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Original Research Article

Indigofera tinctoria Linn (Fabaceae) attenuates cognitive and behavioral deficits in scopolamine-induced amnesic mice

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Abstract

Purpose: *To investigate the cognition-enhancing effects of aqueous extract of Indigofera tinctoria Linn (ITE, Fabaceae) in experimental amnesic mice.*
Methods: *Scopolamine (2 mg/kg, i.p.) was used to induce amnesia in mice. The cognitive-enhancing activity of the ITE (5, 10 and 20 µg/mL) was studied by passive avoidance response, elevated plus maze and Y-maze behavioral paradigm in normal and scopolamine-induced amnesic mice. Antioxidant activities were also determined based on the ability of ITE to inhibit lipid peroxide, superoxide and hydroxyl radicals.*
Results: *Scopolamine-induced cognitive deficits were significantly reversed by ITE ($p < 0.001$ at 20 mg/kg) in a dose-dependent fashion in all the behavioral paradigms tested. Furthermore, ITE dose-dependently scavenged lipid peroxide, superoxide and hydroxyl free radicals with 50 % inhibition concentration (IC_{50}) of 7.28 ± 0.37, 5.25 ± 0.28 and 7.62 ± 0.43 µg/mL, respectively.*
Conclusion: *ITE possesses cognitive-enhancing properties in amnesic mice due to its potent antioxidant action.*

Keywords: *Indigofera tinctoria, Scopolamine, Lipid peroxidation, Amnesia, Antioxidant, Cognition*

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INTRODUCTION

Cognition in a broad sense means the ability of the brain to encode, store and retrieve information [1]. The hippocampus contains the neural circuitry crucial for cognitive functions such as learning and memory, and intellectual aspects of mental functioning [2,3].Cognitive dysfunction, a major health problem in recent times can negatively affect learning and memory skills of individuals leading to the pathogenesis of various neurodegenerative disorders [4,5]. Mounting evidence suggests that oxidative damage in the central nervous system (CNS) is likely the cause of cognitive dysfunction. In particular, brain is believed to be vulnerable to oxidative stress due to a relatively high rate of oxygen free radical generation, with relatively reduced levels of antioxidative defenses [6,7].

Various antioxidant supplements and phytochemical components might be helpful for preserving brain functions and forestalling neurodegeneration [8]. Thus, agents that scavenge free radicals and regulate oxidative defense mechanisms may have potential in the mitigation of cognitive dysfunction seen in neurodegenerative disorders. *Indigofera tinctoria* Linn. (*I. tinctoria*, Fabaceae) a traditional medicinal herb has been widely used for several

years in Indian and Chinese system of Medicine for the treatment of epilepsy, nervous disorders and liver ailments [9]. Experimental evidence suggests that *I. tinctoria* possesses anti-diabetic, anti-inflammatory, hepatoprotective, anti-epileptic, anti-cancer and neuroprotective properties [9-11].

However, the cognitive-enhancing effect of *I. tinctoria* in amnesia conditions has not been studied. In the present study, we evaluated cognitive-enhancing effects of *I. tinctoria* extract in amnesia-induced experimental mice models. We also estimated the antioxidant defense potential of *I. tinctoria* extract.

EXPERIMENTAL

Chemicals

Scopolamine butyl bromide (SBB), 2 deoxy-D-ribose, thiobarbituric acid, nitro blue tetrazolium (NBT) and riboflavin were purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade. Stock solutions of all chemicals were prepared in distilled water and the dilutions were made fresh on the day of the experiment.

Plant material and preparation of *I. tinctoria* extract

Dried aerial part of *I. tinctoria* was obtained from Chemiloids, Vijayawada, India in the month of August 2008. The plant material was authenticated by Dr Vidyadar KN, a taxonomist at VJ's College of Pharmacy and Research Institute, Rajahmundry, India and a voucher specimen (no. ITE-VJ/08) was deposited in Pharmacognosy Department Herbarium of the same institute. The plant material (1 kg) was powdered and extracted with boiling water (5 L) for 30 min using a Soxhlet apparatus. The filtrate was evaporated under vacuum at < 70 °C in a vacuum dryer to give a final yield of 74.12 g. The extract was dissolved in sterile distilled water, filtered through a 0.22 µm filter and stored at -20 °C until use.

Animals

Swiss albino mice of either sex weighing 20 - 25 g were obtained from the animal house of National Institute of Nutrition, Hyderabad, India. The animals were housed in an air conditioned animal room at 23 ± 2 ^{o}C with 12/12 h light/dark photoperiod, and given free access to food and water. The animals were kept for seven days in laboratory for habituation. All animal experiments were performed under the guidelines of Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) [12] and the Institutional Animal Care and Use Committee, Vishnu Institute of Pharmaceutical Education and Research, India (Reg no. 1358/ERe/S/10/ CPCSEA).

Animal grouping and treatments

The mice were divided into six groups (n = 15), i.e., vehicle, scopolamine (2 mg/kg), ITE (20 mg/kg), scopolamine plus ITE (5 mg/kg), scopolamine plus ITE (10 mg/kg) and scopolamine plus ITE (20 mg/kg). Different doses of ITE were prepared freshly by dissolving in distilled water and administered for 15 days through oral gavage. Scopolamine was prepared in normal saline. On the 15th day, after 60 min of administration of doses, acquisition trail for passive avoidance and the transfer latency for elevated plus maze was recorded. On the next day, 60 min after administration of ITE at various doses, amnesia was induced by administration of scopolamine (2 mg/kg, i.p.) and animals were tested for their memory tests 30 min after scopolamine administration. For Y-maze, animals received scopolamine 30 min before performing the test.

Passive avoidance test

Experimental sessions were conducted using GEMINI active and passive avoidance system (San Diego Instruments, San Diego, CA, USA) connected to a computer as described earlier [13]. The instrument has a bright and a dark compartment with a computer-controlled guillotine door between them. Each animal was familiarized with the behavioral apparatus for 2-3 min 24 h before the training session. The delivery of electric shocks, the raising and lowering of the door in the device were automatically controlled by the computer. On the day of training, animals were placed in dark compartment with heads facing the wall. After an acclimatization period of 30 s the guillotine door automatically opens and the animal was subjected to a trial of 270 s. An entry into the dark compartment automatically shuts the door and the subject was punished with single low intensity foot shock of 0.5 mA for 5 s. The latency period at which the animals stepped into the dark from the bright compartment were recorded by the computer. The transfer from one compartment to another was recorded as transfer latency time (TLT) in seconds. The first trial is for acquisition and retention is tested by a second trial after 24 h. The shock is not delivered in the second trial. The criterion for learning was

taken as an increase in the TLT on second trial (retention) relative to first trial (acquisition).

Y-maze task

Immediate working memory performance was assessed by recording spontaneous alternation behavior during a single session in a Y-maze. Each arm was 40 cm long, 12 cm wide and 30 cm high. The procedure was similar as described previously [13]. Each mouse, naïve to the maze, was placed at the end of one arm and allowed to move freely through the maze during an 8 m session, and arm entries were counted. The series of arm entries was recorded visually by person blinded to treatment group and arm entry was considered to be completed when hind paws of the mouse were completely placed in the arm. Alternation was defined as successive entries into the three arms, on overlapping triplet sets. Alternation was calculated as the ratio of actual to possible alternation (defined as the total number of arm entries minus two), expressed a percentage.

Elevated plus maze test

The elevated plus maze serves as exteroceptive behavioral model for evaluating learning and memory in mice. The apparatus consists of two open arms (16 cm × 5 cm) and two covered arms (16 cm × 5 cm × 12 cm). The arms extend from a central platform (5 cm × 5 cm), and the maze is elevated to a height of 25 cm from the floor. On the first day, each animal was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was taken as the time taken by an animal to move into one of the covered arms with all its four legs. TL was recorded on the first day. If the animal did not enter into one of the covered arms within 90 s, it was gently pushed into one of the two covered arms, and TL was assigned as 90 s. The animal was allowed to explore the maze for 10 s and was then returned to its home cage. Memory retention was examined 24 h after the first day trial [14].

Lipid peroxidation inhibition assay

Swiss albino mice of either sex weighing between 20 - 25 g were sacrificed by spinal traction. Whole brain were excised and washed in ice-cold Tris- HCl buffer (0.1 M, pH 7.4), and cytosolic samples of liver and brain homogenate were prepared separately by using a tissue grinder (Thomas Scientific, NJ, USA) and centrifuging at 10,000 rpm for 30 min at 4 °C. The thiobarbituric acid substance (TBARS) assay was performed as described previously [15]. The reaction mixture (0.5 mL) containing mice brain homogenate (0.1 mL, 25 % w/v) in Tris-HCl buffer (40 mM, pH 7.0), KCl (30 mM), ferrous ion (0.16 mM), and ascorbic acid (0.06 mM) was incubated for 1 h at 37 °C in the presence and absence of various dilutions of ITE (1, 5, 10, 15 and 20 µg).

The incubation mixtures (0.4 mL) were treated with sodium dodecyl sulfate (8.1 %, 0.2 mL), thiobarbituric acid (0.8 %, 1.5 mL), and acetic acid (20 %, 1.5 mL, pH 3.5). The total volume was then made up to 4 mL with distilled water and kept in a water bath at 100 °C for 1 h. On cooling, 1 mL of distilled water and 5 mL of a mixture of n-butanol and pyridine (15:1 v/v) were added and vortexed. After centrifugation, the absorbance of the organic layer was measured spectrophotometrically at 532 nm (model UV-1601, Shimadzu). Inhibition of lipid peroxidation was determined by comparing the results of the test compound with those of the control, and expressed as a percentage. The 50 % inhibition values were derived from a plot of ITE (µg) against absorbance.

Superoxide radical assay

Superoxide scavenging activity of ITE was determined by the method described previously [15], which depends on light induced superoxide generation by riboflavin and the corresponding reduction of nitro blue tetrazolium (NBT). The assay mixture contained different concentrations of ITE (1, 5, 10, 15 and 20 µg) and EDTA (6 µM containing 3 µg NaCN), NBT (50 µM), riboflavin (2 µM) and phosphate buffer (58 mM, pH 7.8) to give a total volume of 3 mL. The tubes were uniformly illuminated for 15 min and thereafter the optical density (O.D) was measured at 560 nm using an UV Spectrophotometer (Shimadzu: UV- 1601). The 50 % inhibition values were derived from a plot of ITE tested (µg) against absorbance.

Hydroxyl radical assay

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system (Fenton reaction). The hydroxyl radical attacks deoxyribose which eventually results in TBARS formation. Briefly, the reaction mixture contains deoxyribose (2.8 mM), ferric chloride(0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbic acid (0.1 mM), phosphate buffer (20 mM, pH 7.4) and various dilutions of ITE (1, 5, 10, 15 and 20 µg) in a final volume of 1 mL. The reaction was

incubated for 1 h at 37 °C. Deoxyribose degradation was measured as TBARS by the method described earlier [15]. The 50 % inhibition values were derived from a plot of ITE (µg) against absorbance.

Statistical analysis

The results are expressed as mean ± SEM. Statistical analysis was performed with SAS statistical software (SAS Institute, Cray, NC, USA) using one-way analysis of variance, followed by Dunnett's multiple range tests. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of ITE on scopolamine-induced passive avoidance task in mice

As shown in Fig. 1, TLT was significantly increased on the second trial (221.5 ± 16.9 s) when compared with first trial (15.9 ± 1.3 s) in control-trained group, but in scopolamine-treated group there was no significant increase on second trial (17.05 ± 3.01 s) when compared with first trial (18.21 ± 3.4 s). ITE treated alone group (20 mg/kg) did not influence the TLT and was similar to control group. Pre-treatment with different doses of ITE (5, 10 and 20 mg/kg) for 15 days increased the TLT on second trial (105.52 ± 30.17 s for 5 mg/kg, 154.23 ± 16.72 s for 10 mg/kg and 185.46 ± 22.62 s for 20 mg/kg) when compared with first trial (21.82 ± 6.3 s, 20.2 ± 3.3 s and 23.81 ± 11.51 s), respectively in amnesic mice. The data indicate a dose-dependent effect, with 20 mg/kg showing the highest activity. There was no significant difference in TLT in the first trial among various groups.

Effect of ITE on scopolamine-induced Y-maze task in mice

As shown in Fig 2A, there was no significant difference observed in total arm entry of any group. However, administration of scopolamine (2 mg/kg, i.p) significantly ($p < 0.001$) decreased the percentage alternation (26.12 ± 1.35 s) when tested 30 min after the administration in mice Fig 2B). Pre-treatment with ITE at indicated (5, 10 and 20 mg/kg, p.o.) ameliorated the scopolamine-induced decreased percentage alternation in mice significantly ($p < 0.05$ at 5 and 10 mg/kg and $p < 0.01$ at 20 mg/kg). Results indicated that ITE exhibited a dose dependent activity with 20 mg/kg exhibiting the highest effect (49. 42 ± 0.95 s).

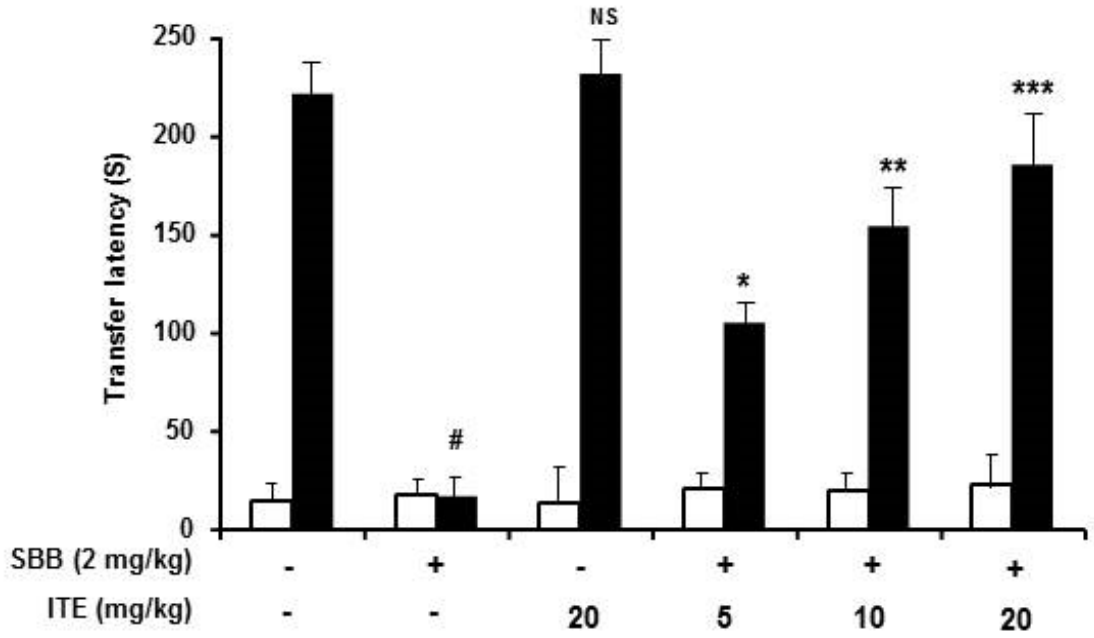

Figure 1: Effect of ITE on scopolamine-induced amnesia in passive avoidance test. Memory impairment was induced by scopolamine (2 mg/kg, *i.p.*) 5 min before acquisition (Trial I), and retention (Trial II) was carried out 24 h after scopolamine treatment. □: Acquisition, ■: Retention. $^{\#}p < 0.001$ compared with control group. NS: Not significant. $^{*}p < 0.05$, $^{**}p<0.01$ and $^{***}p < 0.001$ compared with scopolamine group. Data is expressed as mean ± S.E.M. (n = 5) using one-way ANOVA followed by Tuckey's Multiple comparison test in the Graph Pad Prism v5.01 software. SBB: Scopolamine butyl bromide; ITE: *I. tinctoria* aqueous extract

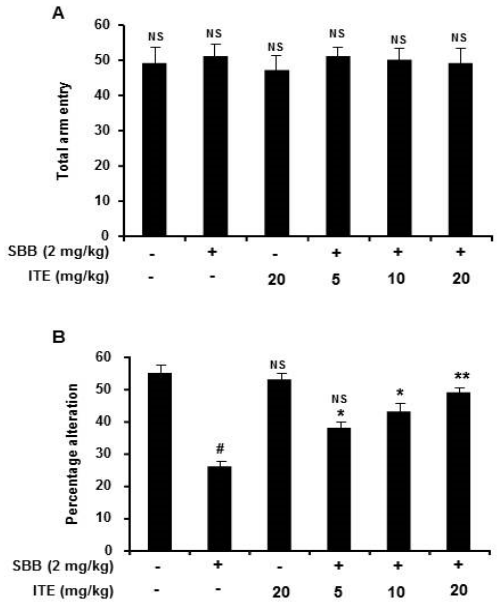

Figure 2: Effect of ITE on scopolamine-induced memory deficits in the Y-maze test. Spatial working memory was assessed using Y- Maze. Memory impairment was induced by scopolamine treatment (2 mg/kg, *i.p.*), and the test was carried out 30 min later. Total Arm entry (A) and percentage alternation (B) was evaluated. $^{\#}p < 0.001$ compared with control group. NS: Not significant. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with scopolamine group. Data is expressed as mean ± SEM (n = 5) using one-way ANOVA followed by Tuckey's Multiple comparison test in the Graph Pad Prism v5.01 software. SBB: Scopolamine butyl bromide; ITE: *I. tinctoria* aqueous extract

Effect of ITE on scopolamine-induced elevated plus maze test in mice

The scopolamine treated group showed a significant increase ($p < 0.05$) in transfer latency values in the acquisition (153.12 ± 10.82 s) as well as the retention period (127.64 ± 8.24 s) over those of the vehicle control mice, indicating impairment in learning and memory (Fig. 3). Pre-treatment with ITE at various doses (5, 10 and 20 mg/kg) for 15 days improved the memory in the elevated plus maze. ITE administration dose-dependently ($p < 0.5$, $p < 0.01$ and $p < 0.001$ at 5, 10 and 20 mg/mL respectively), caused a significant reduction in the acquisition and retention latency in scopolamine-treated group with 20 mg/mL showing the highest effect (acquisition, 75.62 ± 5.21 s and retention, 69.23 ± 4.21 s).

Effect of ITE on lipid peroxidation, superoxide and hydroxyl radical inhibition

ITE at various concentrations (1, 5, 10 and 20 µg) inhibited the lipid peroxide, superoxide and hydroxyl radicals in a dose-dependent manner (Fig. 4). The quantity of ITE (µg) needed for 50 % inhibition was 7.28 ± 0.3, 7.62 ± 0.4 and 5.25 ± 0.4µg/mL, respectively (Fig 4A - C).

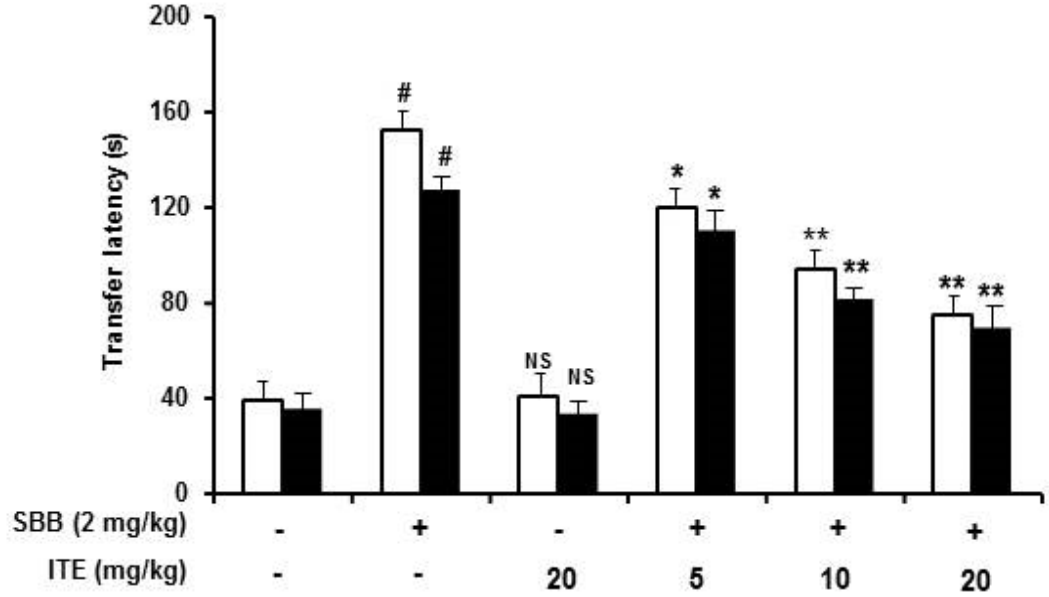

Figure 3: Effect of ITE on scopolamine-induced memory impairment in mice using the elevated plus maze test. Transfer latency in seconds was measured in first trial second trial. □: Acquisition, ■: Retention. $^{\#}p < 0.001$ compared with control group. NS: Not significant. $^{*}p<0.05$ and $^{**}p<0.01$ compared with scopolamine group. Data is expressed as mean ± S.E.M. (n=5) using one-way ANOVA followed by Tuckey's Multiple comparison test in the Graph Pad Prism v5.01 software. SBB: Scopolamine butyl bromide; ITE: *I. tinctoria* aqueous extract

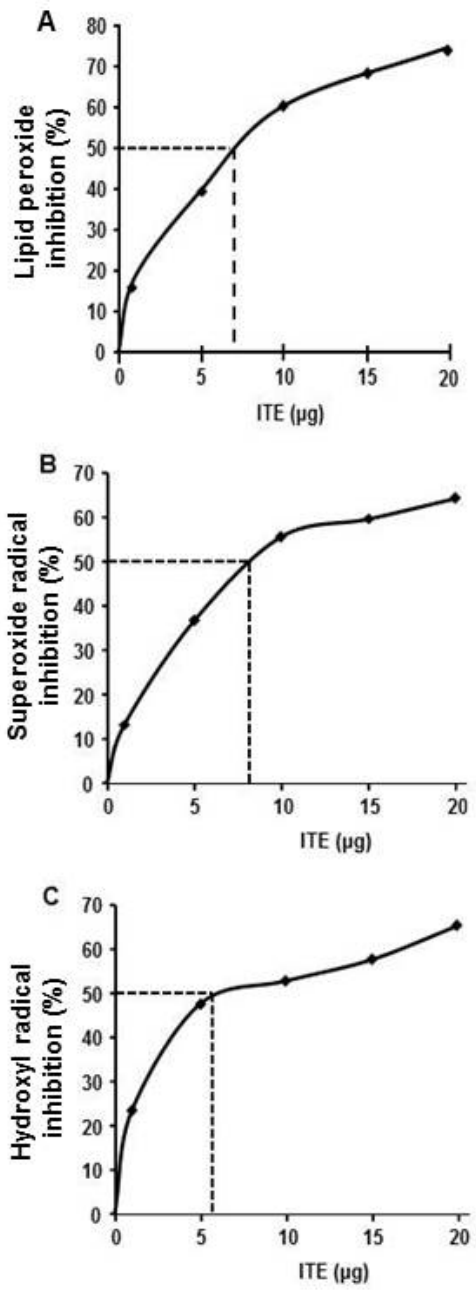

Figure 4: Effect of *ITE* on the inhibition of free radicals. A: Lipid peroxidation inhibition, B: Superoxide radical inhibition and C: Hydroxyl radical inhibition. (n=6). The dotted lines indicate the concentration required for 50 percent inhibition. ITE: *I. tinctoria* aqueous extract

DISCUSSION

In the present investigation ITE showed significant inhibition of cognitive deficits observed in scopolamine-induced amnesic mice. Furthermore, the extract exhibited potent antioxidant action by inhibiting lipid peroxide, superoxide and hydroxyl radicals. Cognitive impairment is one of the major health problems and a characteristic symptom of several neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD) [16]. Mounting evidence suggests that oxidative stress and free radicals might play a critical role in the process of cognitive impairments [17,18]. It is well documented that scopolamine induction impairs retrieval of memory in experimental animals and such amnesia is associated with a significant increase in oxidative stress [17]. Therefore, scopolamine-induced amnesia could be used as a valid model to study the role of antioxidant defense mechanisms in cognitive dysfunctions.

In the present study, the findings from passive avoidance test suggest that ITE at various doses (5, 10 and 20 mg/kg) showed increase in TLT in retention trial of passive avoidance. Further, cognitive behavior evaluated through Y-maze test suggests an improvement in percentage alteration behavior in ITE treated groups against scopolamine-induced amnesia in a dose-dependent manner. Furthermore, elevated plus maze test revealed that ITE showed protective effect in TL against scopolamine-induced amnesia dose-dependently. Moreover, the decrease in TL during retention period indicate the positive response of ITE in attenuating learning and memory deficits induced by scopolamine.

The major active constituents of *I. tinctoria* are the flavonoids, terpenoids, alkaloids, glycosides, methyl paraben, indigotine and indiruben [19]. Some of these constituents are well reported to possess antioxidant effects [20]. Further, it has been demonstrated that certain phenolic antioxidants form *I. tinctoria* attenuate neuronal cell death induced by oxidative stress in mouse PD models exhibiting neuroprotective properties based on antioxidant defense mechanisms [9,18]. In the present study, ITE scavenged the free radicals such as lipid peroxide, superoxide and hydroxyl radical indicating their usefulness in oxidative stress-related cognitive dysfunctions. Importantly, ITE scavenged the lipid peroxides in a concentration-dependent manner in brain homogenates of mice indicating that ITE might play a major role in mitigating CNS oxidative stress.

CONCLUSION

This study provides scientific support for the use of ITE in alleviating cognitive deficits induced by scopolamine in mice which might be due to its antioxidant action. Based on these present results and some traditional medicinal claims, ITE may be developed as a potential therapeutic

agent in attenuating cognitive dysfunctions seen in aging and neurodegenerative diseases.

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