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Research Article

Neuroprotective Effect of Green Synthesized Iron Oxide Nanoparticles Using Aqueous Extract of *Convolvulus Pluricaulis* Plant in The Management of Alzheimer's Disease

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ABSTRACT

To evaluate the neuroprotective effect of green synthesized iron oxide nanoparticles using aqueous extract of whole plant of *Convolvulus pluricaulis* (CPIO) in scopolamine induced amnesia model. CPIO were orally tested at the dose of 100 mg/kg, 200 mg/kg and 400 mg/kg for neuroprotective effect in scopolamine induced amnesia mice. In addition, neurobehavioral studies were carried out using elevated plus maze, Morris water maze to evaluate learning and memory in mice in normal and scopolamine induced amnesia mice. CPIO 400 mg/kg showed a significant improvement in learning and memory in the normal and scopolamine induced amnesia mice in exteroceptive models. Significant differences were observed in lipid peroxidation, catalase and acetylcholinesterase by 400 mg/kg of CPIO treated amnesic animals, when compared with untreated and scopolamine group animals. The highest dose of CPIO exhibited significant neuroprotective effect in normal and scopolamine induced amnesia mice. They also showed significant improvement in learning and memory in exteroceptive and interoceptive models and so might be of value in Alzheimer's treatment.

Keywords: Green synthesis, *Convolvulus pluricaulis*, iron oxide nanoparticles, neurobehavioral, neuroprotective, Alzheimer's

INTRODUCTION

Alzheimer's disease (AD) is the most common disorder of dementia, usually develops slowly and gradually gets worse, in which degeneration and death of the brain cells occur, causing a steady decline in memory and mental function[1]. It affects 13% of people over the age of 65 and 50% of people over the age of 85. AD is the 6th leading cause of death in elderly people of USA[2].

The use of herbal and natural extract in the treatment of AD has been increased tremendously due to their no or less side effects. *Convolvulus pluricaulis* belongs to family "Convolvulaceae" that is commonly referred to as Shankhapushpi. It is a perennial herb seems like morning glory and indigenous to India[3]. It is medicinally used for brain tonic, anti-convulsant, anti-anxiety, neurosis, fatigue, sedative[4].

Convolvulus pluricaulis is used in the treatment of hypertension, neurodegenerative diseases, ulcers, high blood pressure, epilepsy, vomiting, diabetes, sun stroke and bleeding[5]. In addition, the use of plant as brain tonic listed in Ayurvedic literature is also reported as a prominent memory improving drug. The chemical constituents include alkaloids, fatty acids, phenolics, glycosides, triterpenoids, and steroids[6].

The extracts and active compounds showed potential pharmacological effects like antidepressant[7], anti-stress, neuroprotective[8], antiamnesic[9], antioxidant[10], hypolipidemic, Immunomodulatory, anxiolytic[11], anticonvulsant[12], analgesic, antifungal, antibacterial, antidiabetic, antiulcer, and cardiovascular activity[13]. There are also reports on the synthesis of silver nanoparticles using various extracts of *Convolvulus pluricauslis*[14].

In this backdrop, the present study was aimed to synthesize iron oxide nanoparticles using *Convolvulus pluricaulis*, and evaluate their neuroprotective effect on scopolamine induced amnesia model.

MATERIALS AND METHODS

Materials

Convolvulus pluricaulis plant was purchased from authorized medicinal plant dealer in Hyderabad, India. Ferric chloride hexa-hydrate ($FeCl_3.6H_2O$), ferrous chloride tetra-hydrate ($FeCl_2.4H_2O$) were purchased from Sigma-Aldrich and sodium hydroxide (NaOH) was purchased from Merck and the remaining chemicals used in estimation of biochemical parameters were of analytical grade.

Preparation of aqueous extract of Convolvulus pluricaulis whole plant

The whole plant of *Convolvulus pluricaulis* was thoroughly washed in distilled water and dried in shade. The whole plant was made into coarse powder using maple mixer. The extraction was carried out by packing 250g of coarse powder in the soxhlet apparatus using distilled

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water as solvent for 18 hours. The aqueous extract was concentrated using rota evaporator (Heidolph) and crude extract has been stored at 4ºC for future use.

Synthesis of Convolvulus pluricaulis iron oxide nanoparticles

In this study, iron oxide nanoparticles were synthesized by co-precipitation of $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ (1:2 M) and the synthesis is based on the method followed with some modifications like addition of 5 mL of *Convolvulus pluricaulis* aqueous extract, immediately the yellowish colour of the mixture changed to reddish brown colour confirming the green synthesis of iron oxide nanoparticles [15](data not shown).

Experimental Animals

The experiments were performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India on Swiss albino mice of either sex procured from Gentox bioservices (Hyderabad) weighing 20-25g. The study was carried out after getting approval from the Institutional animal ethical committee (approval no: IAEC/1657/CMRCP/T2/PhD-16/47). A week before the neurobehavioural studies animals were acclimatized under standard laboratory conditions (12h light/dark cycle, air conditioned room at 25ºC ± 2ºC temperature) and fed pellet diet and water ad libitum.

Acute oral toxicity studies

Acute oral toxicity studies were carried out according to OECD-423 guidelines utilizing Swiss albino mice (n=3) of either sex and before dosing animals were fasted for 4 hr with free access to water only. The synthesized CPIO nanoparticles were administered orally at a dose of 2000mg/kg, no toxicity, behavioral changes and mortality was observed for 14 days[16].

Preparation of doses

Different doses of CPIO nanoparticles (100, 200, 400mg/kg) were selected based on the acute toxicity studies data. The CPIO are administered orally using distilled water. The standard drug (Donepezil 3mg/kg) was administered orally dissolving in distilled water. Amnesia was induced by administering Scopolamine hydrobromide (3mg/kg) intraperitoneally dissolving in water for injection. Every day fresh drug solutions were prepared before dosing.

Experimental design and treatment schedule

In the present study, the Swiss albino mice (male) were categorized into six groups with minimum of six animals in each and submitted to various interoceptive and exteroceptive models.

Group-I: Normal animals which received distilled water in the dose of 10ml/kg orally for 7 successive days.

Group-II: Animals received Scopolamine Hydrobromide 3mg/kg intraperitoneally(i.p) for 7 successive days.

Group-III: Animals received Donepezil 3mg/kg orally for 7 successive days and after 60 min of last dose administration, amnesia was induced by injecting Scopolamine (3mg/kg, i.p).

Group-IV, V and VI: The animals were pre-treated orally with synthesized CPIO 100 (group IV), 200 (group V), 400mg/kg (group VI) respectively for 7 consecutive days and after 60 min of the last dose administration, amnesia was induced by injecting scopolamine (3mg/kg, i.p) and retention memory was evaluated after 30min of amnesia induction using various exteroceptive models.

Neurobehavioral studies

Elevated plus maze (EPM)

Elevated plus maze served as the exteroceptive model to evaluate short term memory in mice. To study the acquisition and retention of short term memory, EPM was designed of two open arms (16x5cm) and two closed arms (16x5x12cm) extended from a central platform and the maze was raised to a height of 25cm from the floor. On the 7th day of dosing, after 30 min of scopolamine injection, each mice was placed at one end of the open arm facing away from the central platform, to measure the acquisition transfer latency time. Transfer latency was defined as the time taken by the animal to enter from open arm into the enclosed arm with all its four legs. The cutoff time was 90sec if the animal didn't enter the enclosed arm within 90sec.The mouse was allowed to explore the maze for 1min and placed back into its cage. Retention memory is examined after 24 hr i.e., on 8th day of dosing. Significant reduction in transfer latency time is an index of the improved memory[17].

Morris water maze (MWM)

Morris water maze served as the exteroceptive model to evaluate spatial learning and memory in mice. MWM consisted of a circular tank (40cm) filled with water and divided into four quadrants. In one quadrant a platform was hidden 1cm below the water level in the same position throughout the training period and the water was made opaque by adding small quantity of milk. The animal has to memorize the platform location based on various environmental clues as there won't be any clues in and outside the pool. On the 7th day of dosing, after 30 min of scopolamine injection, each mice was placed in the water tank facing the wall from one of the four quadrants and the animal was allowed to swim until it identifies the hidden platform to measure the acquisition escape latency time. Escape latency was defined as the time taken by the animal to climb on to the hidden platform. If the animal didn't identify the hidden platform within 60sec, it is gently placed on the hidden platform and allowed to stay on it for 15sec before placing back into its cage. Retention memory is examined after 24 hr i.e., on 8th day of dosing. Significant reduction in escape latency time is an index of the improved memory[18,19].

Biochemical estimations

Tissue collection and preparation of supernatant

The animals were sacrificed on the 8th day after neuro behavioral studies and the whole brain was excised out carefully and rinsed in 0.9% NaCl to remove blood and homogenized in phosphate buffer pH.7.4, 10%w/v. The homogenates were centrifuged at 15,375 x g at 4ºC for 20 min using Thermo Fischer micro cooling centrifuge.

The protein content in the supernatants was measured by the Lowry method using bovine serum albumin as standard[20].

Estimation of lipid peroxidation

500µl of supernatant was added to freshly prepared 1ml of 10% trichloro acetic acid. The mixture was placed on ice for 30 min with intermittent shaking and centrifuged at 5000 rpm for 10 min at 4ºC. To 1ml of supernatant, 250µl of freshly prepared 0.33% thiobarbituric acid was added and mixed well, boiled for 60min at 95ºC to estimate lipid peroxides. The tubes were immediately cooled by placing them under running tap water and the pink color developed was read at 532nm using Shimadzu UV-visible spectrophotometer[21].

Estimation of catalase

To 10µl of supernatant sample, 1ml of 30mM H_2O_2 in 0.05M mixed phosphate buffer, pH 7.0 was mixed and change in absorbance was monitored for 3min at 30sec intervals[22].

$K_{30} = (2.303/30)* \log (A_1/A_2)$

Where A_1 is the highest OD value and A_2 is the lowest OD value.

Estimation of whole brain AChE

The whole brain AChE activity was estimated following Elman's method with slight modifications. The animals were sacrificed by cervical dislocation and brains were dissected out, weighed and immediately placed in ice-cold saline. The weighed tissue was homogenized in 0.1 M phosphate buffer pH 8 (10% w/v), and centrifuged at 15,375 x g for 10 min. To 2.6 ml phosphate buffer (0.1 M, pH 8) and 100 µl of DTNB, 400 µl aliquot of the supernatant was added, mixed and absorbance was measured at 412 nm. The stable absorbance value was recorded as the basal reading. To this, 20µl of acetylthiocholine substrate was added and the change in OD was recorded for a period of 10 min at 2 min interval. The change in the absorbance per min was determined. AChE activity was calculated using the following equation[23].

$R = A/CO \times 5.74 \times 10^{-4}$

Where, R is the rate in moles of substrate hydrolyzed/ minute/ gram of brain tissue; A is the change in OD/ minute and CO is the original concentration of the tissue in mg/ml.

Statistical Analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Dunnett's test. Values are expressed as Mean ± SEM and $p<0.05$ was considered to be significant and $p>0.05$ was non-significant.

RESULTS AND DISCUSSION

Acute toxicity studies

The synthesized CPIO nanoparticles at a dose of 2000mg/kg were found to be safe upto 14 days and no mortality was observed during the treatment period. The doses selected for the study of neuroprotective effect in the management of Alzheimer's disease were 100, 200 and 400mg/kg per day.

Neurobehavioral studies

The mice showed higher transfer latency (TL) values on 7th day than on 8th day (after 24 hr) indicating impairment in learning and memory due to scopolamine treatment (Fig.1). Donepezil (3mg/kg,p.o) pretreatment for 7 days decreased TL ($p<0.001$) on 7th day and after 24 hrs i.e. on 8th day as compared to Scopolamine group, indicating improvement in both learning and memory. Scopolamine (3mg/kg, i.p) increased TL significantly ($p > 0.05$) in mice on 7th day and 8th day as compared to Normal group, indicating impairment of memory. CPIO (100, 200 and 400 mg/kg) decreased the TL on 7th day and 8th day in mice ($p<0.01$, $p<0.001$ and $p<0.001$ respectively) when compared to scopolamine group. Higher dose of CPIO (400 mg/kg) more significantly enhanced the learning and memory of animals by marked decrease in TL on 7th and 8th day when subjected to elevated plus maze tests. The higher doses of CPIO pretreatment for 7 days successively protected the mice against scopolamine induced amnesia.

The time taken by the experimental groups to escape on to the hidden platform in water maze was shown in (Fig.2). The intraperitoneal injection of scopolamine in mice increased the time to escape on to the platform significantly ($p>0.05$) when compared with the normal control group. Extremely significant ($p<0.001$) decrease in escape latency was indicated in the donepezil treated animals and CPIO treated groups on comparison with amnesia induced group. The low, intermediate and high dose treated groups (100, 200, 400mg/kg) showed an extremely significant ($p<0.001$) reduction in time required to climb on to the hidden platform.

Biochemical estimations

The mice pre-treated with Donepezil (3mg/kg; p.o) ($p<0.001$) and CPIO 400mg/kg ($p<0.001$) were shown extremely significant increase in antioxidant activity when compared to scopolamine group (Fig.3). Administration of Scopolamine (3mg/kg; i.p) increased the brain thiobarbituric acid reactive substance level which was considered as an increase in oxidation activity in brain when compared to control group of animals. Administration of CPIO 400mg/kg ($p<0.001$), 200 mg/kg ($p<0.01$) and 100 mg/kg ($p<0.05$) and Donepezil significantly reversed ($p<0.001$) Scopolamine induced increase in brain thiobarbituric acid reactive substance levels.

The mice pre-treated with Donepezil (3mg/kg; p.o) ($p<0.001$) and CPIO 400mg/kg ($p<0.001$) were shown extremely significant increase in antioxidant activity when comparative to Scopolamine group (Fig.4). Administration of Scopolamine (3mg/kg; i.p) decreased the brain catalase levels ($p<0.001$) which were considered as an increase in oxidation activity in brain when compared to control group of animals. The lowest dose CPIO 100mg/kg non significantly reduced the catalase levels. Administration of CPIO 400mg/kg ($p<0.001$), 200 mg/kg ($p<0.001$) and Donepezil significantly reversed ($p<0.001$) Scopolamine induced decrease in brain catalase levels.

To determine the effect of CPIO in neurotransmitter metabolic enzyme, AChE in the brain tissue was evaluated and shown in (Fig.5). Injection of Scopolamine 3mg/kg; i.p had significantly increased the AChE activity when compared with the normal control group. Among the treatment group, in the low dose (100 mg/kg) treated animals, there was a significant ($p<0.05$) reduction in the enzyme level when compared with the scopolamine group and the group of animals treated with intermediate dose

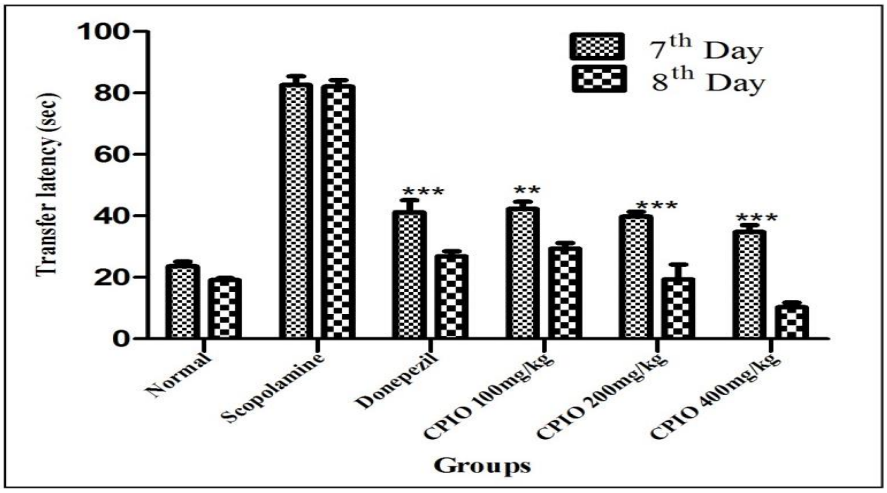

Figure 1: Effect of CPIO nanoparticles on scopolamine induced amnesia model in elevated plus maze task in mice. Each group consists of six animals (n =6). Values are expressed as Mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05 compared with scopolamine group are considered significant.

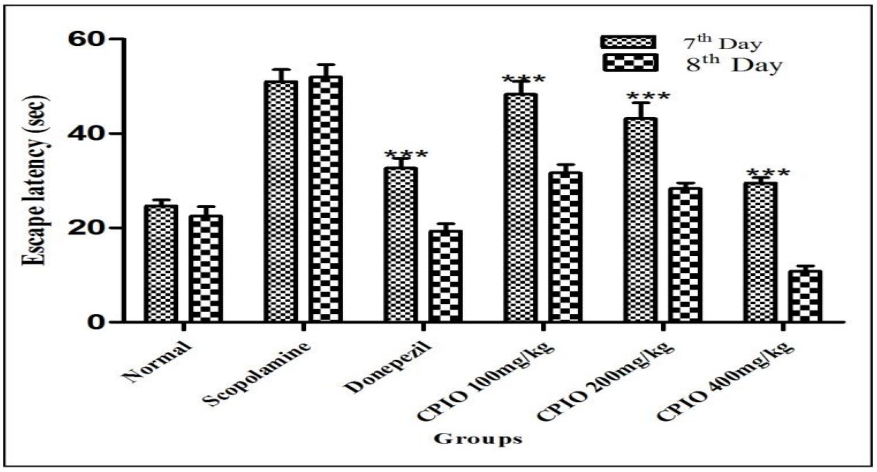

Figure 2: Effect of CPIO nanoparticles on scopolamine induced amnesia model in Morris water maze task in mice. Each group consists of six animals (n=6). Values are expressed as Mean ± SEM. ***p < 0.001 compared with scopolamine group are considered significant.

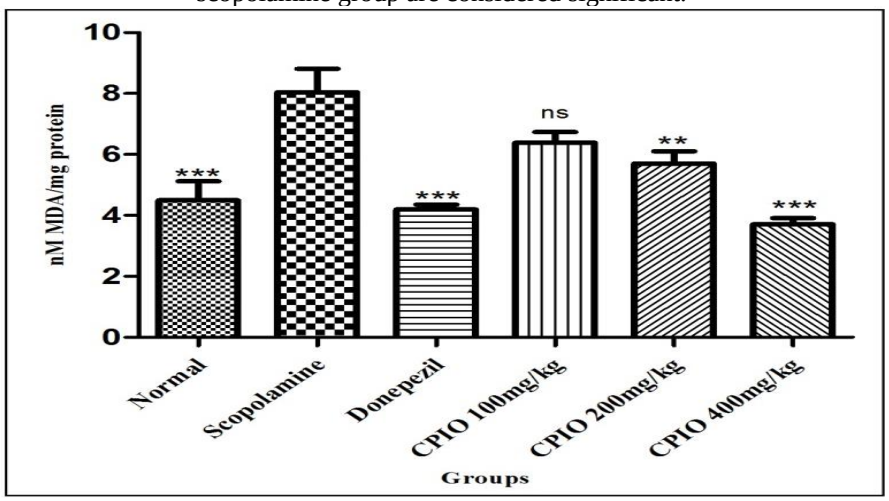

Figure 3: Effect of CPIO nanoparticles in scopolamine induced amnesia model on thiobarbituric reactive species. Each group consists of six animals (n =6). Values are expressed as Mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05 compared with scopolamine group are considered significant and 'ns' as non-significant.

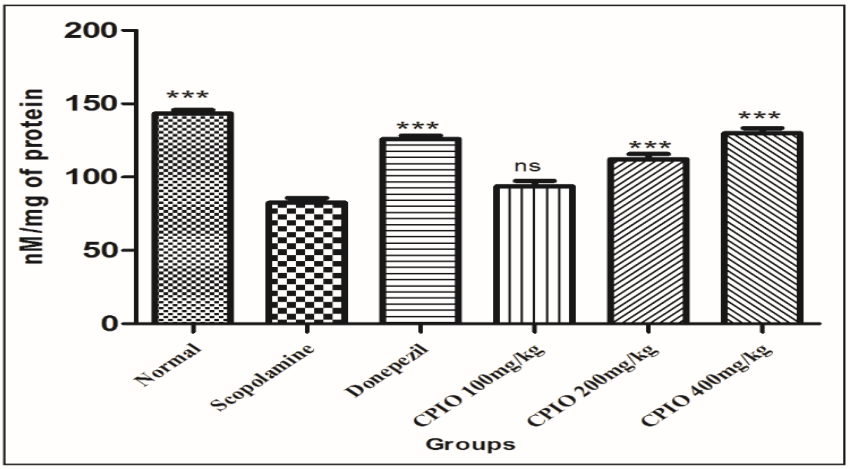

Figure 4: Effect of CPIO nanoparticles in scopolamine induced amnesia model on Catalase levels. Each group consists of six animals (n =6). Values are expressed as Mean ± SEM. ***p < 0.001, compared with scopolamine group are considered significant and 'ns' as non-significant.

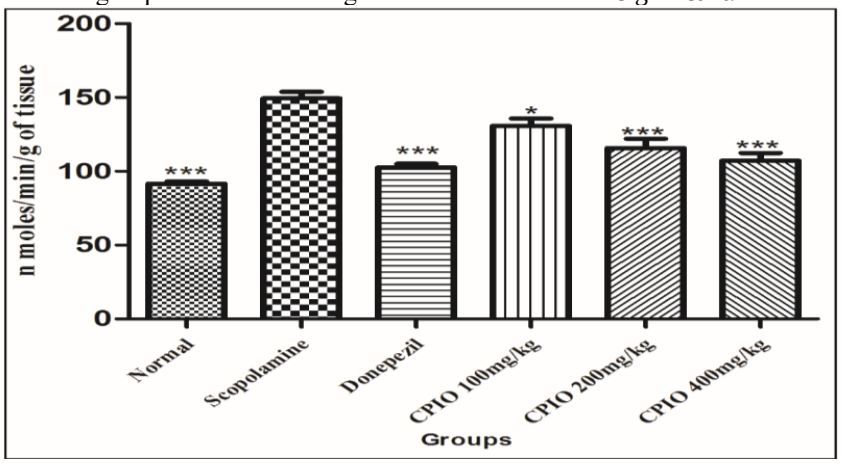

Figure 5: Effect of CPIO nanoparticles in scopolamine induced amnesia model on AChE levels. Each group consists of six animals (n =6). Values are expressed as Mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05 compared with scopolamine group are considered significant and 'ns' as non-significant.

(200 mg/kg) and high dose (400 mg/kg) showed extreme significance (p<0.001) and decrease in the enzyme level compared to the scopolamine group. The dose dependent effect of CPIO on comparing with the respective doses of IV with V, V with VI and IV with VI indicated a significance with p<0.05, p<0.001 and p<0.001 respectively.

DISCUSSION

The major clinical symptoms observed in Alzheimer's related dementia includes the development of multiple cognitive defects which interfere in daily and professional works. In Alzheimer's brain the brain dysfunctions start with neuronal injury, synaptic failure and neuronal death leading to memory impairment. Functional behavior of brain including learning and memory was found to be associated with cholinergic system and acetylcholine levels in the brain of humans and animals[24,25]. So different strategies have adopted to improve cholinergic transmission, by increasing acetylcholine synthesis, pre-synaptic acetylcholine release, employing cholinesterase inhibitors which increases the learning and memory[26,27].

Despite the availability of various treatment strategies till the severity and prevalence of this disease are not yet under control. Hence, in the present study nanoparticles synthesized using herbal extracts containing phytochemicals are being used in the management of Alzheimer's disease.

Scopolamine, is a nonselective muscarinic antagonist that blocks cholinergic signaling and produce memory and cognitive dysfunctions and subsequently causes impairment in learning and memory[28,29]. It also produces amnesic effect in mice and rat model of cognition[30,31].

Scopolamine has been found to increase oxidative stress and impair the anti-oxidative defense system by producing oxygen free radicals responsible for the development of Alzheimer's disease[32,33]. In the present study, this scopolamine significantly increased the thiobarbituric acid reactive substances and decreased the catalase enzyme levels[34,35]. The administration of CPIO nanoparticles in 3 doses (100, 200 and 400 mg/kg) for 7 successive days to mice not only decreased oxidative stress but also prevented the Scopolamine-induced rise in oxidative damage as indicated by the reduced thiobarbituric acid reactive

substances and increased catalase levels as compared to respective control animals.

In the present study, CPIO nanoparticles administered improved learning and memory in both exteroceptive and interoceptive behavior models. The treatment of CPIO and scopolamine showed a significant increase in the cognitive performance assessed by elevated plus maze, water Morris maze and decreased activity of AChE in brains of mice. It is expected that decreased AChE activity may enhance cholinergic activity by raising acetylcholine level, thereby enhancing cognitive functions. In accordance, higher AChE inhibition does not necessarily indicate better cognitive performance and the finding denotes that there is an optimal balance between cholinergic transmission and cognitive performance. No mortality was observed following oral administration of CPIO even with higher dose (2000 mg/kg, p.o.). All the doses of CPIO had no toxic effect on the normal behavior of the mouse.

CONCLUSION

The present study demonstrated that the iron oxide nanoparticles synthesized using Convolvulus pluricaulis aqueous extract has shown promising memory enhancing effects due to its anti-oxidant property at all doses but the highest dose have shown more potential effect as a standard drug (Donepezil) against a scopolamine induced cognitive dysfunction in mice. Moreover, this potent neuroprotective characteristic is well supported by neurochemical findings. Hence, CPIO nanoparticles could be useful in conditions associated with neurodegenerative disorders of Alzheimer's type. Further studies are required to explore the molecular mechanism of neuroprotection of these iron oxide nanoparticles synthesized using *Convolvulus pluricaulis* aqueous extract.

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