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Ameliorative effect of *Amaranthus tricolor* L. leaves on scopolamine-induced cognitive dysfunction and oxidative stress in rats

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ABSTRACT

Ayurveda is a holistic system that uses mostly herbs and minerals in prescriptions that play a crucial role in the treatment of various diseases because of their therapeutic effects, often on multiple targets. In the present study, we assessed the memory-enhancing activity of a leafy vegetable "*Amaranthus tricolor* L." against scopolamine (SCP)-induced cognitive dysfunction and oxidative stress in rats. Initially, we screened *A. tricolor* leaves' ethanolic extract for its acetylcholinesterase (AChE) inhibitory and antioxidant activity using standard procedures. The memory-enhancing activity in rats was performed by using the Morris water maze (MWM), the elevated plus maze (EPM), Actophotometer, and Cook's pole climbing (CPC) apparatus. Biochemical parameters, mainly AChE and butyrylcholinesterase (BChE) enzyme levels measured in the hippocampus and cortex, amyloid beta, reduced glutathione (GSH), superoxide dismutase, and lipid peroxidation (LPO) level, were measured in whole brain tissue. *A. tricolor* leaves' extract showed potent AChE inhibition and antioxidant activity on the scavenging of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH). The EPM, MWM, CPC, and Actophotometer showed that SCP administration increased transfer latency (TL) and decreased escape latency time (ELT) and locomotion, respectively, on day 7 and 14, whereas combined therapy of SCP with Donepezil and SCP with 800 mg/kg *A. tricolor* extract significantly reversed the ELT, TL, and locomotion from day 7 to 14. Moreover, AChE and BChE activities and GSH and Malondialdehyde (MDA) levels were significantly elevated in SCP-treated rats, and treatment with *A. tricolor* elevated the biochemical parameters altered by the SCP administration. *A. tricolor* L. leaves' extract ameliorates SCP-induced cognitive dysfunction and oxidative stress by the restoration of the cholinergic system's function, inhibiting Amyloid precursor protein (APP) deposition, and via antioxidant potency, i.e. increasing GSH level, by inhibiting the LPO in the brain. The current study suggests that *A. tricolor* L. leaves could be a valuable source for the treatment of cognitive dysfunction and oxidative stress associated with Alzheimer's disease.

INTRODUCTION

During the twentieth century, the significant increase in life expectancy made Alzheimer's disease (AD) the most widely perceived progressive neurodegenerative disorder (NDD) with high incidence and multifaceted pathogenesis. AD is a polygenic disease that involves multi-protein and multi-pathways in its progression (Uddin *et al*., 2016). AD is characterized by the presence of extreme amounts of extracellular deposits containing insoluble amyloid-β plaques, neurofibrillary tangles, and cholinergic deficits. The etiology of AD remains obscure because this disease is perplexingly caused by aging in concert with a complex interaction of both genetic factors and environmental risk. Numerous studies have reported that central cholinergic neurons are involved in learning and memory. During aging, the elevated central cholinergic system is associated with the decline in memory storage, ultimately impairing the retention of newly assimilated information (Huang *et al*., 2009). Hence, AD is an age-related progressive NDD that involves unavoidable loss of cognition, accounting for ~ 60% of all cases, and over 35M people

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are diagnosed with AD worldwide and about 7.7M new cases are diagnosed every year (Ali *et al*., 2015).

Currently, there is no satisfactory treatment available for AD. The current AD therapy includes utilization of conventional acetylcholinesterase (AChE) inhibitors, viz. tacrine, physostigmine, and donepezil. However, extreme utilization of these agents is associated with negative effects (Asaduzzaman *et al*., 2014). In this way, it investigates the use of conventional spices as an elective resource for the treatment or management of various cognitive disorders. Herbal medicines are widely utilized as a major source for the treatment of cognitive dysfunctions due to the presence of various chemical constituents, antioxidant properties, and are associated with less toxicity compared to modern synthetic medicines. Furthermore, antioxidants have endogenous and exogenous properties which control the oxidation of neuron cells and act against oxidative stress-related harmful effects. The major role of antioxidants is scavenging free radicals and improving the antioxidant status (Uttara *et al*., 2009). In order to regulate the development of free radical-mediated oxidative stress in the brain, antioxidant defense enzymes, such as reduced glutathione (GSH), catalase, and lipid peroxidation (LPO), play an important role (Sharma *et al*., 2013).

Amaranthus tricolor L. belongs to the family Amaranthaceae. *A. tricolor* is also known as "Tambdi Bhaji/Lal Saag" and is native to a large part of India. The leaves are rich in antioxidant compounds, act as a free radical scavengers (defense mechanism against oxidation), reduce oxidative stress, and act as an anti-inflammatory herb. The alkaloids, carbohydrates, cardiac glycosides, flavonoids, phenol, amino acids/proteins, saponins, tannins, terpenoids, pterocarpans, triterpenes, steroids, quinones, resins, and "coumarins" are found to be the major chemical constituents of the leaves. *A. tricolor* leaves were utilized as traditional medicine and for neuroprotection, as an astringent, to strengthen the liver and improve vision, as a hepatoprotective and anti-nociceptive, and as an anti-inflammatory, anti-cancer, and anti-arthritic (Al-Dosari *et al*., 2010; Amicarelli *et al*., 2012; Amornrit *et al*., 2016; Sable *et al*., 2017). Therefore, the present study aimed to evaluate the effect of *A. tricolor* leaves' ethanolic extract on scopolamine (SCP)-induced cognitive dysfunction and oxidative stress in rats.

MATERIALS AND METHODS

Chemicals and equipment

SCP HBr (Vital Laboratories Pvt. Ltd, Gujarat), Donepezil (Apotex pharmaceuticals, Bengaluru), Acetylthiocholine iodide (Sigma, St. Louis, MO), 5,5'-dithio-bis-(2-nitrobenzoic acid) (Sigma, St. Louis, MO), and distilled water were used wherever mentioned. Double distilled ethanol was used for extraction. Enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Scientific Multiskan GO version 1.00.40), centrifuge (Remi), elevated plus maze (EPM), Morris' water maze (MWM), Actophotometer (INCO, Ambala, India), Cook's pole climbing (CPC) apparatus (INCO), electronic weighing balance, rotary evaporator, refrigerator, etc. were used in the current study.

Plant material

Amaranthus tricolor fresh leaves were collected from Dhamane (15.797209, 74.545502), Belagavi, Karnataka, India. The plant leaves were authenticated by Dr. Harsha Hegde, Scientist E, ICMR-National Institute of Traditional Medicine, Belagavi. Accession number: RMRC-1440. The herbarium was prepared and stored for further reference. The fresh leaves were washed under distilled water to remove adhered dirty material, shade dried, coarsely powdered, and subjected to maceration using 95% *v/v* ethanol for 7 days with occasional shaking. After maceration, the powder obtained was further subjected to Soxhlet extraction. The mixture was filtered and concentrated using a rotary evaporator under reduced pressure. The extract was stored in an airtight light resistance glass container for further use.

In vitro antioxidant assay

DPPH radical scavenging assay

The DPPH radical scavenging assay was analyzed by using the Blois method (Blois *et al*., 1958). A stock solution of 1 mg/ml plant extract, 1 mg/ml Ascorbic acid, 5.85 mg DPPH in 150 ml methanol was prepared and stored in the light resistance area. Briefly, 0.4 ml plant extract (50–800 µg/ml) and the standard drug were mixed with a 3.6 ml methanolic solution of DPPH (0.1 mM). An equal amount of methanol (0.4 ml) was used as a blank with a DPPH solution (3.6 ml). The above-mentioned mixture was vortexed for 1 minute and incubated for 30 minutes at 37°C. After incubation, the absorbance of each sample against methanol as blank by using the ELISA microplate reader at 517 nm was noted. The percentage of DPPH scavenging was calculated by using the following formula: DPPH inhibition (%) = $(A_{control} - A_{sample}/A_{control}) \times 100$. The results were reported as IC_{50} value.

In vitro AChE enzyme assay

AChE enzyme inhibition activity was performed according to Ellman's method (Ellman *et al*., 1961). The AChE enzyme of 6.67 U/ml^{-1} stock solution was prepared by dissolving it in 20 mM sodium Phosphate-buffered saline (PBS) (pH 7.6), and the solutions were stored at −80°C for further use. Acetylthiocholine iodide was used as a substrate. In a test tube, 1.7 ml of 50 mM Tris HCl buffer (pH 8.0) and 250 µl of different concentrations of plant extract (10, 20, 40, 80, 160, and 320 µg/ml) and donepezil (1, 2, 4, 8, 16, and 32 µg/ml) were added. To the above mixture, 10 µl AChE enzyme and 20 µl DTNB were added. The mixture was incubated for 15 minutes and 10 µl acetylthiocholine iodide was added; immediately, the absorbance was read at 412 nm for every 45 seconds for 3 minutes. The enzyme inhibition was calculated from the rate of change in absorbance with time.

$$\% \text{ Inhibition} = 100 - (\text{Change of sample Abs} / \text{Change of blank Abs}) \times 100$$

Experimental animals

Wistar rats (either sex, 180 ± 20 g) were acquired from Invivo Biosciences, Bengaluru, housed in a clean and transparent polypropylene cage, and were divided into six groups (n = 6). Animals were maintained under the 12 hours light-dark cycle at 23°C ± 2°C. Relative humidity was maintained at 45%−55%. The study protocol was reviewed and approved by the Institutional Animal Ethical Committee, KLE College of Pharmacy, Belagavi, Resolution No. KLECOP/CPCSEA – Reg.No.221/Po/Re/S/2000/

CPCSEA, Res.28-12/10/2019. The animal experiments were carried out as per CPCSEA guidelines.

Experimental procedure

The dose of the extract was selected based on the previously reported acute toxicity study reports. Ethanolic extract of leaves showed no mortality at 2,000 mg/kg, thus we selected the 1/2.5th, 1/5th, 1/10th doses of the LD_{50} value as therapeutic doses. The extract was administered orally using Carboxymethyl cellulose (CMC) as a suspending agent (Al-Dosari *et al*., 2010). Group I received food and water. Group II (negative control) received SCP (1 mg/kg/day i.p.) for 14 days (Zahra *et al*., 2015). Group III (positive control) received SCP (1 mg/kg/day; i.p.) with donepezil HCl (3 mg/kg/day; p.o.) for 14 days (Zahra *et al*., 2015). Group IV, V, and VI received SCP (1 mg/kg/day; i.p.) with *A. tricolor* L. extract of 200, 400, and 800 mg/kg/day, p.o., respectively, for 14 days. All the animals were subjected to exteroceptive behavioral models of memory on day 0, 7, and 14, and brain tissue samples for biochemical estimation.

***In vivo* screening models for memory (exteroceptive behavioral models)**

Elevated plus maze

Short-term memory was evaluated by using Bhuvanendran *et al*.'s (2018) method. The EPM consists of two open arms crossed over two closed arms having dimensions of 50 × 10 cm. Four arms were connected using a central square having 10 × 10 cm dimensions. During the assessment of memory via the EPM, the rats were individually placed at one end of the open arm facing away from the central stage (platform), and the time taken for the rat to move from an open arm to a closed arm (initial transfer latency) was recorded using a stopwatch. The animals were allowed to explore the maze for 120 seconds. If the animal did not enter the closed arms within 120 seconds, it was directed on the back into one of the closed arms and the transfer latency (TL) was given as 120 seconds. Later, the rat was allowed to explore the maze for 30 seconds to become familiar with the maze and was then returned to its cage. Each group was tested on day 0, 7, and 14.

Morris water maze

Escape latency time (ELT) was assessed using Ishola *et al*.'s (2018) method. It consists of a large circular water tank having 110 cm diameter and 60 cm height. It is made of black opaque polyvinyl chloride or hardboard coated with fiberglass or resin with a white surface and filled with water (26°C ± 2°C) to a depth of 30 cm. The floor of the circular tank was marked into four equal quadrants. In all trials, 2 cm below the surface of the water, a black round platform of 10 cm diameter was placed in a constant position. The cut-off time given to individual rats was 120 seconds. The time taken for the rat to locate the escape platform was noted. If the rat was unable to find/locate the platform within the cut-off time, it was directed to the stage (platform) and was permitted to stay on it for 10 seconds. Each group was tested on day 0, 7, and 14.

Actophotometer

Photoactometer is utilized to access locomotion in the experimental animals. The apparatus functions based on photoelectric cells that are connected to the circuit with a counter. A motion count was recorded when the beam of light falling on the photocell was cut off by the rat. The cut-off time given to individual rats was 120 seconds. Each rat's locomotor activity was noted. Each time the apparatus was wiped with 10% ethanol to avoid intervention due to animal odor. Each group was tested on day 0, 7, and 14 (Bhosale *et al*., 2011).

Cook's pole climbing

Cook's pole climbing method is a conditional avoidance/escape latency procedure used to screen Central nervous system (CNS) activity. The apparatus' floor acts as a source of shock. The experimental rats were subjected to training under a well-closed, electrical, grid-less, light chamber with a buzzer. This was carried routinely. Animals were subjected to training in such a way that the animal learned to climb the pole within 120 seconds. Each group was tested on day 0, 7, and 14 (Kadian *et al*., 2014).

Biochemical estimation

Cerebral cortex and hippocampus AChE and Butyrylcholinesterase (BChE) enzymes estimation

At the end of the study, the rats are decapitated; their brains are removed quickly and kept in ice-cold saline. The cerebral cortex and hippocampus were isolated, weighed, and homogenized in 0.1M (pH 8) PBS. Briefly, 0.4 ml of the homogenate was added to a test tube containing 2.6 ml PBS (0.1M, pH 8). Furthermore, 100 µl of DTNB reagent, followed by *t* 20 µl of acetylthiocholine iodide solution (butyrylthiocholine iodide for BChE enzyme estimation), was added. The absorbance was then noted at 412 nm for 5 minutes and change in absorbance per minute was also noted (Ellman *et al*., 1961). The "rate of moles of substrate hydrolyzed per minute per gram of tissue" was calculated by the following equation: $R = 5.74 \times 10^{-4}(\Delta A/Co)$. Where R = rate in moles of substrate hydrolyzed per minute per gram of tissue; ΔA= change in absorbance per minute; and Co= original concentration of tissue (mg/ml).

Estimation of amyloid beta ($A\beta_{1-42}$)

The capacity of rat brain $A\beta_{1-42}$ levels was estimated according to the sandwich ELISA kit for rat $A\beta_{1-42}$ manual (YH Bioresearch Laboratory, Shanghai, China) (Pattanashatti *et al*., 2017). The weighed brain tissue sample was homogenized in 0.1M PBS (pH 7.4) and centrifuged at 5,000 rpm. The supernatant was used for the detection of $A\beta_{1-42}$. All reagents, standard solutions, and samples were prepared as per instructions and methodology given in the manual. Reagents were kept at room temperature before use (assay solutions should be at room temperature). The standard curve analysis was run in parallel to the test samples. The absorbance was measured in the multi-scan spectrum spectrophotometer (Thermo Scientific, Waltham, MA, Multiskan GO) at 450 nm. All the readings were performed in triplicate.

***In vivo* antioxidants**

Rat brain was removed, weighed, and homogenized in cold 0.05M PBS (pH 7.4). Furthermore, homogenates were centrifuged at 10,000 rpm for 10 minutes at 4ºC (MPW-350 R, Korea) and post-mitochondrial supernatant was utilized for the

estimation of Total protein (TP) and LPO. The supernatant was further centrifuged at 15,000 rpm for 1 hour at 4ºC. The supernatant obtained was utilized to estimate superoxide dismutase (SOD) and GSH (Shalavadi *et al*., 2012).

Estimation of LPO in rat brain

Thiobarbituric acid reactive substances in the homogenate were estimated by the method described by Shalavadi *et al*. (2012). Briefly, 0.5 ml of 10% homogenate was incubated with 15% Trichloroacetic acid (TCA), 0.375% 2-Thiobarbituric acid (TBA), and 5 N HCl at 95ºC for 15 minutes; the mixture was cooled, centrifuged, and the absorbance of the supernatant was measured at 512 nm against an appropriate blank. The LPO levels were determined by using the following equation: $\varepsilon = 1.56 \times 105\ M^{-1}cm^{-1}$.

Estimation of reduced GSH

5% tissue homogenate was prepared in 20 mM Ethylenediaminetetraacetic acid (EDTA) (pH 4.7). Briefly, 100 µl of the homogenate was added to 1 ml 0.2 M Tris-EDTA buffer (pH 8.2) and 0.9 ml 20 Mm EDTA (pH 4.7), followed by 20 µl of Ellman's reagent (10 mmol/l DTNB in methanol), and incubated for 30 minutes. Samples were further centrifuged and the absorbance was noted at 412 nm.

Estimation of SOD

SOD activity was estimated based on the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH. Briefly, 25 µl of the supernatant obtained from the centrifuged brain homogenate was added to a mixture of 0.1 mM epinephrine in carbonate buffer (pH 10.2). The formation of adrenochrome was measured at 295 nm. The SOD activity (U/mg of protein) was calculated by using the standard plot.

Statistical analysis

The results were expressed as Mean ± SEM. The difference among the group was determined using one-way and two-way analyses of variance (ANOVA), followed by Tukey's multiple comparison test and Bonferroni's multi-comparison test as *post hoc* tests using GraphPad Prism software version 5.0.

RESULTS

Plant extraction

The percentage yield (*w/w*) of *A. tricolor* L. leaves' ethanolic extract by maceration method was found to be 5.2%, followed by the Soxhlet extraction method, which was found to be 2.3%.

***In vitro* antioxidant assay**

DPPH radical scavenging assay

Ascorbic acid was used as the reference compound. The IC_{50} value of ascorbic acid was found to be 67.12 µg/ml, whereas IC_{50} value of *A. tricolor* extract was found to be 236.42 µg/ml. The results suggest that the ascorbic acid has a more radical scavenging activity compared to the *A. tricolor* ethanolic extract. The IC_{50} value is shown in Figure 1a and Table 1.

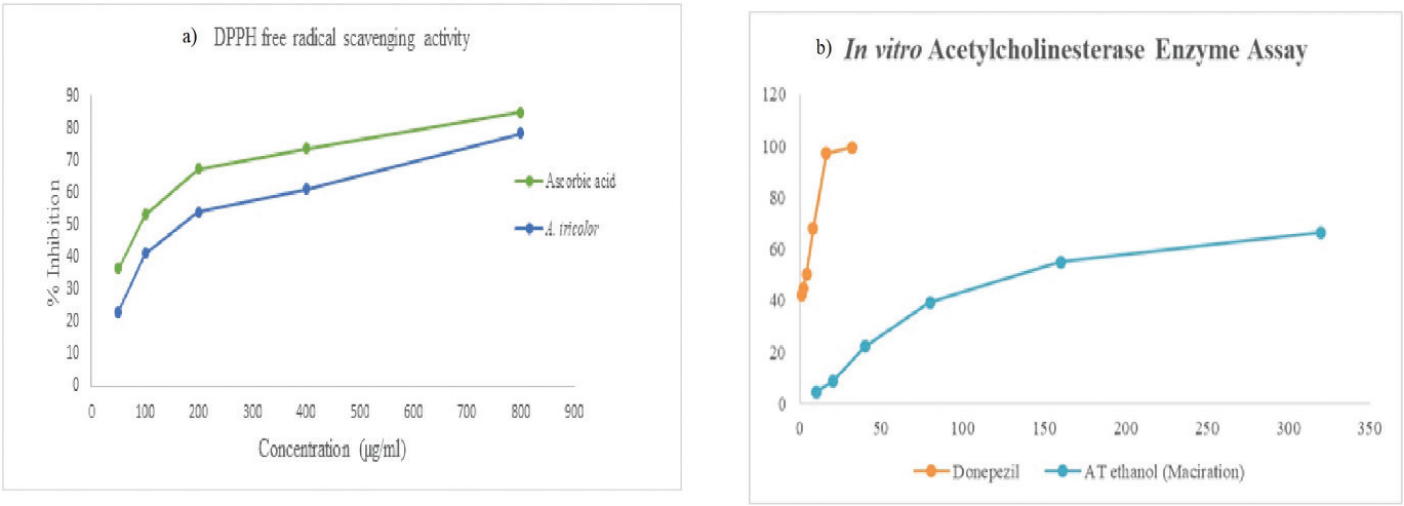

Figure 1. The effect of *A. tricolor* leaves' extract on (a) DPPH radical scavenging and (b) AChE enzyme.

Table 1. Effect of *A. tricolor* on free radical scavenging assay by using DPPH and AChE enzyme.

Test agent	DPPH radical scavenging assay		AChE enzyme assay	
	$y = mx + C$	IC_{50} (µg/ml)	$y = mx + C$	IC_{50} (µg/ml)
Ascorbic acid	$y = 0.0542x + 46.362\ R^2 = 0.773$	67.12177	–	–
A. tricolor	$y = 0.0649x + 34.656\ R^2 = 0.7847$	236.4253	$y = 0.1943x + 12.325\ R^2 = 0.8461$	193.9011837
Donepezil	–	–	$y = 1.9808x + 46.102\ R^2 = 0.8238$	1.967891761

In vitro AChE enzyme assay

The AChE enzyme inhibitory activity of *A. tricolor* extract and clinically proven drug donepezil are shown in Table 1. The IC_{50} value of *A. tricolor* was found to be 193.9 µg/ml and donepezil was found to be 1.96 µg/ml. This suggests that donepezil is 100 times more potent than *A. tricolor* extract. The IC_{50} value and standard calibration curve are shown in Figure 1b and Table 1, respectively.

In vivo studies

Effect of A. tricolor extract on TL using EPM

The rats were exposed to the EPM to assess the TL retrieval of memory. The TL of the SCP group was significantly decreased on day 7 ($p < 0.05$) and 14 ($p < 0.001$) compared to the normal group, whereas SCP with Donepezil 3 mg/kg was significantly increased in TL on day 7 ($p < 0.01$) and 14 ($p < 0.001$) compared to SCP alone (Group II). However, the administration of SCP with *Amaranthus tricolor* L. (AT) 400 mg/kg and SCP + AT 800 mg/kg was significantly increased TL on day 14 ($p < 0.01$ and $p < 0.001$, respectively) compared with SCP alone (Group II). The effect of *A. tricolor* on TL is shown in Figure 2a and Table 2a.

Effect of A. tricolor extract on ELT using MWM

The ELT was evaluated on day 0, 7, and 14 from the duration of the treatment period. Group II (SCP 1 mg/kg/day) showed significantly increased ELT on day 7 and 14 compared to the normal group ($p < 0.001$), whereas treatment of SCP with Donepezil 3 mg/kg significantly decreased ELT on day 7 and 14 ($p < 0.001$) compared to SCP alone. However, SCP with AT 200, 400, and 800 mg/kg/day showed a significant decrease in ELT on day 7 and 14 ($p < 0.001$) compared to SCP alone. The effect of *A. tricolor* on ELT is shown in Figure 2b and Table 2b.

Effect of A. tricolor extract on locomotor activity using Actophotometer

The locomotion of the SCP group was significantly decreased on day 7 and 14 ($p < 0.001$) compared to the normal group. Treatment of SCP with Donepezil 3 mg/kg and SCP with AT 400 and 800 mg/kg group was significant increased ($p < 0.001$) on

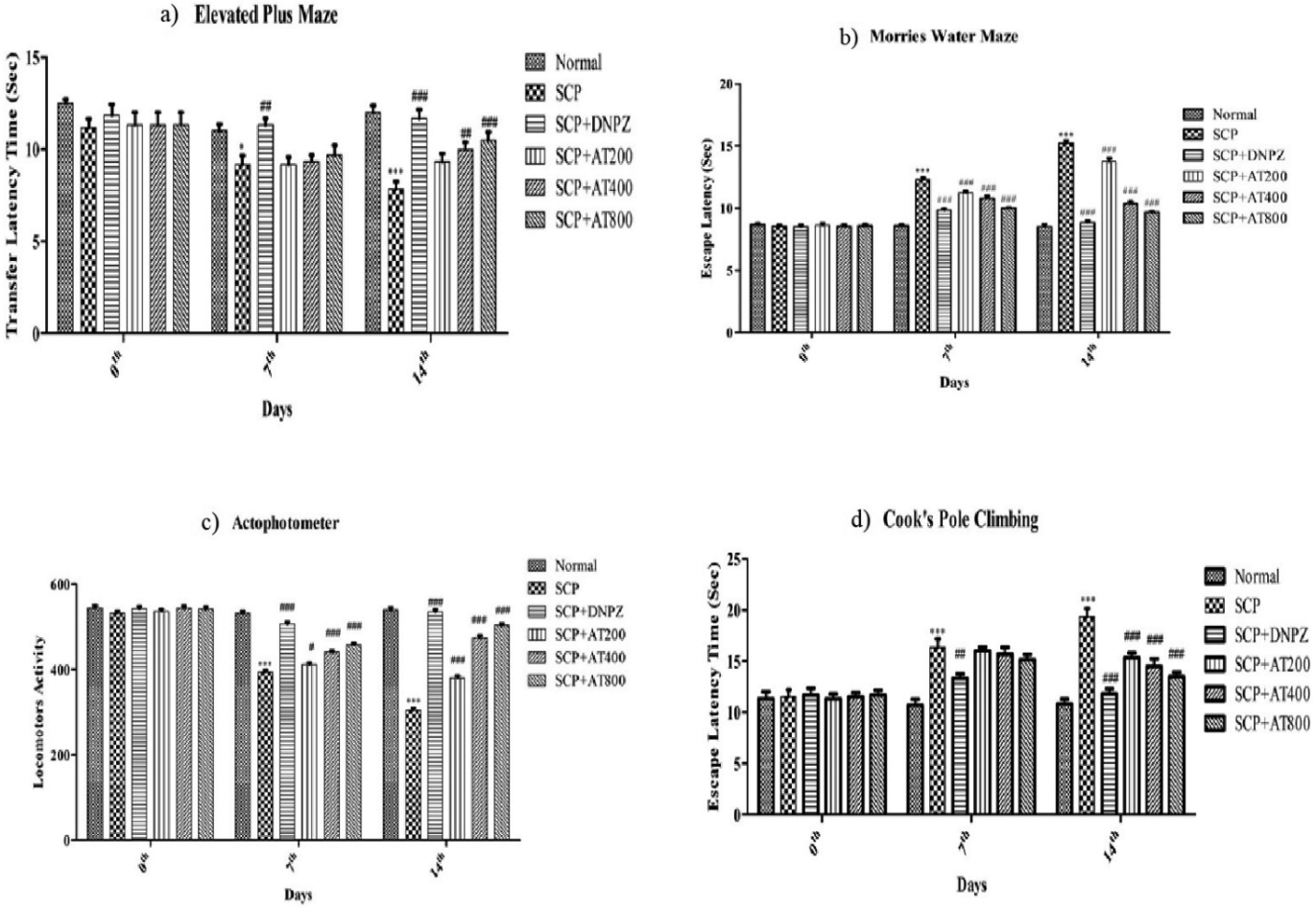

Figure 2. Effect of *A. tricolor* leaves' extract on exteroceptive behavioral models: (a) EPM, (b) MWM, (c) Actophotometer, and (d) CPC. Number of rats in each group is $n = 6$. All the data were analyzed by two-way ANOVA, followed by Bonferroni's multi-comparison test as *post hoc* test and expressed in Mean ± SEM. *$p < 0.05$; ***$p < 0.001$ compared to normal group, #$p < 0.05$; ##$p < 0.01$; ### $p < 0.001$ compared to SCP group.

Table 2a. Effect of *A. tricolor* on TL using EPM.

Treatment groups	TL		
	0th day	7th day	14th day
Normal	12.50 ± 0.224	11.00 ± 0.365	12.00 ± 0.365
SCP	11.16 ± 0.477	09.16 ± 0.477*	07.83 ± 0.401***
SCP+DNPZ	11.83 ± 0.601	11.33 ± 0.333$^{\#\#}$	11.66 ± 0.494$^{\#\#\#}$
SCP+AT200	11.33 ± 0.667	09.16 ± 0.401	09.33 ± 0.422
SCP+AT400	11.33 ± 0.667	09.33 ± 0.333	10.00 ± 0.365$^{\#\#}$
SCP+AT800	11.33 ± 0.667	09.67 ± 0.558	10.50 ± 0.428$^{\#\#\#}$

Values are expressed in mean ± SEM; (n = 6); Analyzed by two-way ANOVA, followed by Bonferroni's multi-comparison test as *post hoc* test.
$^{*}p < 0.05$; $^{***}p < 0.001$ compared to normal group, $\#\#p < 0.01$; $\#\#\#p < 0.001$ compared to SCP group.

Table 2b. Effect of *A. tricolor* on ELT using MWM.

Treatment groups	ELT		
	0th day	7th day	14th day
Normal	08.66 ± 0.124	08.58 ± 0.124	08.50 ± 0.214
SCP	08.54 ± 0.119	12.29 ± 0.176***	15.20 ± 0.218***
SCP + DNPZ	08.50 ± 0.171	09.83 ± 0.124$^{\#\#\#}$	08.83 ± 0.167$^{\#\#\#}$
SCP + AT200	08.62 ± 0.168	11.25 ± 0.129$^{\#\#\#}$	13.75 ± 0.266$^{\#\#\#}$
SCP + AT400	08.54 ± 0.164	10.79 ± 0.198$^{\#\#\#}$	10.37 ± 0.180$^{\#\#\#}$
SCP + AT800	08.58 ± 0.139	10.00 ± 0.065$^{\#\#\#}$	09.66 ± 0.053$^{\#\#\#}$

Values are expressed in mean ± SEM; (n = 6); Analyzed by two-way ANOVA, followed by Bonferroni's multi-comparison test as *post hoc* test.
$^{***}p < 0.001$ compared to normal group, $\#\#\#p < 0.001$ compared to SCP group.

Table 2c. Effect of *A. tricolor* on locomotion using Actophotometer.

Treatment groups	Locomotor Activity		
	0th day	7th day	14th day
Normal	544.33 ± 5.608	531.83 ± 5.023	539.50 ± 5.476
SCP	531.83 ± 5.023	393.00 ± 4.789***	303.83 ± 5.394***
SCP + DNPZ	542.00 ± 5.317	506.00 ± 5.317$^{\#\#\#}$	534.83 ± 5.689$^{\#\#\#}$
SCP + AT200	536.16 ± 5.102	411.16 ± 4.534$^{\#}$	379.83 ± 5.570$^{\#\#\#}$
SCP + AT400	543.66 ± 5.232	440.83 ± 4.061$^{\#\#\#}$	473.16 ± 5.357$^{\#\#\#}$
SCP + AT800	541.66 ± 5.136	458.00 ± 3.759$^{\#\#\#}$	503.16 ± 4.110$^{\#\#\#}$

Values are expressed in mean ± SEM; (n = 6); Analyzed by two-way ANOVA, followed by Bonferroni's multi-comparison test as *post hoc* test.
$^{***}p < 0.001$ compared to normal group, $\#p < 0.05$; $\#\#\#p < 0.001$ compared to SCP group.

Table 2d. Effect of *A. tricolor* on ELT using CPC.

Treatment groups	ELT		
	0th day	7th day	14th day
Normal	11.33 ± 0.667	10.66 ± 0.615	10.83 ± 0.477
SCP	11.50 ± 0.719	16.33 ± 0.843***	19.33 ± 0.803***
SCP + DNPZ	11.66 ± 0.667	13.33 ± 0.422$^{\#\#}$	11.83 ± 0.477$^{\#\#\#}$
SCP + AT200	11.33 ± 0.494	16.00 ± 0.365	15.33 ± 0.494$^{\#\#\#}$
SCP + AT400	11.50 ± 0.428	15.66 ± 0.667	14.50 ± 70.719$^{\#\#\#}$
SCP + AT800	11.66 ± 0.494	15.16 ± 0.477	13.50 ± 0.428$^{\#\#\#}$

Values are expressed in mean ± SEM; (n = 6); Analyzed by two-way ANOVA, followed by Bonferroni's multi-comparison test as *post hoc* test.
$^{***}p < 0.001$ compared to normal group, $\#\#p < 0.01$, $\#\#\#p < 0.001$ compared to SCP group.

day 7 and day 14 and SCP with AT 200 mg/kg showed increased locomotion on day 14 ($p < 0.001$) compared to SCP group. The effect of *A. tricolor* on locomotion is shown in Figure 2c and Table 2c.

Effect of A. tricolor extract on ELT using CPC apparatus

The ELT was evaluated on day 0, 7, and 14 of treatment using CPC apparatus. SCP 1 mg/kg/day group showed a significant increase ($p < 0.001$) in ELT on day 7 and 14 compared to the normal group. Treatment of SCP with Donepezil 3 mg/kg showed a significant decrease in ELT on day 7 ($p < 0.01$) and 14 ($p < 0.001$) compared to SCP alone. However, treatment of AT 200, 400, and 800 mg/kg showed a significant reduction in ELT on day 14 ($p < 0.001$) compared to SCP alone. The effect of *A. tricolor* on ELT is shown in Figure 2d and Table 2d.

In vivo biochemical studies

Effect of A. tricolor extract on AChE and BChE levels in rat hippocampus and cerebral cortex

The SCP group showed a significant increase in AChE enzyme activity compared to the normal group ($p < 0.001$) in the hippocampus and cerebral cortex. Treatment of SCP with Donepezil (DNPZ) showed significantly reduced levels of AChE activity in the hippocampus and cortex ($p < 0.001$). However, no significant change in AChE activity was observed in the hippocampus and cortex on the treatment of SCP with AT 200 mg/kg, whereas the treatment of SCP with AT 400 mg/kg showed significantly reduced AChE activity in the cortex ($p < 0.01$). Treatment of SCP with 800 mg/kg showed a significant change in AChE activity in the hippocampus ($p < 0.05$) and cortex ($p < 0.001$). The effect of *A. tricolor* on the hippocampus and cerebral cortex AChE enzyme activity is shown in Table 3 and Figure 3a and b.

The SCP group showed a significant decrease in BChE enzyme activity compared to the normal group ($p < 0.001$) in the hippocampus and cerebral cortex. However, a significant increase in BChE activity was observed in the rat hippocampus and cortex treated of SCP with DNPZ ($p < 0.01$) compared to the SCP group. BChE enzyme activity is shown in Table 3 and Figure 3c and d.

Effect of A. tricolor extract on Amyloid-beta (1–42) level in rat whole brain

The SCP group showed a significant increase in the level of Amyloid-beta (1–42) compared to the normal group($p < 0.001$) in the whole rat brain. However, the treatment of SCP with Donepezil showed a significant decrease in Amyloid beta (1–42) in the whole brain ($p < 0.001$) compared to SCP alone. However, the treatment of SCP with AT 400 mg/kg showed a significantly reduced level of Amyloid-beta (1–42) ($p < 0.01$) and SCP with AT 800 mg/kg ($p < 0.001$) compared to SCP alone. The effect of *A. tricolor* on rat brain Amyloid beta (1–42) level is shown in Table 4 and Figure 4a.

In vivo antioxidants

Effect of A. tricolor extract on MDA level in the brain

The SCP group showed a significantly increased level of MDA ($p < 0.001$) compared to the normal group. Treatment of SCP with DNPZ showed a significant decline in the MDA level (p

Table 3. Effect of *A. tricolor* on the hippocampus and cerebral cortex AChE and BChE enzymes.

Treatment groups	AChE enzyme activity (in moles × 10^{-6}/minutes/g of tissue)		BChE enzyme activity (in moles × 10^{-6}/minutes/g of tissue)	
	Hippocampus	Cerebral cortex	Hippocampus	Cerebral cortex
Normal	06.06 ± 0.251	01.55 ± 0.356	11.10 ± 0.321	08.97 ± 1.332
SCP	13.77 ± 0.688***	10.83 ± 0.240***	04.38 ± 0.656***	01.91 ± 0.141***
SCP + DNPZ	06.29 ± 1.297$^{\#\#\#}$	03.38 ± 0.85$^{\#\#\#}$	10.02 ± 1.746$^{\#\#}$	07.10 ± 0.325$^{\#\#}$
SCP + AT200	12.63 ± 0.664	08.72 ± 0.077	05.13 ± 0.242	02.70 ± 0.293
SCP + AT400	10.14 ± 1.141	06.82 ± 0.147$^{\#\#}$	06.32 ± 0.455	03.31 ± 0.797
SCP + AT800	08.98 ± 0.710$^{\#}$	05.08 ± 0.733$^{\#\#\#}$	07.72±0.313	03.57±0.211

Values are expressed in mean ± SEM: (n = 6); Analyzed by one-way ANOVA, followed by Tukey's multiple comparison test.
*** $p < 0.001$ compared to normal group, #$p < 0.05$; ##$p < 0.01$; ### $p < 0.001$ compared to SCP group.

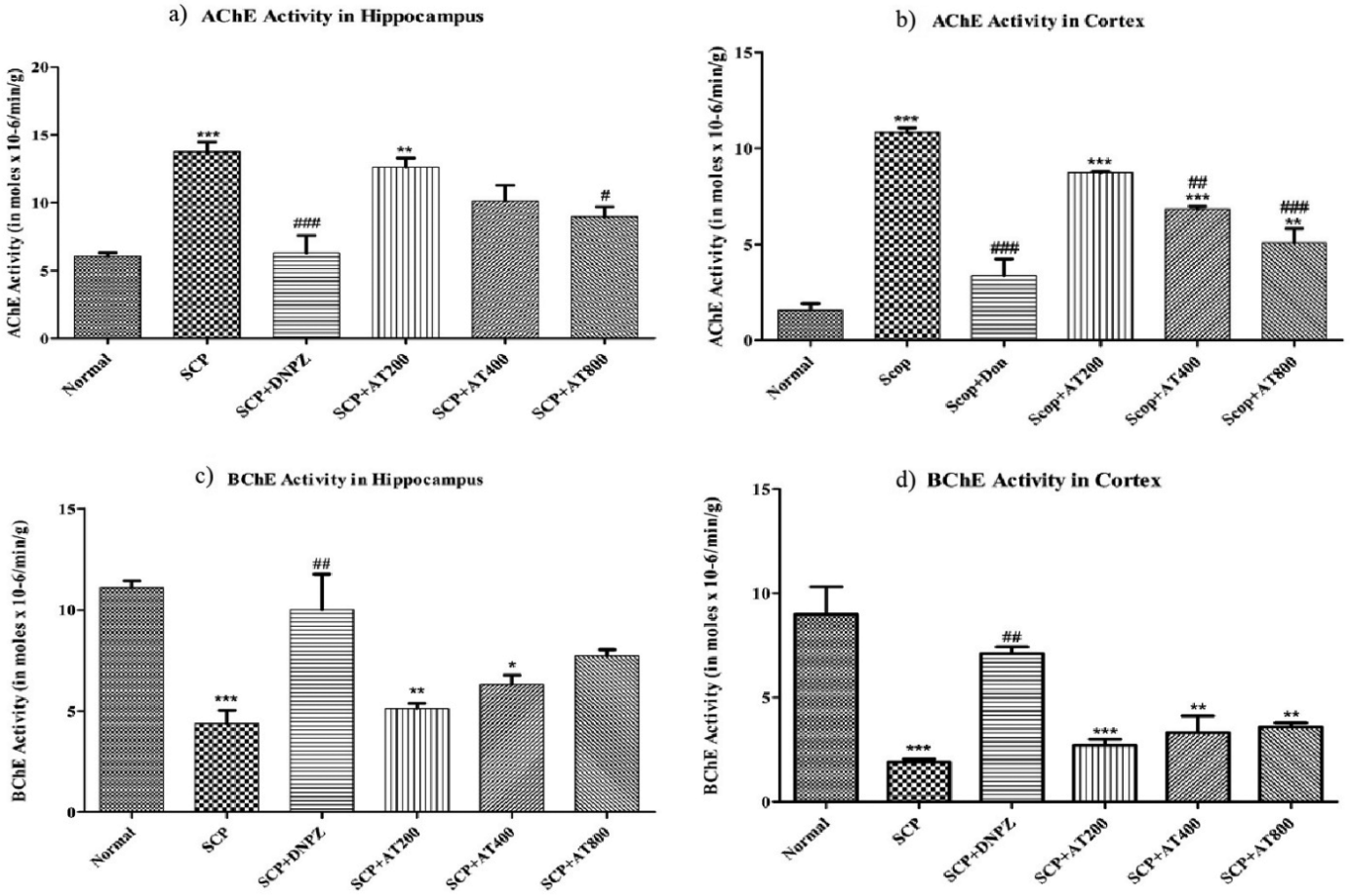

Figure 3. Effect of *A. tricolor* leaves; extract on hippocampus and cerebral cortex AChE and BChE levels. All the data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test and expressed in Mean ± SEM. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$ compared to normal group, #$p < 0.05$; ##$p < 0.01$; ### $p < 0.001$ compared to SCP group.

< 0.001) compared to the SCP group, whereas SCP with AT 800 mg/kg showed a significant decrease in the MDA level ($p < 0.01$) compared to SCP group. The effect of *A. tricolor* on the MDA level is shown in Figure 4b and Table 4.

Effect of A. tricolor extract on reduced GSH

The SCP group showed a significantly decreased level of GSH ($p < 0.001$) compared to the normal group. Treatment of SCP with the DNPZ group indicated a significant increase in the reduced GSH level ($p < 0.001$), whereas treatment of SCP with AT 400 mg/kg ($p < 0.01$) and SCP with AT 800 mg/kg ($p < 0.001$) showed a significant increase in GSH level. However, no significant change was observed in SCP with AT 200 mg/kg. The effect of *A. tricolor* on a reduced GSH level is shown in Figure 4c and Table 4.

Effect of A. tricolor extract on reduced SOD

The SCP group showed a significant decline in the SOD level ($p < 0.001$) compared to the normal group. Treatment of

Table 4. Effect of *A. tricolor* on amyloid-beta (1-42), LPO, GSH, and SDO on rat whole brain.

Treatment groups	Amyloid-beta (1–42) concentration in pg/ml	LPO (in nmoles of MDA/mg protein)	GSH (in µmoles/mg of protein)	SOD (units / mg of protein)
Normal	1.07 ± 0.049	07.00 ± 1.186	9.41 ± 0.657	288.1 ± 21.99
SCP	2.15 ± 0.155***	20.11 ± 1.341***	1.34 ± 0.166***	107.3 ± 05.83***
SCP + DNPZ	1.15 ± 0.043###	07.83 ± 1.106###	7.00 ± 0.423###	264.7 ± 28.83##
SCP + AT200	2.10 ± 0.049	17.13 ± 0.233	2.33 ± 0.332	131.7 ± 12.67
SCP + AT400	1.63 ± 0.021##	14.86 ± 1.398	4.14 ± 0.300##	185.8 ± 19.47
SCP + AT800	1.39 ± 0.026###	10.99 ± 1.070##	5.88 ± 0.335###	212.1 ± 28.65#

Values are expressed in mean ± SEM: (n = 6); Analyzed by one-way ANOVA, followed by Tukey's multiple comparison test.
***p < 0.001 compared to normal group, #p < 0.05; ##p < 0.01; ###p < 0.001 compared to SCP group.

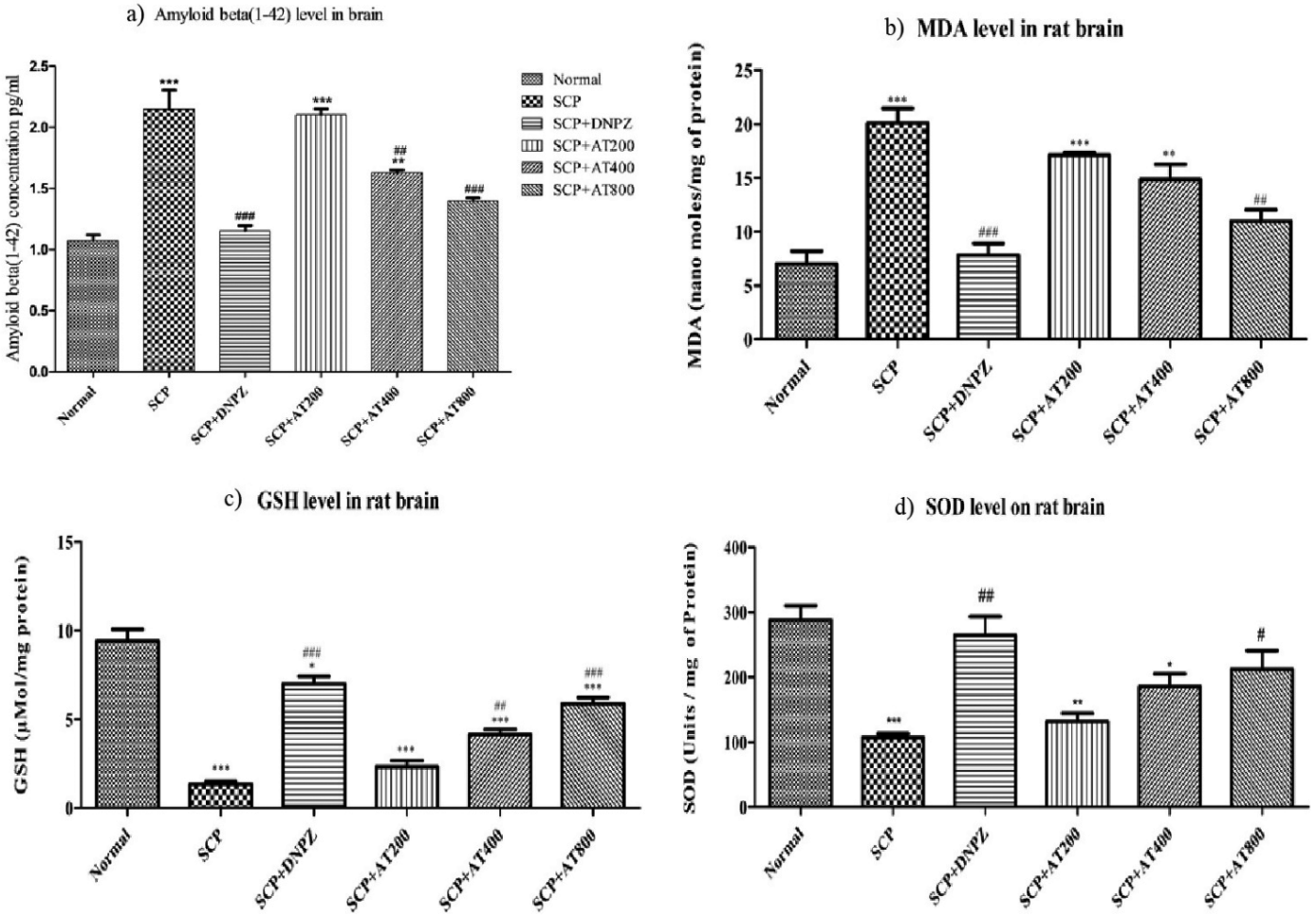

Figure 4. Effect of *A. tricolor* leaves' extract on (a) β–Amyloid ($A\beta_{1-42}$) (b) LPO /Malondialdehyde, (c) GSH, and (d) SOD. All the data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test and expressed in Mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared to normal group, #p < 0.05; ##p < 0.01; ### p < 0.001 compared to SCP group.

SCP with DNPZ and SCP with AT 800 mg/kg group indicated a significant increase in the reduced SOD level (p < 0.01 and p < 0.05, respectively) compared to SCP alone. The effect of *A. tricolor* on a reduced SOD level is shown in Figure 4d and Table 4.

DISCUSSION

Ayurveda is an ancient system of medicine that originated and was practiced in India since ages (Sheeja Malar *et al*., 2017). It is a holistic system that uses mostly herbal plants, vegetables, and minerals in prescriptions that play a crucial role in the treatment of various diseases because of their antioxidants properties and therapeutic effects. The primary central goal in neuroscience is the advancement of pharmacologically successful medicines which ease neurodegenerative issues. The high event of dementia, in AD, in old individuals gives solid inspiration to look and build up the new substances with the possibility to invert the deficit in

short and long-term memory. The current pharmacotherapy of AD includes the extreme utilization of conventional AChE inhibitors, i.e. donepezil, rivastigmine, etc., which is apparent to cause a verity of adverse effect and also lose the effect as the disease progresses (Waldemar *et al*., 2007; Mayo Clinic, 2018). *A. tricolor* is a leafy vegetable and has a high content of antioxidants and secondary metabolites. One of the studies conducted on *A. tricolor* L. showed a neuroprotective effect on gene expression of Receptor for advanced glycation endproducts (RAGE) during oxidative stress in SH-SY5Y cells. However, the potential of biochemicals regulated by the bioactive phytoconstituents from *A. tricolor* L. in the management of cognitive impairment has not been elucidated clearly.

SCP-induced memory impairment is one of the best models for assessment of drug candidates for their memory-enhancing potency by utilizing various models such as MWM, EPM, Actophotometer, CPC, and also for determining the level of ACh, BCh, MDA, and GSH in the hippocampus and cerebrum. In the current study, to assess the neuroprotective activity of *A. tricolor*, we administered SCP alone and in combination with 200, 400, and 800 mg/kg/day *A. tricolor* extract to the rats and examined their behavior by utilizing MWM, EPM, Actophotometer, and CPC, followed by determining the level of AChE and BChE enzyme activity and reduced antioxidant defence mechanism, i.e. reduced GSH and SOD levels and MDA level in the rat brain. A clinically approved drug "Donepezil" is used as an internal standard at 3 mg/kg/day dose.

An imbalance in the free radical and Reactive oxygen species (ROS) generation decreases the antioxidant defense mechanism leading to cell damage and inflammation (Manoharan *et al*., 2016). Furthermore, generation (production) of ROS lead to various unwanted reactions, including DNA damage, lipid abnormalities, and damage to the proteins (Khansari *et al*., 2009). Researchers demonstrated that plant extracts with higher flavonoid contents usually have the best free radical scavenging property (antioxidant activity). In the current study, the ethanolic extract exhibited strong DPPH scavenging activity and the extract treated in combination with the SCP significantly reversed the oxidative stress mediated by the SCP in rat brain by reducing the MDA level and increasing GSH and SOD levels ($p < 0.001$).

The memory-improving activity of *A. tricolor* against SCP-induced cognitive impairment in rats was examined by utilizing the MWM, EPM, CPC, Actophotometer, and by assessing the biochemical from the cerebrum. The short-term memory was tested utilizing MWM and CPC apparatus. In the current study, animals treated with SCP elevated the brain function by altering the ELT, TL, and locomotion. However, combined therapy of SCP and *A. tricolor* for 14 days showed a dose-dependent decline in the ELT and an increase in the TL and locomotion in rats. Among the selected doses, 800 mg/kg showed better activity compared to the clinically approved drug. The outcomes of the results propose that the administration of *A. tricolor* changes the neuronal performance of rats from day 7 to 14 and reduced the risk of development of AD by acting as an antioxidant and altering the disease-associated proteins and pathways. Furthermore, we examined the cholinergic function and beta-amyloid level of the brain to elucidate the memory improvement mechanism of *A. tricolor*. ACh and BCh neurotransmitters involved in the memory improvement, the concentration of ACh is reduced, and BCh is increased in AD due to rapid hydrolysis by AChE and BChE enzyme activity, respectively (Jung *et al*., 2009). In the current study, initially, we examined *A. tricolor* on the AChE enzyme and *A. tricolor* extract showed good inhibitory activity. The *in vivo* hippocampus and cerebral cortex AChE and BChE inhibitory role of *A. tricolor* was performed at the end of the study, and the resulting outcome showed the dose-dependent significant decline in AChE and BChE enzyme activity on the treatment of 200, 400, and 800 mg/kg of *A. tricolor* extract compared to SCP alone. The results suggest that the administration of 400 and 800 mg/kg of *A. tricolor* extract could be the potent inhibitory dose compared to Donepezil. Amyloid beta (1–42) level on the whole brain of the rat was examined using a sandwich ELISA kit. Results showed that *A. tricolor* 800 mg/kg significantly reduced the level of deposition of amyloid-beta in the brain compared to the SCP. Therefore, *A. tricolor* leaves' ethanolic extract could be the potent treatment regimen against oxidative stress-mediated AD.

CONCLUSION

In conclusion, the current study describes the molecular mechanism of *A. tricolor* on cognitive improvement via *in vitro* and *in vivo* studies. *Amaranthus tricolor* ameliorates the SCP-induced cognitive dysfunction and oxidative stress by the restoration of the cholinergic function, reducing APP deposition, by inhibiting the LPO, increasing GSH, and SOD function in the brain. The study also demonstrated the improvement of memory by utilizing MWM, EPM, Actophotometer, Cock's pole climbing behavioral models. Therefore, the present study helps the multipronged solutions to tackle AD in the future.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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