Memory enhancing potential of Euphorbia prostrata through antioxidant, anti-inflammatory, and anti acetylcholinesterase effect against Scopolamine -induced Alzheimer’s disease in Wistar albino rats

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ABSTRACT: Alzheimer’s disease (AD) is an irreversible multi-factorial disease that marks the most common neuro-degenerative disorder in the elderly population having cognitive decline as a primary clinical attribute. The present study pursued the evaluation of Euphorbia prostrata (E. prostrata) against scopolamine-induced Alzheimer’s disease in Wistar albino rats. Total five groups of animals (having 90 rats) were included in the present study to access the therapeutic impact of E. prostrata. Morris water maze, radial arm maze, and elevated plus maze were evaluated for estimating learning and memory activity. The diverse parameters including oxidative stress, inflammatory cytokines, and acetylcholinesterase assay were assessed to estimate the mechanism of action. The consequences of the present investigation revealed the development of experimental dementia by administration of scopolamine. Whereas the treatment of E. prostrata (100 and 200 mg/kg, per oral (p.o.) and donepezil (1 mg/kg, p.o.) significantly improved the learning and memory ability in scopolamine treated rats. Incomparable to donepezil, the treatment of a higher dose of E. prostrata (200 mg/kg, p.o.) was more effective than compared to the low dose of E. prostrata (100 mg/kg, p.o.). Treatment of donepezil and a higher dose of E. prostrata (200 mg/kg, p.o.) produced comparable results for anti-inflammatory, anti-oxidative, and anti-acetylcholinesterase activity. The outcomes of the present investigation showed the memory-enhancing activity of E. prostrata (100 and 200 mg/kg, p.o.) against scopolamine–induced amnesia in rats. This effect of E. prostrata may be due to the inhibition of brain acetylcholinesterase activity, through the involvement of an anti-inflammatory pathway and due to its antioxidant potential.

KEYWORDS: Euphorbia prostrata; Euphorbiaceae; Alzheimer’s Disease; Oxidative stress; Inflammatory cytokines; Acetylcholinesterase activity.

1. INTRODUCTION

Memory plays a dynamic role in creating a stable sense of self besides guiding the behaviors or facilitating social interaction. It is a psychological process that aids in preserving and recovering all information. In humans, memory loss at an older age is a characteristic feature of Alzheimer’s disease (AD) [1,2]. According to an epidemiological report approximately 50 million people are affected with AD globally and are expected to rise exponentially and reach about 120 million by 2050. It has also been reported as the 6th major reason for mortality [3]. Pathology of AD is simple superficially but complex deceptively. AD is marked by degenerative variations in the neuro-transmission system that results in the accumulation of B-amyloid and neuro-fibrillar tangles and faulty mitochondrial, biochemical, and molecular abnormalities [4]. The affected population suffers from progressive cognitive disorders, the tremendous decline in memory combined with behavioral and neuro-psychiatric manifestations [5]. Although the etiology of the disease is poorly understood, age, head injuries, hypertension, depression, and vascular deficits are the most common risk factors associated with the disease [6]. Conversely, there have been many therapeutic strategies suggested that include several combination therapies and monotherapies, but all this fail to effectively combat the malignancy [2,7–9]. Numerous pro cholinergic agents have been employed for the treatment, some of them...
include rivastigmine, tacrine, donepezil, and metrifonate, but these also show several adverse effects [7,10]. Furthermore, herbal drugs have shown promising results in managing the prognosis of numerous fatal diseases. Diverse genera of Euphorbia have been investigated for their possible therapeutic action against different pathological conditions including neurological diseases [11]. Of which, some of them remain under the bridge such as Euphorbia prostrata (E. prostrata). E. prostrata is an annual herb belonging to the Euphorbiaceae family and is found lavishly in India and Africa [11,12]. The different parts of this herb have been reported to have numerous pharmacologically active constituents including phytosterols, flavonoids, polyphenols, and phenolic compounds [11,12]. Contemporary research reveals that phytoconstituents of the plant endorse the numerous conventional therapeutic uses of E. prostrata against warts, gonorrhea, skin infections, migraines, parasitic infection, and viral diseases along with its previously reported therapeutic actions such as anthelmintic activity [13], anticanical activity [14], analgesic [15], wound healing [16], antioxidant [12], insecticidal activity, antihyperglycemic activity, hypolipidemic effect [17], and antitumor activity [18]. Despite such a diverse pharmacological arena, the plausible therapeutic action in neurodegenerative disorders by endorsing traditional therapies including phytoconstituents and homeopathy, the underlying molecular mechanism is still a challenge now a days [19]. The phytoconstituents in E. prostrata have a massive opportunity to interact with numerous pathophysiological cascades and could lead to an advancement in the health system. However, the missing link of molecular interaction of E. prostrata with the biological system limits its therapeutic usage and lacks the confirmation of an accountable molecular pathway as a preferred therapeutic approach [20,21]. Hence, this study is a novel attempt to decipher the therapeutic impact with plausible molecular interaction of E. prostrata against AD.

2. RESULTS

2.1 Effect of E. prostrata on escape latency (EL) and time spent in the target quadrant (TSTQ) of rats using Morris water maze

E. prostrata (100 and 200 mg/kg, p.o.) and Donepezil (1 mg/kg, p.o.) significantly decreased EL of rats on the 8th day and increased TSTQ by rats on the 8th day as compared to the control group, thus showed significant improvement of learning and memory. Scopolamine (1 mg/kg, i.p.) significantly increased EL and decreased TSTQ in rats, indicating their amnesic effects (Figure 1A and 1B). The low dose of E. prostrata (100 mg/kg, p.o.) did not significantly decrease EL or increase TSTQ as compared to Scopolamine treated group. E. prostrata (200 mg/kg, p.o.) significantly reversed scopolamine-induced learning and memory impairment in rats as compared to respective scopolamine-treated groups (Figure 1A and 1B).

2.2 Effect of E. prostrata on working memory error, reference memory error, and retrieval latency of rats using radial arm maze

A radial arm was used to access working memory error, reference memory error, and retrieval latency which estimate the therapeutic potential of E. prostrata (100 and 200 mg/kg, p.o.) and Donepezil (1 mg/kg, p.o.) against scopolamine (1 mg/kg, i.p.) induced dementia. Working memory errors, reference memory errors, and retrieval latency were significantly increased in scopolamine-treated animals. Whereas the treatment of low and high doses of E. prostrata (100 mg/kg, p.o.) produced significant reductions in working memory error, reference memory error, and retrieval latency respectively (Figure 1C, 1D, and 1E). Although, the low dose E. prostrata extract (100 mg/kg, p.o.) produces a moderate reduction of working memory error, reference memory error, and retrieval latency time. Further, the consequences describe the comparable potency of E. prostrata extract (200 mg/kg, p.o.) with Donepezil (1 mg/kg, p.o.).

2.3 Effect of E. prostrata on retrieval transfer latency of rats using the elevated plus maze

E. prostrata (100 and 200 mg/kg, p.o.) and Donepezil (1 mg/kg, p.o.) administered for 7 successive days did not significantly affect the retrieval TL of rats on the 7th day (learning) as compared to the control group. But E. prostrata (100 and 200 mg/kg, p.o.) and Donepezil (1 mg/kg, p.o.) significantly decreased retrieval TL in rats on the 8th day (memory) as compared to the control group, thus showing significant memory enhancing activity (Figure 1F). The low dose of E. prostrata (100 mg/kg, p.o.) moderately reduced retrieval TL of rats on the 8th day as compared to Donepezil (1 mg/kg, p.o.) treated group. Scopolamine (1 mg/kg, i.p.) significantly increased retrieval TL in rats, indicating its amnesic effect (Figure 1F). E. prostrata (100 and 200 mg/kg, p.o.) and Donepezil (1 mg/kg, p.o.) significantly reversed scopolamine-induced memory impairment in rats.
2.4 Effect of E. prostrata on oxidative stress of rats

The oxidative stress in rats having diverse interventions was measured by accessing SOD, CAT, GSH, and TBARS concentrations in brain tissue. Levels of SOD, CAT, and GSH were significantly decreased in Scopolamine (1 mg/kg, i.p.) treatment as compared to normal control. The treatment of E. prostrata (100 and 200 mg/kg, p.o.) and Donepezil (1 mg/kg, p.o.) significantly upsurge SOD, CAT, and GSH levels. Furthermore, the increased level of TBARS was comparable that indicate the therapeutic potential of E. prostrata (100 mg/kg, p.o.) and Donepezil (1 mg/kg, p.o.) for oxidative stress level (Figure 2A, 2B, and 2C). Although the increment in low dose E. prostrata (200 mg/kg, p.o.) was greater than that in Donepezil (1 mg/kg, p.o.) for oxidative stress level (Figure 2D). Figure 1: The figure describes the effect of E. prostrata against Alzheimer’s disease on cognitive paradigms by measuring: escape latency (EL) as shown in [A]; time spent in target quadrant (TSTQ) as shown in [B] using Morris water maze; working memory error as shown in [C]; reference memory error as shown in [D]; retrieval latency of rats using radial arm maze as shown in [E]; and retrieval transfer latency of rats as shown in [F] using elevated plus maze. All values were expressed as mean ± SD whereas, ‘a’ represents p < 0.001 vs Normal Control; ‘b’ represents p < 0.001 vs disease control (Scopolamine group).

2.5 Effect of E. prostrata on inflammatory cytokines of rats

The anti-inflammatory potential of E. prostrata was measured by accessing inflammatory cytokines. The concentration of inflammatory biomarkers (IL-6 and TNF-α) was significantly raised in Scopolamine (1 mg/kg, i.p.) treated animals as compared to animals in the normal control group. Treatment of E. prostrata (100 and 200 mg/kg, p.o.) and Donepezil (1 mg/kg, p.o.) significantly diminished the increased level of IL-6 and TNF-α in Scopolamine (1 mg/kg, i.p.) treated animals (Figure 3A and 3B). This reduction of inflammatory cytokines was dose-dependent as the treatment of higher dose E. prostrata (200 mg/kg, p.o.) was greater than the reduction of inflammatory cytokines by low dose E. prostrata (100 mg/kg, p.o.) as shown in Figure 3A and 3B respectively.

2.6 Effect of E. prostrata on Brain Acetyl Cholinesterase (AChE) Activity in Rats

Scopolamine (1 mg/kg, i.p.) administration significantly increased AChE activity as compared to normal control. Treatment of E. prostrata (100 and 200 mg/kg, p.o.) and Donepezil (1 mg/kg, p.o.) for 7 consecutive days produced a significant decrease in brain AChE activity as compared to Scopolamine (1 mg/kg, i.p.).
low dose of *E. prostrata* (100 mg/kg, p.o.) did not produce a significant decrease in AChE activity as compared to the standard control group (Donepezil (1 mg/kg, p.o.)) (Figure 4). Conversely, the reduction of AChE activity by *E. prostrata* (200 mg/kg, p.o.) was comparable to Donepezil (1 mg/kg, p.o.) treatment.

**Figure 2:** The figure describes the effect of *E. prostrata* on brain oxidative stress of rats by measuring: SOD activity as shown in (A), CAT activity as shown in (B), GSH level as shown in (C), and TBARS as shown in (D). All values were expressed as mean ± SD whereas, ‘a’ represents p < 0.001 vs Normal Control; ‘b’ represents p < 0.001 vs disease control (Scopolamine control) group.

### 2.7 Effect of *E. prostrata* on brain histology

Histological sections of brain tissue of respective groups showed diverse structural variations in the histology of small pyramidal cells of the hippocampus (CA1) region with vesicular nuclei. Section of the normal control group showed granule cells of the dentate gyrus and glial cells. The brain histology of scopolamine (1 mg/kg, i.p.) administered to rats showed vacuolization and cell death. The predominance of granule cells of the dentate gyrus and glial cells was significantly reduced in the scopolamine (1 mg/kg, i.p.) treated group. The 7 days of consecutive treatment of donepezil (1 mg/kg, p.o.), and *E. prostrata* (100 and 200 mg/kg, p.o.) significantly reversed pathological changes as compared to the histology of scopolamine (1 mg/kg, i.p.) treated group. The structural arrangements of brain tissue of animals having a higher dose of *E. prostrata* (200 mg/kg, p.o.) was comparable with the animal tissue of donepezil (1 mg/kg, p.o.) treatment (Figure 5).
3. DISCUSSION

Alzheimer’s disease (AD) is a common progressive neurodegenerative disorder at an older age which gradually increased worldwide. One of the initial symptoms of AD and other dementias is frequently memory loss that interferes with everyday activities, such as getting lost in a familiar location or having trouble doing familiar duties at home, as well as diminished or poor judgement as well as mood or behaviour changes. Although numerous pharmacological interventions are available, have limited therapeutic impact [3]. *E. prostrata* is an annual herb belonging to the Euphorbiaceae family and is found lavishly in India and Africa [11]. The different parts of this herb have been described to keep the numerous pharmaceutically active constituents including phytosterols (cholesterol, stigmasterol, campesterol, beta-sitosterol), flavonoids (Apigenin, apigenin-7-glucoside, luteolin, luteolin-7-glucoside), polyphenols (ellagic acid, tannic acid), and phenolic compound (gallic acid) [11,12]. Although the growing evidence unveils the promising pharmacological influence of *E. prostrata*, the plausible therapeutic effect against AD with molecular mechanism is uncertain. In the present study, *E. prostrata* (100 and 200 mg/kg, p.o.) administered for 7 successive days showed a significant memory-improving effect in rats. This is the first report that shows memory enhancing potential of *E. prostrata* in rats. Behavioral models like Morris water maze, radial arm maze, and elevated plus maze were employed in the current study to investigate learning and memory. These models are extensively used for assessing the drug potential on learning and memory [24–26]. The consequences of the Morris water maze model revealed that treatment of *E. prostrata* leads to a significant reduction in escape latency during training and an increase in time spent in the target quadrant during retrieval showed an increase in learning and memory respectively; and vice versa. The existing evidence suggest strong correlations between the Morris water maze test and hippocampus synaptic plasticity [24]. In the radial arm maze, a considerable decrease in working memory error, reference memory error, and retrieval
Yadav et al.
Euphorbia prostrata against Alzheimer’s disease

Figure 5: Figure showed the effect of E. prostrata on brain histology by identifying the small pyramidal cells of the hippocampus (CA1) region with vesicular nuclei in different groups of treatments using an inverted microscope (Cosmo Laboratory Equipment). The black arrow shows granule cells of the dentate gyrus. The red arrow shows vacuolization and cell death, and the green arrow shows glial cells.

Latency was observed by E. prostrata treatment that signifies the recovery of learning and memory respectively in Scopolamine (1 mg/kg, i.p.) treated rats. These effects of the radial maze provide more proof that there may be a considerable recovery in brain regions, which may increase the outputs of these systems and increase competition for behavioural control. Moreover, the outcomes of the elevated plus maze model also confirmed a significant decrease in retrieval transfer latency which indicated improvement of memory. Although out of the two effective doses of E. prostrata (100 and 200 mg/kg, p.o.), the higher dose (200 mg/kg) produced significantly better memory-enhancing potential in Scopolamine (1 mg/kg, i.p.) treated rats as compared to the lower dose (100 mg/kg) treatment in all behavioral models, hence the memory enhancing the potential of E. prostrata probably dose dependant. AD is also described by the gradual formation of insoluble amyloid plaques and the deposition of amyloid beta-peptide in the brain [27]. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is a potent drug target for the treatment of AD as prolonged inhibition of BACE1 restricts the structural and functional synaptic plasticity in rats (altering the metabolism of BACE1 substrates) [28,29]. The memory-boosting action of E. prostrata is also supported by its BACE 1 inhibiting property. Although the over production of reactive oxygen species itself the root cause of diverse pathological conditions including neuronal diseases [26,30,31]. Oxidative stress can significantly affect amyloid-beta generation in the pathogenesis of AD through upregulation of BACE 1 gene transcription by oxidative stress [32]. Conversely, E. prostrata showed significant antioxidant activity as reported by the consequences of the present study. Thus, E. prostrata induced significantly enhancing memory in rats may be due to its antioxidant potential and resulting in the protection of vulnerable neuronal cells from oxidative stress. Moreover, the study also finds that inflammation in the brain cells leads to the progression from the presence of amyloid plaque and tau tangles to the onset of dementia and AD [33,34]. The constant stimulation of the brain’s macrophages (microglia) may serve as a link in the progression of the pathogenesis of AD by exacerbating amyloid and tau proteins [34]. Some studies already revealed the anti-inflammatory potential of E. prostrata [35–37]. The consequences of the present study showed the significant anti-inflammatory potential of E. prostrata measured by the reduced level of inflammatory cytokines (IL-6 and TNF-α) in Scopolamine (1 mg/kg, i.p.) treated rats. Furthermore, the central cholinergic system also plays a foremost role in the management of the cognitive function. Acetylcholine is an important neurohumoral transmitter that regulates cognitive functions, whereas acetylcholinesterase is responsible for the breakdown the acetylcholine in the synapse. Impaired cholinergic transmission due to hyperactivation of acetylcholinesterase can leads to cognitive dysfunction and senile dementia [38,39]. Thus, the drugs which can improve cholinergic function either by diminishing
acetylcholinesterase levels or by increasing cholinergic transmission could be a promising treatment for dementia and AD. The administration of donepezil (1 mg/kg, p.o.) for 7 successive days significantly improved the memory of rats in the present study. Donepezil is widely used and reported for its memory enhancement activity. The therapeutic effect of *E. prostrata* (100 and 200 mg/kg, p.o.) was studied in comparison to donepezil. Muscarinic receptor antagonist like scopolamine diminishes cholinergic function and produce amnesia in laboratory animals [40]. The present study showed similar results and produced significant impairment in memory of rats measured by various behavioral models and increased level of AChE. The administration of *E. prostrata* (100 and 200 mg/kg, p.o.) for 7 successive days significantly reversed scopolamine-induced amnesia in rats. This reversal of scopolamine-induced amnesia by *E. prostrata* indicated the possible facilitation of cholinergic transmission. Treatment of *E. prostrata* (100 and 200 mg/kg, p.o.) also significantly lessened brain AChE activity in scopolamine (1 mg/kg, i.p.) treated rats as compared to the diseases group. These consequences suggest the memory-enhancing potential of the effect of *E. prostrata* which may be triggered by an upsurge level of brain acetylcholine through antagonizing of AChE. Additionally, the histological study confirmed the pathophysiological changes in brain tissue with or without treatment. The predominance of granule cells of the dentate gyrus and glial cells was significantly upsurged in the issue section of treated rats as compared to scopolamine (1 mg/kg, i.p.) treated rats.

4. CONCLUSION

Conclusively, the outcomes of the present investigation showed the memory-enhancing activity of *E. prostrata* (100 and 200 mg/kg, p.o.) against scopolamine-induced amnesia in rats. This effect of *E. prostrata* may be due to the inhibition of brain acetylcholinesterase activity, through the involvement of an anti-inflammatory pathway, and due to its antioxidant potential.

5. MATERIALS AND METHODS

Male Wistar albino rats of 250-280 g were used in the present study. Experimentation on animals was approved by IAEC of Chitkara College of Pharmacy, Chandigarh, India, and CPCSEA, New Delhi, India (IAEC/CCP/22/01/PR-18). All animals were acclimatized for 15 days with access to food and water ad libitum, environmental conditions were maintained at temp 20 ± 2 °C, relative humidity 60 ± 10 %.

5.1 Experimental Design and animals

A total of 90 rats were divided into five groups including normal control, disease control, standard treatment, and drug treatment with low and high doses. All five groups were given various interventions daily for 7 days. Animals of normal control received normal saline 0.9% (10 ml/kg intraperitonially (i.p) for 7 days). Scopolamine (1 mg/kg i.p.) was given to induce dementia. Donepezil (1 mg/kg i.p.) was used as standard treatment whereas low (100 mg/kg i.p.) [22] and high doses (200 mg/kg i.p) [23] of hydroalcoholic extract of test drug (HAE *E. prostrata*) were given as test drugs against diseases and standard treatment. Scopolamine (1 mg/kg i.p.) was administered in groups II, III, IV, and V. In groups III, IV, and V, it was administered after 30 minutes of intervention administration from Day 4 to Day 7. The cognitive paradigms were evaluated 30 minutes after scopolamine administration from Day 4 to Day 7. On day 8, retention tests were performed followed by biochemical estimations.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Group Name</th>
<th>Interventions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal Control</td>
<td>Normal Saline 0.9% (10 ml/kg i.p.) (for 7 days)</td>
</tr>
<tr>
<td>2.</td>
<td>Disease Control</td>
<td>Scopolamine (1 mg/kg i.p.)</td>
</tr>
<tr>
<td>3.</td>
<td>Standard</td>
<td>Donepezil (1 mg/kg per oral (p.o) + Scopolamine (1 mg/kg i.p.)</td>
</tr>
<tr>
<td>4.</td>
<td>Treatment 1</td>
<td><em>E. prostrata</em> (100 mg/kg p.o.) + Scopolamine (1 mg/kg i.p.)</td>
</tr>
<tr>
<td>5.</td>
<td>Treatment 2</td>
<td><em>E. prostrata</em> (200 mg/kg p.o.) + Scopolamine (1 mg/kg i.p.)</td>
</tr>
</tbody>
</table>
5.2 Morris water maze (MWM) test
A circular pool having a radius of 110 cm and a height of 52 cm, divided into four quadrants was used. An elevated platform is placed centrally to be located by the rats. Water was made misty by using talc. A maximum time of 120 sec was considered as the cut-off time. Now, the time taken by each rat of a different group to locate the elevated platform is noted. This is escape latency time. Also, the time spent in each quadrant was recorded.

5.3 Radial arm maze (RAM) test
A set up of eight wooden arms, connected centrally with food items placed in alternate arms was used. Working memory of each animal is tested by their entry into the food-containing arms. A complete entry was considered as the one, in which the rat has entered all four paws in the arm. The time spent on each arm was also observed. % Latency time was calculated.

5.4 Elevated plus maze (EPM) test
EPM test is a tool to measure anxiety & memory in lab animals. A four-armed wooden apparatus connected centrally is used. Two arms are open and two are closed with an elevated wooden block on opposite sides. Each animal is placed on a central platform and was allowed to explore for 90 seconds. The movement of animals in the open arm from the closed arm is taken as a sign of working memory.

5.5 Estimation of oxidative stress biomarkers
Animals were sacrificed after anesthetizing with isoflurane followed by cervical dislocation. The Animal’s brain was carefully isolated and treated with normal saline. A 10% w/v homogenate tissue was prepared in 0.03M phosphate buffer (pH 7.4) by centrifuging this mixture for 15 minutes at 3000 g. The supernatant was collected for further biochemical estimation.

5.6 Estimation of superoxide dismutase (SOD)
0.1 ml of brain homogenate was added to a mixture of 2.8 ml of potassium phosphate buffer (0.1M, pH 7.4) & 0.1 ml of pyrogallol solution. The formed mixture was used to measure the absorbance at 325nm with the UV spectrophotometer.

\[
\text{Catalase activity} = \frac{\delta \text{OD}}{E \times \text{Vol of sample (ml)} \times \text{mg of protein}}
\]

Where $\delta \text{OD}$ = change in absorbance per minute
E= Extinction coefficient of hydrogen peroxide (0.071mmol cm$^{-1}$).

5.7 Measurement of catalase activity (CAT)
0.05ml of tissue homogenate was added to 1ml of hydrogen peroxide (H2O2) and 1.95ml of phosphate buffer (50Mm, pH 7).

5.8 Measurement of malondialdehyde (MDA)
The solution of 3ml of thiobarbituric acid (TBA) reagent (which contained 5N HCL and trichloroacetic acid; TCA) was added to 1 ml of brain homogenate to make a reaction mixture. This mixture was heated for 15 minutes at 90° C. After cooling the heated solution, it was centrifuged at 3500 g for 10 minutes. The pink-colored supernatant was used to measure the absorbance at 532nm.

1011
The MDA level was calculated by the following equation

\[ \text{conc.of MDA} = \frac{\text{Abs}_{532} \times 100 \times V_t}{(1.56 \times 10^5) \times W_t \times V_u} \]

Where Abs_{532}= absorbance, V_t= total mixture volume, 1.56\times10^5= molar extinction coefficient, W_t= weight of the brain and V_u=aliquot volume.

5.9 Measurement of reduced glutathione (GSH) Level
The 1ml brain homogenate solution was mixed with the 1ml of 10% trichloroacetic acid and centrifuged at 2000 g for 10 minutes. The resultant supernatant was separated in which 2ml of phosphate buffer (pH 8.4) and 0.5ml of DTNB reagents were added and absorbance was measured at 412nm.

The equation used to measure the GSH level are as follow:

\[ \text{GSH Level} = \frac{Y - 0.00314}{0.0314} \times \frac{D_F \times 1}{B_T \times V_u} \]

Here, y= absorbance at 412nm, DF= dilution factor BT= brain tissue homogenate volume (1ml), and VU= volume of aliquot (1ml)

5.10 Estimation of inflammatory biomarkers
The treated animals with diverse interventions were accessed for pro-inflammatory (IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines. Inflammatory cytokines were estimated from the rat serum sample at the end of the protocol by performing an enzyme-linked immunosorbent assay (sandwiched ELISA kit, Ray Biotech, USA).

5.11 Estimation of acetylcholinesterase activity
100µl of DTNB, 2.6ml of phosphate buffer; 0.4ml tissue homogenate were mixed thoroughly. The absorbance was measured at 412nm by using a UV spectrophotometer. The stable reading was recorded as the basal reading. In this mixture of 20µl, acetylthiocholine iodide was added as the substrate to change the absorbance. Further, the change in absorbance was measured for 10 minutes at an interval of 2 minutes. The following formula helps in calculating the average change in absorbance and the activity of acetylcholinesterase (AChE) was expressed as µM/min/gm of tissue.

\[ R = 5.74 \times 10^{-4} \times \frac{\Delta A}{C_o} \]

Here, R=rate in moles substrate hydrolysed per minutes per gram of tissue, ΔA= change in absorbance per minute, and C_o= original concentration of tissue in mg/ml.

5.12 Histological Assessment
Animals from each group were sacrificed on the 8th day and brain were isolated to for histological assessment. Sections of brain sample were prepared and stained with haematoxylin and eosin staining.

5.13 Statistical analysis
Data for all biochemical markers were expressed as mean ± SD and statistically analysed by one-way ANOVA followed by post-hoc Tukey’s test using Sigma Plot version 11.0 (Systat Software, Inc., San Jose California USA).

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