates the conversion of superoxide anion to hydrogen peroxide [37]. According to our data, the SOD activity in the PD condition was significantly lower than in the control group. In contrast to the PD groups, PD mice that received BME both pre and post-treatment showed a substantial increase in SOD activity. Catalase is a crucial enzyme that facilitates the detoxification of H_2O_2 by catalyzing its two-electron dismutation, resulting in the conversion of H_2O_2 into O_2 and H_2O . We have discovered that CAT activity is decreased in

individuals with PD. However, when BME is administered before PD development, it can enhance the activity of catalase. Furthermore, significant increases in CAT activity were identified in mice treated with post-BME, although the rise was not as pronounced as in the pre-BME-treated group.

We have found that PD mice exhibit increased oxidative stress, which may contribute to neurodegeneration. It is clear that dopaminergic neuronal degeneration occurs in Parkinson's disease [38]. In light of this, we have examined the reduction of hippocampus neuronal density in order to investigate the relationship between Parkinson's disease and memory impairment. Our Nissl's findings demonstrated that, in PD animals, the density of live neurons in the CA1, CA3, and DG areas of the hippocampus drastically decreased, but the treatment with BME (CDRI-08) greatly increased the density of live neurons in various hippocampal regions. This showed the ability of BME to keep the number of neurons stable by reducing oxidative stress in neurons. Further administration of BME in the PD groups led to an increase in neuronal cell density, and helped preserve the synaptic networks in the hippocampus. As a result, memory consolidation and retrieval were accelerated. Our findings align with multiple studies indicating that the active chemical components of *Bacopa monnieri* extract, specifically bacosides A and B, are thought to repair impaired neurons by increasing kinase activity [39] and neuronal synthesis associated with the restoration of synaptic activity. This ultimately results in enhanced transmission of nerve impulses. Therefore, the evidence indicates that CDRI-08 has the ability to protect and treat neurodegeneration generated by Parkinson's disease in the hippocampus.

To investigate the cause of neuronal death, we examined the expression of cleaved caspase-3 at both the transcript and protein levels. Additionally, we assessed DNA damage in the hippocampus subregion using the TUNEL assay.

Our data on caspase 3 expression reveals that PD mice exhibit elevated levels of active caspase 3 both at protein level. This suggests an increased rate of apoptosis, as active caspase 3 is involved in the degradation of DNA-dependent protein kinase (DNA-PK) [40]. This degradation leads to a decrease in the cell's capacity for DNA repair and subsequently promotes the characteristic DNA cleavage that occurs during apoptosis. Caspase-3 induces chromatin condensation and nuclear fragmentation by activating protein kinase C delta through proteolysis [41]. BME inhibited the expression of caspase 3 in PD mice, either as a preventative strategy or as a potential therapeutic medication, resulting in reduced apoptosis. Recent investigations have shown that α -Synuclein also has a significant role in increasing oxidative stress and apoptosis. Fibrillar aggregation is a common occurrence in the formation of Lewy bodies, which are a key characteristic of Parkinson's disease. Aggregates and inclusions of α -Synuclein are observed in the brains of individuals with Parkinson's disease, as well as in rodents and cells that have been exposed to mitochondrial poisons [42]. The buildup of normal α -Synuclein in dopaminergic neurons results in reduced functioning of mitochondrial complex I and heightened production of reactive oxygen species. An investigation conducted on rat mitochondria demonstrated that the excessive production of α -Synuclein resulted in oxidative stress and the discharge of cytochrome c into the cytoplasm, specifically in vitro systems. After being released into the cytoplasm, cytochrome c interacts with pro-survival, antiapoptotic proteins, which then initiate mitochondria-mediated apoptosis [43–44].

As the level of caspase 3 expression rises, there is a corresponding increase in the rate of DNA fragmentation. We measured this using the TUNEL assay in various regions of the hippocampus, specifically the DG, CA3, and CA1. Our findings indicate that there is a higher number of TUNEL-positive cells in PD mice, suggesting increased apoptosis in Parkinson's disease. However, treatment with BME largely mitigates this effect, offering some protection against neuronal apoptosis. So far, it has been observed that BME has a more potent neuroprotective role compared to its therapeutic impact. This trend is consistent with the findings that both pre-BME mice and post-BME treated mice exhibit a considerable reduction in apoptosis. The impact of PD on neuronal morphology was assessed by measuring the dendritic arborization and spine density of hippocampal CA1 pyramidal neurons using the Golgi-cox method. The data shows a significant reduction in the total dendritic length, the total number of branching points, and the dendritic spine density in PD mice. However, both pre- and post-treatment with BME restored these parameters. This phenomenon may be attributed to the ability of BME to enhance and nourish the neurons, hence mitigating the apparent severity of the disease. Furthermore, the post-treatment effects of BME may appear in a manner that either reduces harm or completely prevents any loss, or both. Therefore, BME demonstrates strong therapeutic and neuroprotective properties in mitigating the symptoms of Parkinson's disease, by reducing oxidative stress, DNA fragmentation, apoptosis, and neuronal degeneration and damage.

Conclusion

In conclusion, findings of this study showed that PD exhibited a decline in their ability to remember spatial working memory, which can be correlated with high oxidative stress. This oxidative stress resulted in a drop in the density of neurons due to increased apoptosis/cell death, leading to a loss of dendritic length, branching, and dendritic spine density. Administering BME (CDRI-08) orally effectively alleviates the illness and additionally improves neuroprotection by strengthening and nourishing neurons by maintaining the hippocampal oxido-apoptotic balance. Collectively, these findings indicate that *Bacopa monnieri* extract can be utilized as a herbal remedy to treat memory impairment induced by PD in which pre-treatment approach is more promising. .

Declarations

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Authors' Contributions

Vartika Gupta: Planned and carried out experiments, captured images, processed data, carried out statistical analysis of the result, and prepared draft manuscript.

S. Prasad: Generated funds, developed concept and planned experiment, Interpreted the results, Edited and finalized the manuscript.

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Data Availability

The datasets analyzed during the study are available from the corresponding author upon reasonable request.

Ethics Approval

The present study was performed according to the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), and the Institutional Animal Ethical Committee of Banaras Hindu University, Varanasi, approved all experimental procedures (Approval no.: BHU/DOZ/IAEC/2021-2022/007, Dated 15/02/2022).

Consent to Participate

Not Applicable

Consent for Publication

Not Applicable

Competing Interests

The authors declare no competing interests.

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Figures

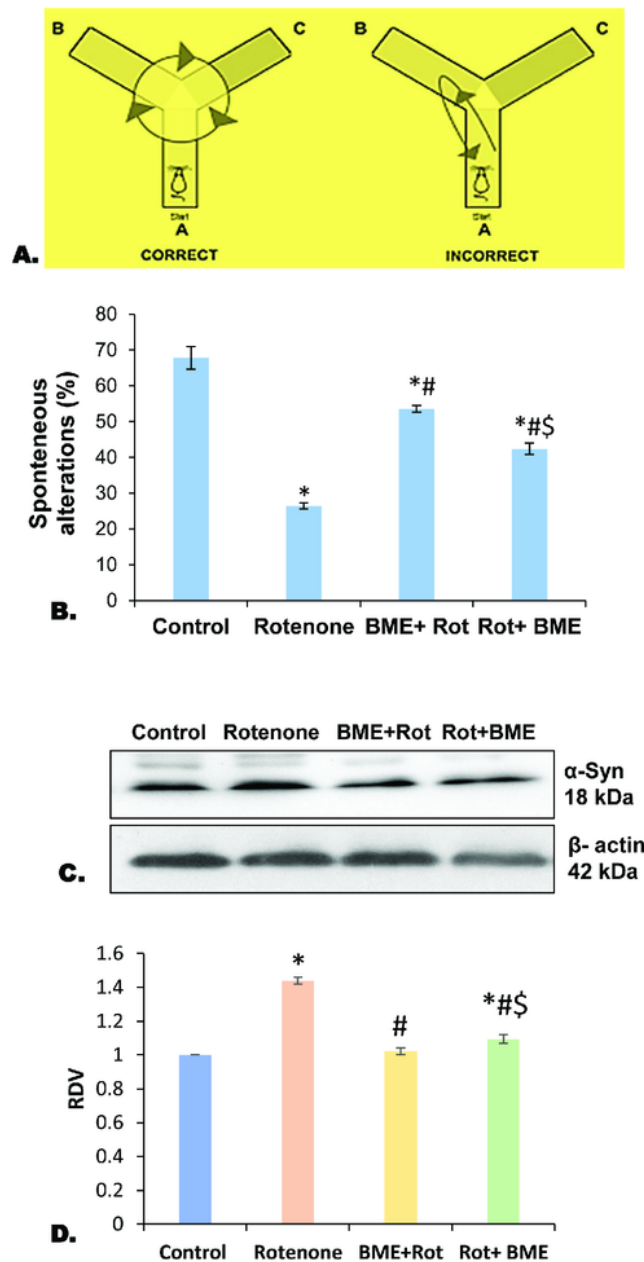


Fig. 1

Figure 1

Effect of PD and BME (CDRI-08) treatment on spatial memory by Y maze test and α -Synuclein expression. Representative image of Y-maze test (A), histogram represents % of spontaneous alterations (B). Western blot of α -Synuclein and β -actin (C) Histogram represents the level of α -Synuclein expression as Mean $\pm$ S.E.M. of RDV after its normalization with integrated density value (IDV) of β -actin as internal

control (D). Values are represented as mean $\pm$ SEM; * indicates $p < 0.05$ vs control, # Indicates $p < 0.05$ vs PD and \$ Indicates $p < 0.05$ BME+Rot vs Rot+BME.

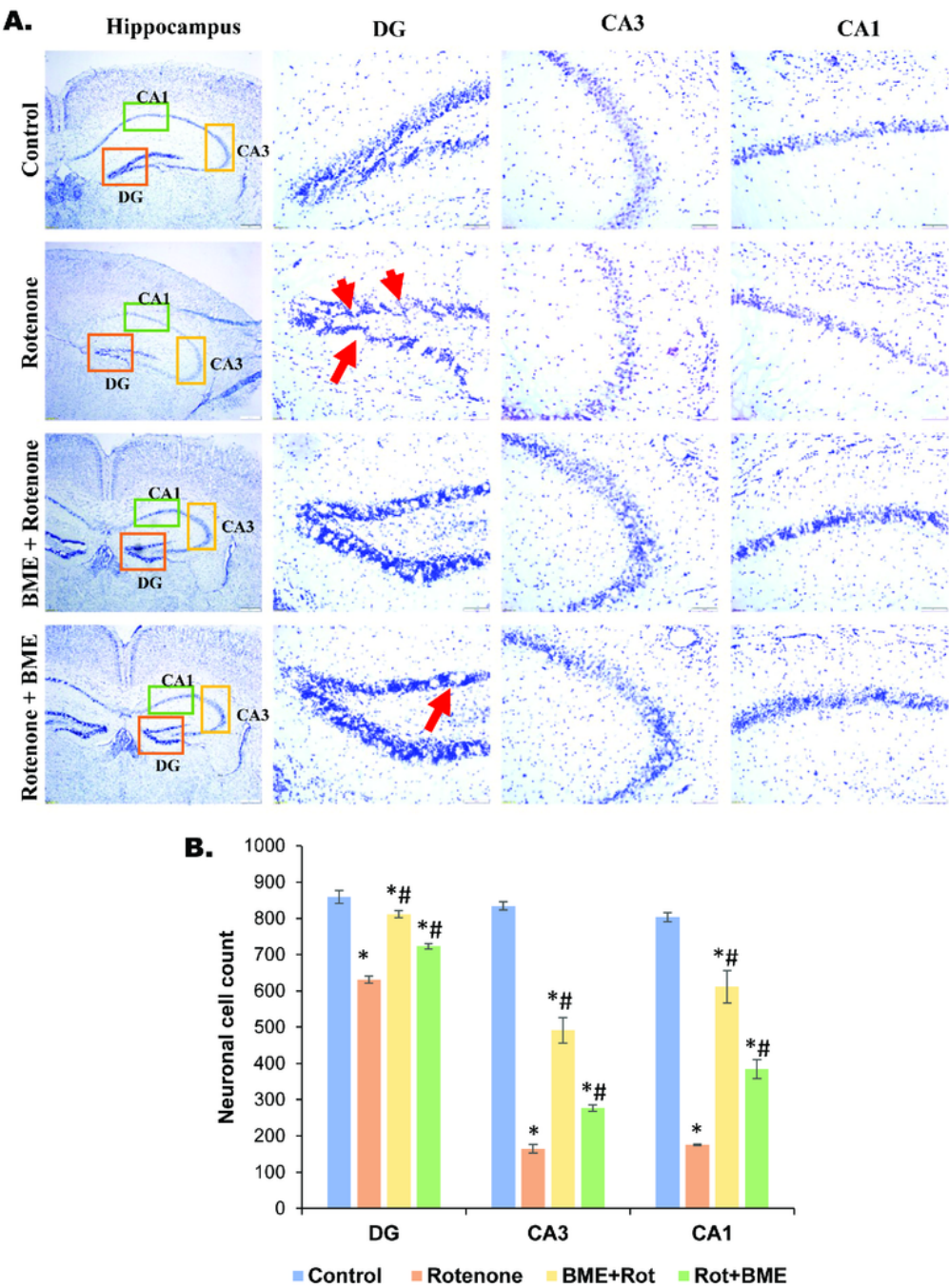


Fig. 2

Figure 2

Effect of PD and BME (CDRI-08) treatment on neuronal cell density. Microphotographs show Nissl's staining in the whole hippocampus, DG, CA3, and CA1 regions of the hippocampus (A). Histogram

represents no. of neuronal cells/50μm sq. (B). Red arrow indicating the neurodegeneration in DG region. Values are represented as mean ± SEM. * indicates p<0.05 vs control, # Indicates p<0.05 vs PD.

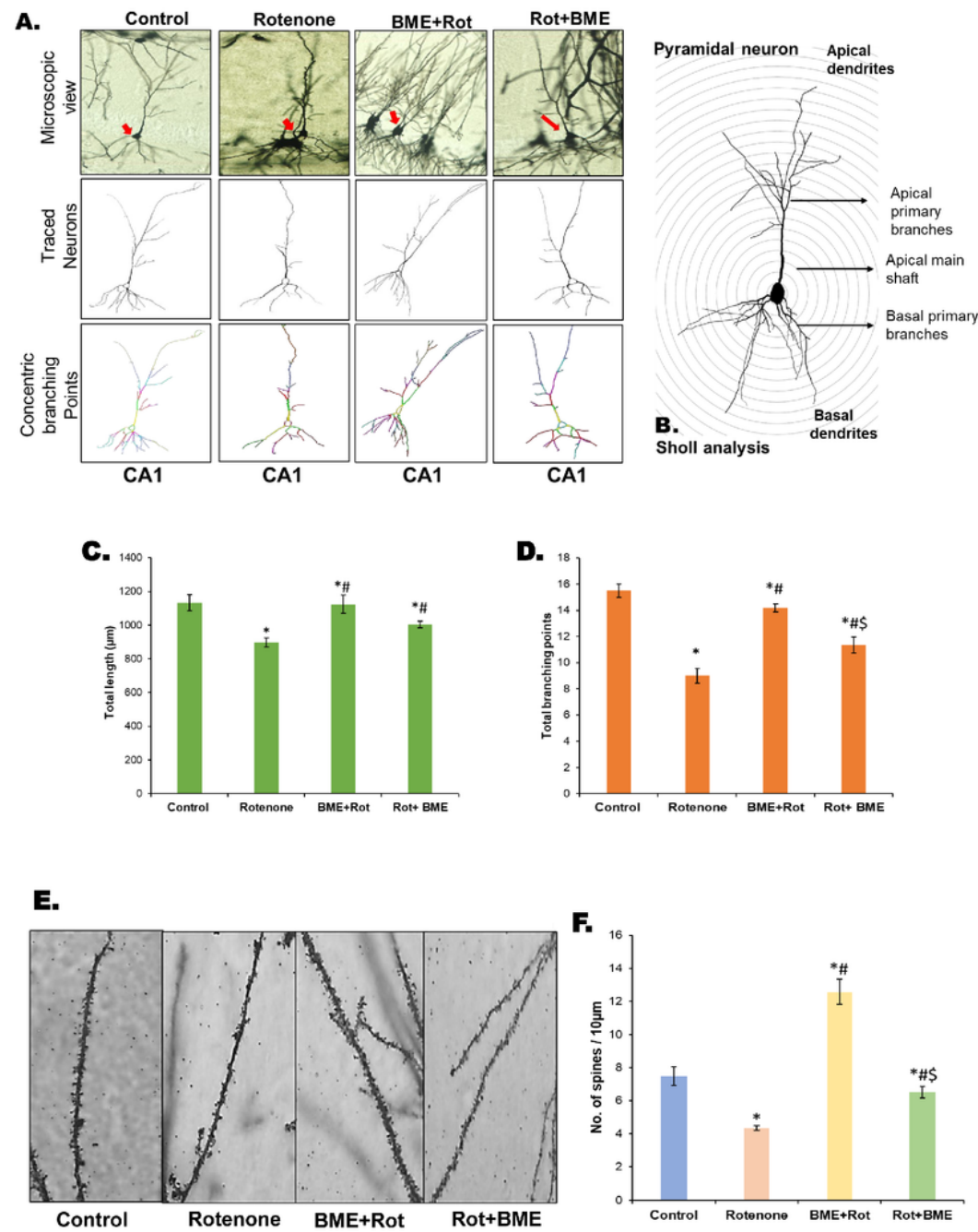


Fig. 3

Figure 3

Effect of PD and BME (CDRI-08) treatment on neuronal dendritic arborization in CA1 pyramidal neurons of the hippocampus. Microphotographs show microscopic view of pyramidal neurons, neurolucida-traced

neurons and traced neurons with secondary and tertiary branching (A). Sholl profile of traced neurons (B). Total length of apical dendrites (C) and total branching point of apical dendrites (D). Dendritic spine density in CA1 pyramidal neurons of the hippocampus: Z-stacked image of apical dendrite (E), Histogram showing the number of spines per 10µm (F). Values are represented as mean ± SEM. * indicates p<0.05 vs control, # Indicates p<0.05 vs PD and \$ Indicates p<0.05 BME+Rot vs Rot+BME.

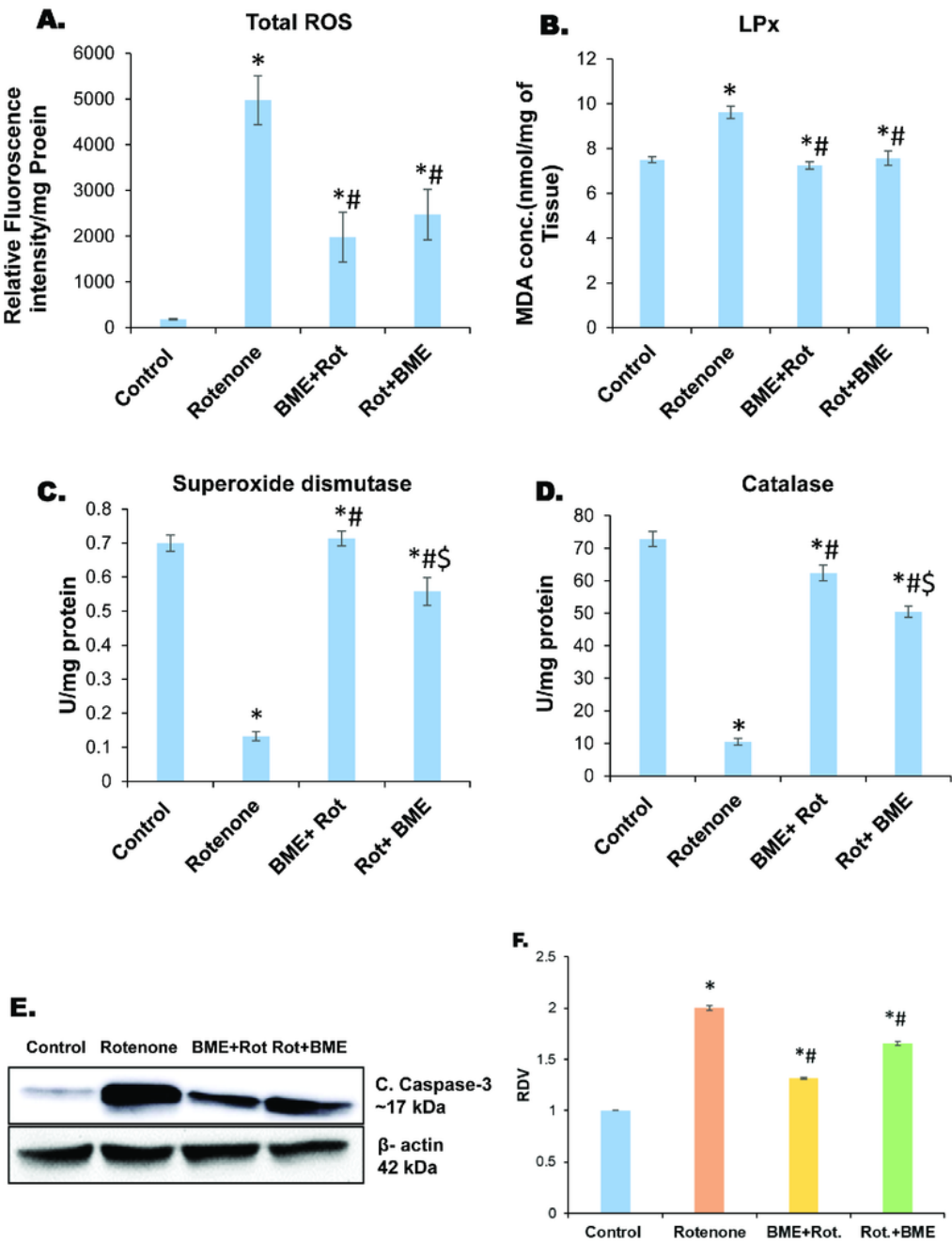


Fig. 4

Figure 4

Effect of PD and BME (CDRI-08) treatment on total ROS level, lipid peroxidation and antioxidant enzymes in the hippocampus; Histogram represents total ROS in RFI/mg of protein (A) and MDA in nmol/mg of protein (B). Activity of SOD (C) and catalase (D) in the hippocampus: Histogram represents Unit/mg protein. Western blot of cleaved caspase-3 and β -actin (E) Histogram represents the level of α -Synuclein expression as Mean $\pm$ S.E.M. of RDV after its normalization with integrated density value (IDV) of β -actin as internal control (F). Values are represented as mean $\pm$ SEM; * indicates $p < 0.05$ vs control, # Indicates $p < 0.05$ vs PD and \$ Indicates $p < 0.05$ BME+Rot vs Rot+BME.

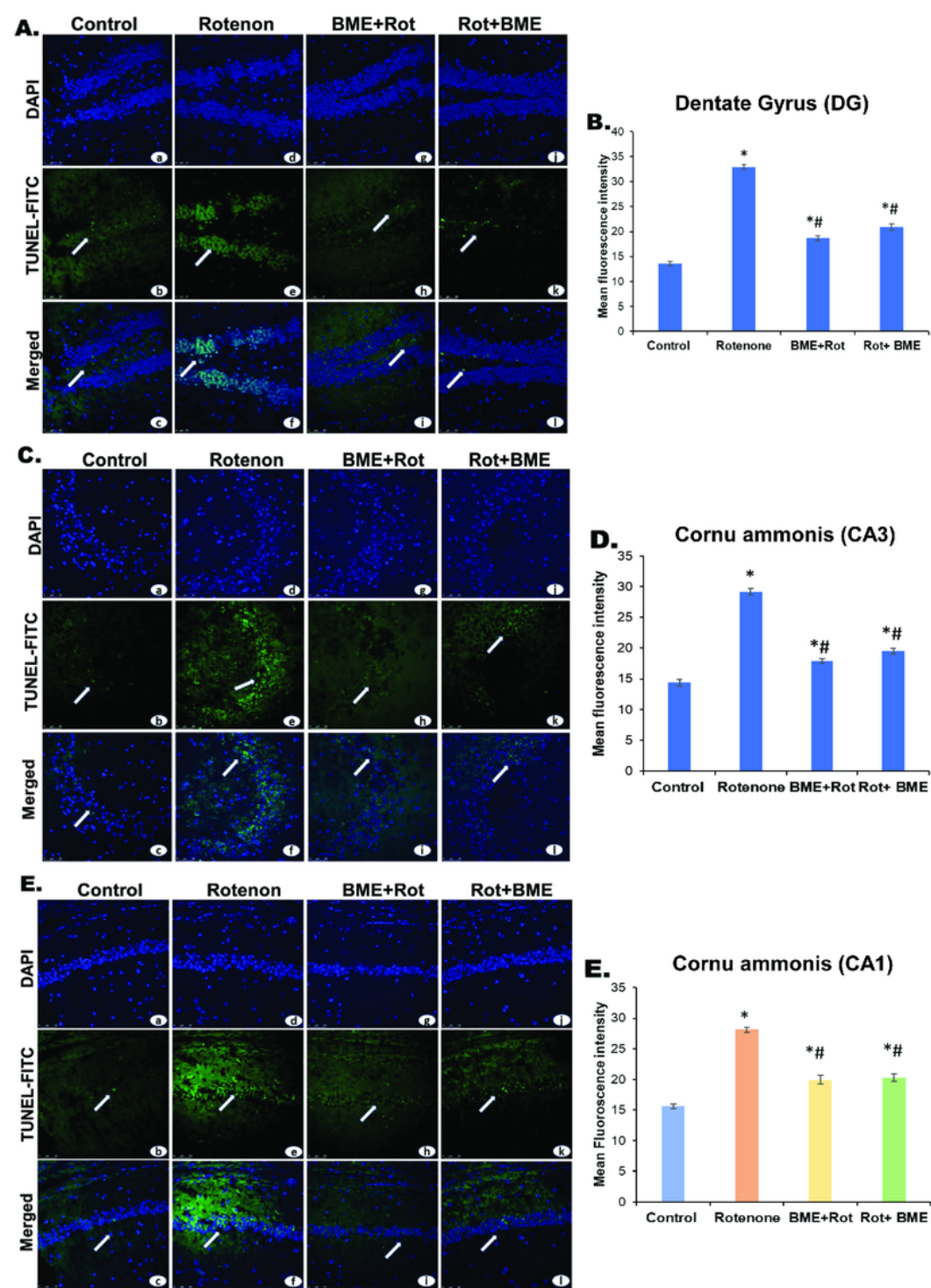


Fig. 5

Figure 5

Effect of PD and BME(CDRI-08)treatment on apoptotic neuronal death. Microphotographs showing TUNEL positive cell (Magnification 20X) in DG (A), CA3 (C) and CA (E) regions of the hippocampus. The histogram represents the corresponding mean fluorescence intensity (B, D and F). Arrow representing the TUNEL positive cells. Values are represented as mean $\pm$ SEM; * indicates $p < 0.05$ vs control, and # Indicates $p < 0.05$ vs PD.