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Original Article

Neuroprotective Effect of Pirfenidone on Scopolamine Induced Cognitive Impairment and Oxidative Stress

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Abstract

Dementia is a decline of cognitive functions, Studies on Alzheimer's have led to major revaluation of concept such as 'Neuroinflammation'. Glial- derived proinflammatory cytokines set a spectrum of signaling events which influence in neurological disease and disorder. Pirfenidone has well-established antifibrotic and anti-inflammatory,It modulates a variety of cytokines and suppresses translation of TNF-α. **Materials and Methods:** In the present study, the effects of Pirfenidone (anti fibrotic) on scopolamine-induced learning and memory impairments in mice were investigated. Morris water maze test, Locomotor activity and elevated plus mazewere conducted to evaluate the learning and memory parameters. Various biochemical parameters such as TBARS assay, catalase activity were also assessed. **Results:** The present study demonstrates that Pirfenidone had potential therapeutic effects on improving the antiamnesic activity in mice through inhibiting lipid peroxidation, augmenting endogenous antioxidant enzymes and decreasing proinflammatory cytokines. **Conclusion:** The memory enhancing capacity of the drug was very significant when compared to disease control ($P<0.001$).

Introduction

Dementia is an umbrella term describing a variety of diseases and conditions that develop when nerve cells in the brain die or no longer function normally. The death or malfunction of neurons causes changes in one's memory, behaviour and ability to think clearly (1). Alzheimer's disease is the most common type of dementia. Patients with dementia are unable to generate coherent speech or understand spoken or written languages. They have inability to recognise or identify objects (agnosia) and have difficulties in executing motor activities (apraxia). But the most common phrase Apraxia and Agnosia mostly occur later in course of illness (2).

Both on cellular and clinical levels neuroinflammation plays active role in the pathophysiology of Alzheimer's Disease leading to dementia (7). Chronic inflammation precipitates sustained release of the various proinflammatory cytokines such as IL-1, IL-2, IL-1β, IL-6, and TNF-α,chemokines, pentraxins and prostaglandins which leads to dysfunctioning of neuronal cell.

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Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a small molecule that inhibits progression of fibrosis in vivo in a variety of animal models of lung (8) kidney (9), hepatic (10) and cardiac fibrosis (11, 12). In vitro studies have shown that pirfenidone inhibits proliferation and/or activation of a wide range of cell types including human lung fibroblasts (13), human myometrial and leiomyoma cells, human Tenon's fibroblasts (14), human T cells, rat hepatic stellate cells (15), and rat renal fibroblasts. In addition, pirfenidone modulates a variety of cytokines, and it has been shown that it decreases levels of intercellular adhesion molecule-1 in cultured human synovial fibroblasts, inhibits heat shock protein 47 expression in human lung fibroblasts (16), down-regulates TGF-β in human Tenon's fibroblasts (17), and suppresses translation of TNF-α in a murine macrophage-like cell line.

Understanding of inflammation in AD brain will open more targets for its therapy. Focusing on the integrity of the cellular players and the molecular pathways which causes glial activation may provide more targeted treatments that specifically resolve the harmful aspects of neuroinflammation while retaining its innate benefit. However, the present study has been designed to study the effect of pirfenidone in scopolamine induced cognitive impairment.

Material and Methods

Experimental animals

Swiss albino mice, weighing 20-30 grams were employed in the present study (procured from Central Research Institute (CRI), Kasauli). They were maintained on standard laboratory diet (Aashirwad feeds Ltd., Chandigarh, India) and tap water *ad libitum*. They were housed in the animal house of Rayat and Bahra Institute of Pharmacy (RBIP), Sahauran and were exposed to natural photoperiod. The experiments were conducted in a semi sound proof laboratory between 10:00 am to 5:00 pm. The experimental protocol of the study was duly approved by Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Reg. No. 1380/a/10/CPCSEA).

Drugs and chemicals

All the drug solutions were freshly prepared before use. Pirfenidone was a purchased from Cipla Ltd., Baddi. Donepezil was purchased from Cipla Ltd. Scopolamine was purchased from Sigma-Aldrich Cooperation, India. All the reagents used in this study were of analytical grade. All the drug solutions were freshly prepared before use. Scopolamine and donepezil were dissolved in normal saline (0.9% NaCl). Pirfenidone was dissolved in CMC (0.05% in 10ml). Scopolamine, CMC were injected intraperitoneally (i.p.). Donepezil and Pirfenidone were administered orally with the help of an oral tube (cannula).

Induction of experimental memory impairment by i.p administration of Scopolamine in mice:

Scopolamine (0.75 mg/kg), a cholinergic muscarinic receptor antagonist, was used for memory impairment. It was dissolved in normal saline (0.9% NaCl) and administered intraperitoneally (i.p) 1 hour after drug administration (donepezil (5 mg/kg, p.o) and pirfenidone (200 mg/kg, p.o) administration in all groups except vehicle control. Mice were subjected to behavioral testing from day 1 to day 5, five minutes after scopolamine injection.

Assessment of learning and memory using morris water maze (MWM):

The MWM test was employed to assess the learning and memory of the animals (Morris, 1984; Sharma and Singh, 2010). It is a swimming based model where the animal learns to escape on to a hidden platform. It consisted of a large circular pool (120 cm in diameter, 50 cm in height, filled to a depth of 30 cm with water maintained at 26±2°C). The water was made opaque with white colour non-toxic dye. The tank was divided into four equal quadrants with the help of two threads fixed at right angles to each other on the rim of the pool. A submerged platform (10 cm^2), painted in white, was placed inside the target quadrants of this pool, 1 cm below the surface of water. The position of platform was kept unaltered

throughout the training session. From day 1 to day 5, each animal was subjected to four consecutive training trails on each day with inter trial gap of 5 minutes, The trials were conducted after 10 minutes of drugs (Donepezil 5 mg/kg, p.o and Pirfenidone 200 mg/kg, p.o) administration for all the four consecutive days. The mouse was gently placed in the water between quadrants, facing the wall of pool with the drop location changing for each trial, and allowed 90 s to locate submerged platform. Day 4 escape latency time (ELT) to locate the hidden platform in water maze was noted as an index of acquisition or learning. The starting position was changed with each exposure as mentioned below and the target quadrant (Q4) remained constant throughout the training period.

Day1 Q1 Q2 Q3 Q4

Day2 Q2 Q3 Q4 Q1

Day3 Q3 Q4 Q1 Q2

Day4 Q4 Q1 Q2 Q3

During Probe trial on day 5, the platform was removed and each mouse was allowed to explore the pool for 60 s. The mean time spent in all four quadrants was noted. The mean time spent by the animal in the target quadrant searching for the hidden platform was noted as an index of retrieval or memory. Care was taken regarding the relative location of the water maze with respect to other objects in the laboratory so that prominent visual clues were not disturbed during the total duration of study. All of the trials were completed between 10:00 am and 5:00 pm. (Fig. 1)

Assessment of memory using elevated plus maze (EPM):

Transfer latency (TL) of each animal is measured by employing the elevated plus maze test. The plus-maze consists of two open (16×5 cm^2) and two closed (16×5×12 cm^3) arms, connected by a central platform of 5×5 cm^2. The apparatus has to be elevated to a height of 25 cm above the floor. A fine line has to be drawn in the middle of the floor of each closed arm. All the animals were then given a single trial on the plus-maze. Each mouse has to be individually placed at the end of an open arm facing away from the central platform of the maze. TL is then taken as the time taken by the mouse to move from an open arm to any one of the closed arms with all its four legs crossing the middle line. In case, the animal did not enter the closed arm within 90 seconds it is gently pushed into the closed arm and a transfer latency of 90 seconds has to be assigned to it.After an interval of 24 hours each animal is again subjected to elevated plus-maze test. TL measured on plus-maze on day 4 serves as an index of learning and acquisition (basal TL), whereas TL on day 5 serves as an index of retrieval and memory (final TL) (17). (Fig. 2)

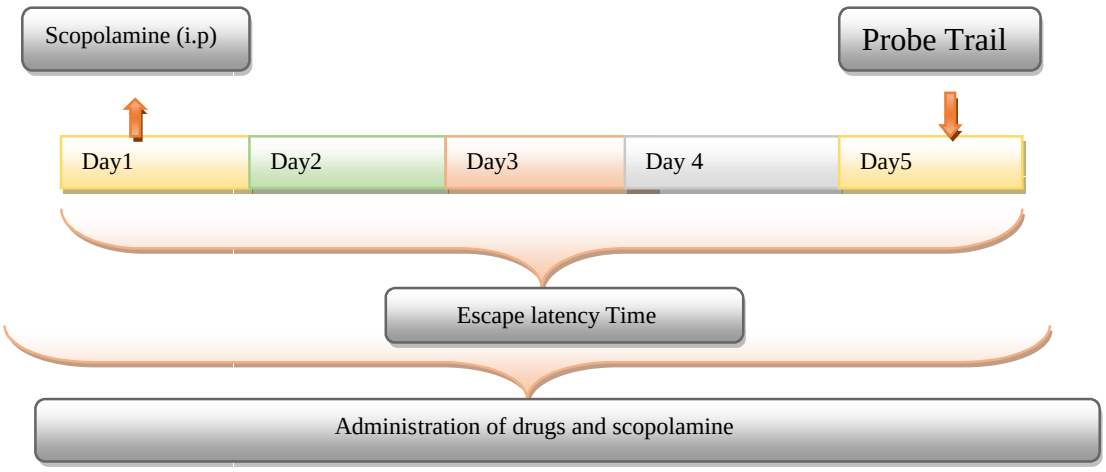


Fig. 1 : Diagrammatic representation of experimental protocol for drug administration and behavioral study (Morris water maze).

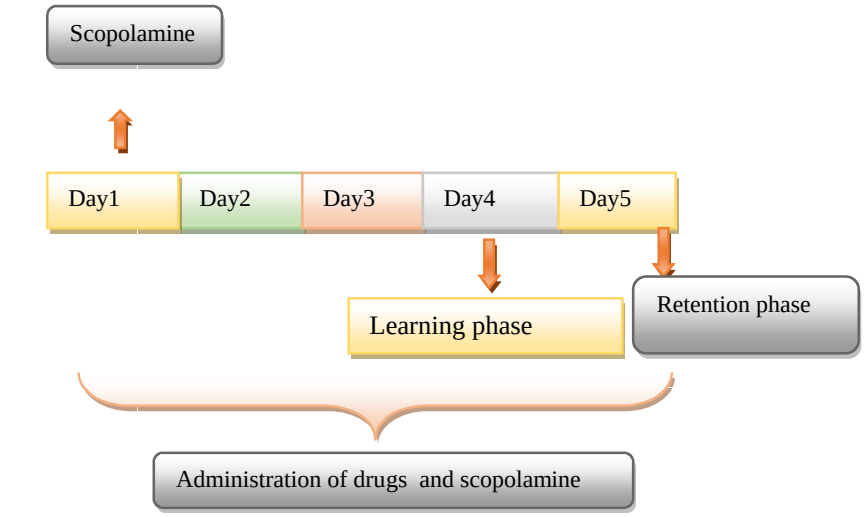


Fig. 2 : Diagrammatic representation of experimental protocol for drug administration and behavioral study (elevated plus maze).

Assessment of locomotor activity using actophotometer:

The locomotor activity was monitored by using actophotometer. The actophotometer employed to 288 assess the locomotor activity in mice had a test chamber of the 289 dimensions 18 inch (length) × 18 inch (width) × 12 inch (height). Two of the adjoining lateral walls of the test chamber consisted of six sources of light at a lower level (1 inch from base) and six sources of light at a higher level (2inch from base).Other two walls consisted of photo-cell-based receptors, present opposite to each source, receiving the light. Therefore, the total number of light beams assessing the movement of the mice. Each time an animal interrupted the light, the same was recorded by the apparatus. For locomotor activity trial, animals were individually placed in the activity meter, and total activity count was registered for every 10 min of observation period (Reddy and Kulkarni 1998) (Fig. 3)

Sample preparation and biochemical estimation

a) Sample preparation and estimation biochemical parameters.

After behavioral assessments, animals were scariûed by cervical dislocation. The brains were removed. Each brain was separately put on ice and rinsed with ice-cold isotonic saline. A (10% w/v)

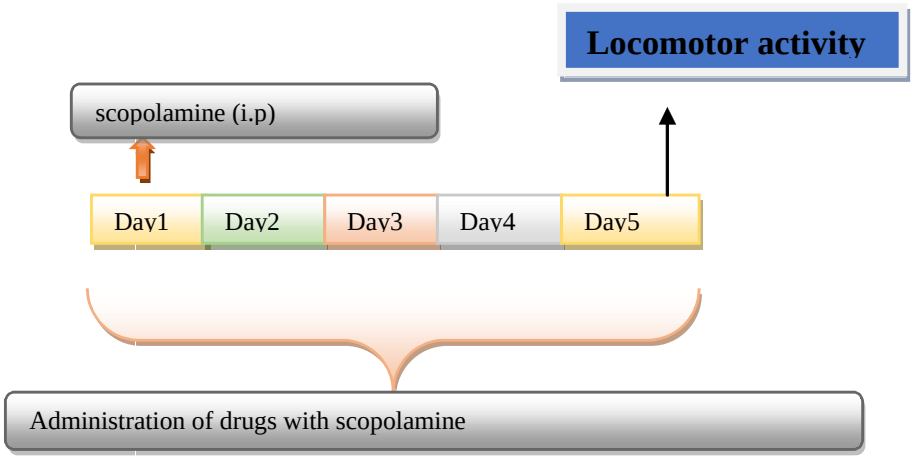


Fig. 3 : Diagrammatic representation of experimental protocol for drug administration and locomotor activity.

homogenate was prepared in 0.1M phosphate buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm for 15 minutes and aliquots of supernatant were separated and used for biochemical estimation.

b) Assessment of Biochemical parameters:

- **Thiobarbituric Acid Reactive Substances (TBARS) Assay:**

This assay was used to determine the lipid peroxidation. To 0.1 ml of prepared homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid solution, 1.5 ml of 0.8% thiobarbituric acid was added (pH>3 was adjusted by adding NaOH). This mixture was then stirred with a vortex. The mixture was then boiled in distilled water at 95°C for 60 minutes. Then it was cooled, and the following were added to the mixture: 1 ml of distilled water, 5 ml of n-butanol and pyridine (15:l, v/v), and the mixture was rinsed. The resulting mixture was spun at 4,000 rpm for 10 minutes. A sample from the upper layer of the mixture was taken, and absorbance at 532 nm was measured spectrophotometrically.

- **Catalase Activity:**

Catalase activity is measure of the breakdown of hydrogen peroxides. To 0.05 ml of prepared tissue homogenate added 3ml of H_2O_2phosphate buffer was added. The absorbance was recorded at 240 nm spectrophotometer. The results were expressed as micromoles of H_2O_2 decomposed per minute per mg protein (19).

4.3.6 Histopathological Studies

After behavioural and biochemical studies on day 5, the brains of different groups were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and post fixed in the same fixative overnight at 48°C. The brains were embedded in paraffin and stained with Hematoxylin-Eosin. The hippocampus lesions were assessed microscopically at 40 magnifications.

Preparation of Reagents:

1. 0.1M Phosphate buffer:

Solution A: 5.22g of K_2HPO_4 and 4.68 g of NaH_2PO_4were dissolved in 150 ml of distilled water.

Solution B: 6.2g NaOH was dissolved in 150ml of distilled water. Solution B was added to solution A to get the desired pH (pH 8.0 or 7.0) and then finally the volume was made up to 300ml with distilled water.

2. Hydrogen phosphate buffer:

Hydrogen peroxide (H_2O_2) solution: add about 0.75 ml of 30% $H_2O_2$was added to 100 ml of phosphate buffer.

4.4 Experimental design

Four groups were employed in the present study and each group consisted of five Swiss albino mice. Dosage of the drugs was selected on the basis of the previous reports.

Group I: Vehicle control group:

Mice were administered with vehicle of scopolamine (normal saline,i.p) one hour after administration of vehicle of Pirfenidone (CMC 0.05% w/v, 10 ml/kg, i.p) from day 1 to day 5. Behavioral studies were carried out from day 1 to day 5 in which mice were exposed to Morris water maze from day 1 to day 5, actophotometer for locomotor activity on day 5 and elevated plus maze on day 4 and day 5.

Group II: Scopolamine treated group:

Mice were administered scopolamine (0.75 mg/kg, i.p) one hour after vehicle of pirfenidone (0.5% w/v CMC) for 5 days. Behavioral studies were carried out from day 1 to day 5 in which mice were exposed to Morris water maze from day 1 to day 5, actophotometer for locomotor activity on day 5 and elevated plus maze on day 4 and day 5.

Group III: Donepezil and scopolamine treated group

Mice were administered scopolamine (0.75 mg/kg) one hour after donepezil (5 mg/kg, p.o) from day 1 to day 5. Behavioral studies were carried out from day 1 to day 5 in which mice were exposed to Morris water maze from day 1 to day 5, actophotometer for locomotor activity on day 5 and elevated plus maze on day 4 and day 5.

Group IV: Pirfenidone and scopolamine treated group

Micewere administered Pirfenidone (200 mg/kg, i.p) for 5 days before regular administration of scopolamine.Behavioral studies were carried out from day 1 to day 5 in which mice were exposed to Morris water maze from day 1 to day 5, actophotometer for locomotor activity on day 5 and elevated plus maze on day 4 and day 5.

Statistical analysis:

All the results were expressed as mean± std error of mean (SEM) followed by one way ANOVA along with Tukey's multiple comparison test and Dunnett's test. The $p<0.05$ was considered to be statistically significant.

Results and Discussion

5.1 Results

5.1.1 Morris Water Maze

Effect of various interventions on escape latency time (ELT):

Acquisition (learning) trials were conducted in animals of each group from day 1 to day 4 in Morris water maze (MWM). Mice in vehicle treated group, administered with vehicle of pirfenidone i.e. CMC (0.05%, i.p.) and vehicle of scopolamine i.e. normal saline (0.9% NaCl, i.p) for five days , showed a downward trend in their escape latency time (ELT). Scopolamine (0.75 mg/kg, i.p) treated mice showed significant difference in ELT from day 1 to day 4. Also, scopolamine treated group showed significant increase in day 4 ELT when compared to day 4 ELT of vehicle treated group. In donepezil treated group, where mice were administered with donepezil (5 mg/kg, p.o) and scopolamine (0.75 mg/kg, i.p.) (1 hr after donepezil) for five days, a decrease in ELT on day 4 as compared to the ELT of day 1 was observed. Also, donepezil treated mice showed significant decrease in day 4 ELT when compared to day 4 ELT of scopolamine treated group. In pirfenidone treated group, where pirfenidone (200 mg/kg, p.o) and scopolamine (0.75 mg/kg, i.p.) was administered for five days, a decrease in day 4 ELT as compared to day 1 ELT was observed. Pirfenidone treatment significantlyprevented a scopolamine induced rise in day 4 ELT indicating reversal of scopolamine induced impairment of acquisition.

Also, donepezil treated mice showed significant decrease in day 4 ELT when compared to day 4 ELT of scopolamine treated group. In pirfenidone treated group, where pirfenidone (200 mg/kg, p.o) was administered for five days and a dose of scopolamine (0.75 mg/kg, i.p.) was injected (1 hour after pirfenidone administration), a decrease in day 4 ELT as compared to day 1 ELT was observed. Pirfenidone treatment significantlyprevented a scopolamine induced rise in day 4 ELT indicating reversal of scopolamine induced impairment of acquisition (Table I) (Fig. 4).

B. Effect of various interventions on probe trial:

Probe trial was conducted on day 5, in mice of all groups. During probe trial, the time spent in the target quadrant (Q4) (TSTQ) and other quadrants (Q1, Q2, Q3) (TSOQ) was calculated. Mice in vehicle treated group, administered with vehicle of pirfenidone i.e. CMC (0.05% w/v, i.p.) for five days and vehicle of scopolamine i.e. normal saline (NaCl, 0.9% w/v, i.p.) (1 hour after CMC administration), significantly spent more time in target quadrant (Q4), in search of the missing platform as compared to time spent in other three non-target quadrants (Q1, Q2, Q3), indicating memory or retrieval. Scopolamine treated mice showed no significant difference in time spent in target quadrant (TSTQ) as compared to other quadrants (TSOQ). Donepezil treated mice, administered with donepezil (5 mg/kg, p.o.) and scopolamine (0.75 mg/kg, i.p) for five days (1 hour

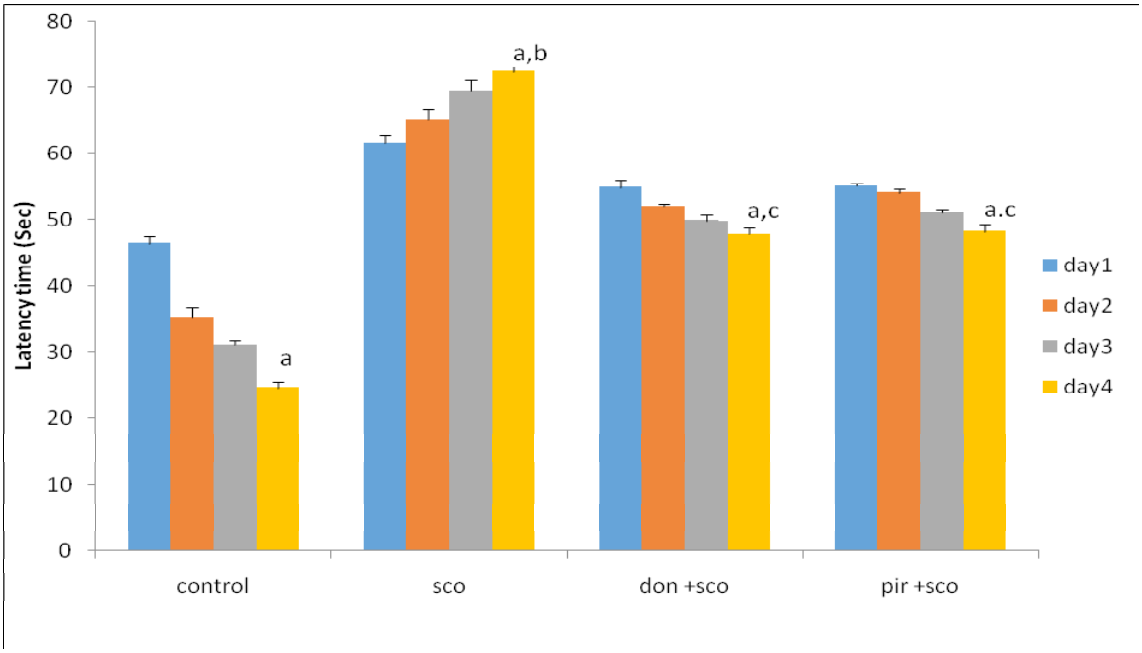


Fig. 4 : Graph effect of Pirfenidone on escape latency time (ELT) using Morris water maze.
Sco: scopolamine; Don: donepezil; Pir: Pirfenidone. For all groups n=5, all the data for ELT are represented as Mean±Standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple camparison test.
[a]P<0.05 versus day 1 ELT in respective groups.
[b]P<0.05 versus day 4 ELT in Vehicle treated group.
[c]P<0.05 versus day 4 ELT in Scopolamine treated group.

TABLE I : Effect of pirfenidone on memory using Morris water maze.

Groups	*Day 1 (sec)*	*Day 2 (sec)*	*Day 3 (sec)*	*Day 4 (sec)*
Control	46.4±1.166	35.200± 1.463	31.000±0.7071	24.600±0.678
Sco	61.6±1.208	65.000± 1.643	69.400±1.691	72.400±1.372
Don + Sco	54.8±1.0770	40.600± 0.400	37.200±0.969	29.800±0.489
Pir+ Sco	55±0.4472	45.800± 0.583‘	41.600±0.600	34.200±0.860

Sco: scopolamine; Don: donepezil; Pir: Pirfenidone. For all groups n=5, all the data for ELT are represented as Mean±Standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

after donepezil administration), exhibited improved search accuracy as indicated by significantly longer time spentin target quadrant (Q4) in comparison to that of other quadrants (Q1, Q2, Q3) (TSOQ). Also, significant increase in time spent in target quadrant (TSTQ) was observed as compared to the time spent in target quadrant (TSTQ) of scopolamine treated group. Mice in pirfenidone treated group, administered with pirfenidone (200 mg/kg, i.p) for five days and a scopolamine dose (0.75 mg/kg, i.p) for five days (1 hour after pirfenidone administration) for five days, significantly spent more time in target quadrant (Q4) searching for the missing platform as compared to the time spent in other three non-target quadrants (Q1, Q2, Q3). Also, increase in time spent in target quadrant (TSTQ) was observed when compared with time spent in target quadrant of scopolamine treated group, thereby indicating improvement in memory and retrieval as mice spent more time in other quadrants as compared to target quadrant in search of missing platform (Table II) (Fig. 5).

Donepezil treated mice, administered with donepezil (5mg/kg, p.o.) for and scopolamine (0.75 mg/kg, i.p) (1 hour after donepezil administration) for five days, exhibited improved search accuracy as indicated by

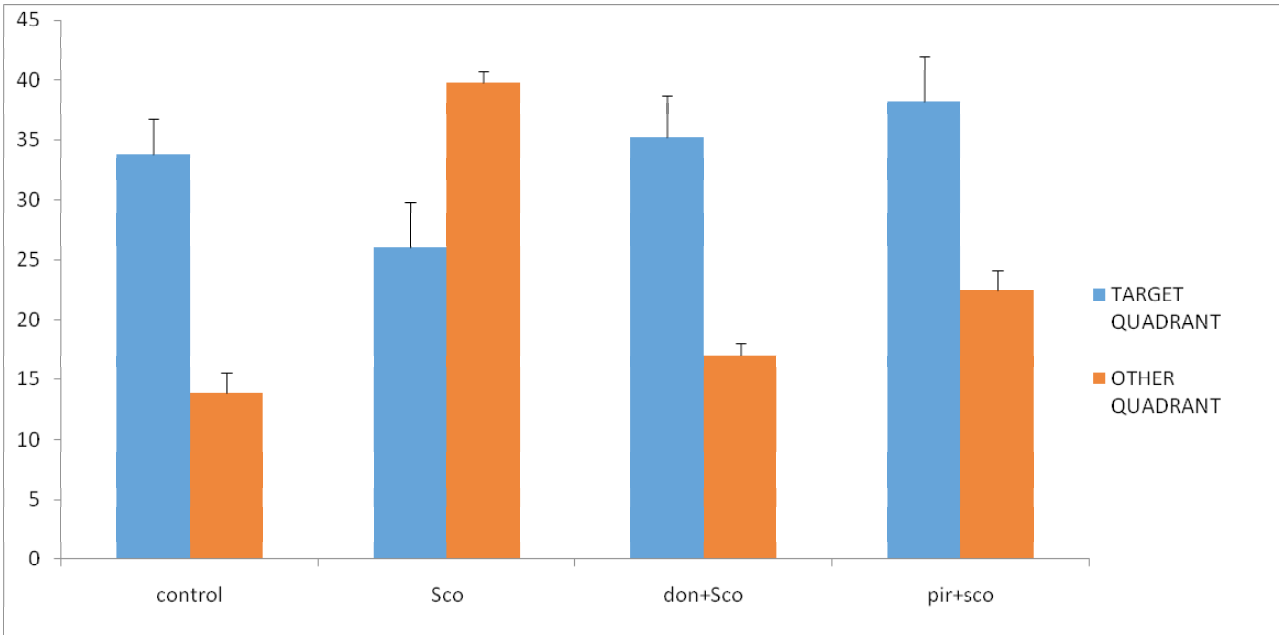


Fig. 5 : Effect of Pirfenidone on mean time spent in target quadrant (TSTQ) during probe trial using the Morris water maze. Sco: scopolamine; Don: donepezil; Pir: Pirfenidone. For all groups n=5, all the data for TSTQ are represented as Mean±Standard error of the mean (SEM) and were statistically analyzedusing one way analysis of variance (ANOVA) followed by Tukey's multiple camparison test.
[a]P<0.05 versus mean time spent in other quadrants in respective groups.
[b]P<0.05 versus mean TSTQ in Vehicle treated group.
[c]P<0.05 versus mean TSTQ in Scopolamine treated group.

TABLE II : Effect of Pirfenidone on probe trial performance in MWM.

Groups	*TSTQ (sec)*	*TSOQ (sec)*
Control	33.800±2.950	13.800±1.772
Sco	26.000±3.742	39.800±0.8602
Don+Sco	35.200±3.493	17.000±1.049
Pir+Sco	38.200±3.768	22.400±1.720

Sco: scopolamine; Don: donepezil; Pir: Pirfenidone, TSTQ: Time spent in target quadrant; TSOQ: Time spent in other quadrantsFor all groups n=5, all the data for probe trial are represented as Mean±Standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's's multiple comparison test.

significantly longer time spentin target quadrant (Q4) in comparison to that of other quadrants (Q1, Q2, Q3) (TSOQ) and also significant increase in time spent in target quadrant (TSTQ) was observed as compared to the time spent in target quadrant (TSTQ) of scopolamine treated group. Mice in pirfenidone treated group, administered with pirfenidone (200 mg/kg, p.o) and a scopolamine dose (0.75 mg/kg, p.o) for five days (1 hour after pirfenidone administration), significantly spent more time in target quadrant (Q4) searching for the missing platform as compared to the time spent in other three non-target quadrants (Q1, Q2, Q3) and also increase in time spent in target quadrant (TSTQ) was observed when compared with time spent in target quadrant of scopolamine treated group, thereby indicating improvement in memory and retrieval.

Elevated plus maze

A. Effect of various interventions on transfer latency (TL)

Transfer latency is the time taken by the mouse to move from an open arm to any one of the closed arms with all its four legs crossing the middle line. Mice were exposed to elevated plus maze on day 4 for learning and on day 5 for retrieval. Scopolamine (0.75 mg/kg, i.p) treated mice showed higher transfer latency (TL) values on day 4 and on day 5 (after 24 h) as compared to mice in vehicle treated group, where mice were treated with vehicles of pirfenidone i.e. CMC (0.05%) for five days and scopolamine vehicle i.e. normal saline (0.09%) for 5 days, indicating impairment in learning and memory. Donepezil (5 mg/kg, p.o) pre-treatment for 5 days

decreased transfer latency on day 4 and after 24 h, i.e. on day 5 as compared to vehicle treated group, indicating improvement in both learning and memory. Pre-treatment with pirfenidone (200 mg/kg, p.o) also decreased transfer latency on day 4 and on day 5 as compared to scopolamine treated group thereby indicating improvement in learning and memory (Table III).

Locomotor activity

Spontaneous locomotor activity was tested using actophotometer in mice of all groups on day 5 after session of Morris water maze test. No significant change in locomotor activity was observed as compared to control, donepezil and pirfenidone group (P>0.05) (Table IV).

TABLE III : Effect of pirfenidone on memory using elevated plus maze.

Groups	*Day 4 (Learning) (sec)*	*Day 5 (Retrieval) (sec)*
Control	50.800±2.131	38.600±1.833
Sco Treated	59.800±2.059	46.200±1.744
Don+Sco	48.200±1.020	32.400±1.364
Pir+sco	48.600±1.364	34.400±2.993

Sco: scopolamine; Don: donepezil; Pir: Pirfenidone, For all groups n=5, all the data for probe trial are represented as Mean±Standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's's multiple comparison test.

TABLE IV : Effect of pirfenidone on memory using Actophotometer.

Groups	*Locomotor activity*
Control	177.40±1.965
Sco	168.00±1.028
Done+Sco	177.80±1.655
Pir+Sco	176.6±1.568

Sco: scopolamine; Don: donepezil; Pir: Pirfenidone, For all groups n=5, all the data for probe trial are represented as Mean±Standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Dunnet's comparison test.

Biochemical Estimation

A. Effect of various intervention on MDA levels

Scopolamine (0.75 mg/kg, i.p) treatment for five days signicantly increased the brain MDA level compared to vehicle- control group, where mice were treated with vehicles of pirfenidone i.e. CMC (0.05%) and scopolamine vehicle i.e. normal saline (0.09%) for 5 days, (Fig. 5.5). Donepezil treated mice, administered with donepezil (5mg/kg, p.o.) for and scopolamine (0.75 mg/kg, i.p) (1 hour after donepezil administration) for five days, signicantly (P<0.05) decreased brain MDA level compared to their corresponding scopolamine treated group. In pirfenidone treated group, where pirfenidone (200 mg/kg, p.o) was administered for five days and a dose of scopolamine (0.75 mg/kg, i.p.) was injected (1 hour after pirfenidone administration), signicantly (P<0.05) decreased brain MDA level compared to their corresponding scopolamine treated group (Table V).

TABLE V : Effect of TBARS on mice brain.

Groups	*TBARS*
Control	9.8±0.00518
Scopolamine	13.21±0.004410
Don+sco	10.87±0.002476
Pir+sco	11.275±0.003935

Sco: scopolamine; Don: donepezil; Pir: Pirfenidone, TSTQ: Time spent in target quadrant; TSOQ: Time spent in other quadrantsFor all groups n=5, all the data for probe trial are represented as Mean±standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Dunnet's comparison test.

B. Effect of various intervention on Catalase levels

Scopolamine (0.75 mg/kg, i.p) treatment for five days signicantly decreased the brain catalase level compared to vehicle-control group, where mice were treated with vehicles of pirfenidone i.e. CMC (0.05%) and scopolamine vehicle i.e. normal saline (0.09%) for 5 days. Donepezil treated mice, administered with donepezil (5 mg/kg, p.o.) for and scopolamine (0.75 mg/kg, i.p) (1 hour after donepezil administration) for five days, signicantly (P<0.05) increased brain catalase level compared to their corresponding scopolamine treated group. In pirfenidone treated group, where pirfenidone (200 mg/kg, p.o) was administered for five days and a dose of scopolamine (0.75 mg/kg, i.p.) was injected (1 hour after pirfenidone administration), signicantly (P<0.05) increased brain catalase level compared to their

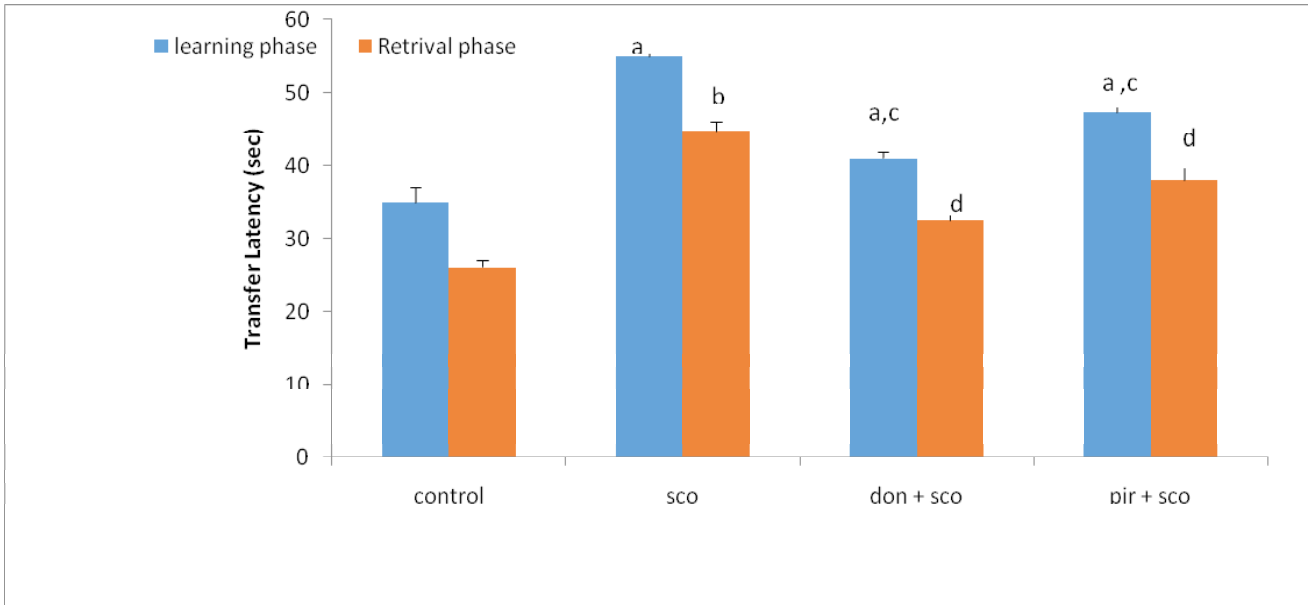


Fig. 6 : Effect of Pirfenidone on TL in elevated plus maze.
Sco: scopolamine; Don: donepezil; Pir: Pirfenidone. For all groups n=5, all the data for ELT are represented as Mean±Standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple camparison test.
[a]P<0.05: compared to vehicle versus all in learning phase.
[b]P<0.05: compared to vehicle versus scopolamine in retrival phase.
[c]P<0.05:Scopolamine versus all in lerning phase.
[d]P<0.05:Scopolamine versus all in retrival phase.

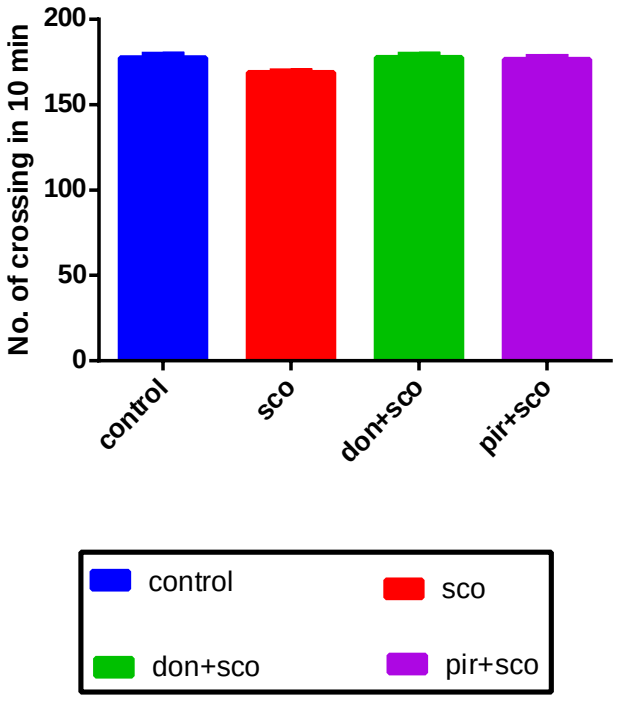


Fig. 7 : Effect of Pirfenidone on locomotor activity in mice.
Sco: scopolamine; Don: donepezil; Pir: Pirfenidone. For all groups n=5, all the data for locomotor activity are represented as Mean±Standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Dunnet's test.
[a]P>0.05: scopolamine versus control group.
[b]P>0.05:scopolamine versus don and pir group.

corresponding scopolamine treated group as shown in Table VI.

Histopathology studies:

The hippocampal lesions assessed microscopically at 40 magnification revealed significant decrease in the lesion size with Pirfenidone treated group when compared with Scopolamine treated group. (Figs. 10, 11, 12, 13) were normal control, scopolamine (disease control), donepezil (standard), Pirfenidone respectively, representing the histological sections of the brain tissue showing neurological lesions (Figs. 10-13).

TABLE VI : Effect of Catalase on mice brain.

Groups	*Catalase (% H_2O_2 scavenging activity)*
Control	71.3
Scopolamine treated	30.3
Don+sco treated	66.2
Pir+sco treated	65.5

Sco: scopolamine; Don: donepezil; Pir: Pirfenidone, For all groups n=5, all the data for probe trial are represented as Mean±Standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Dunnet's comparison test.

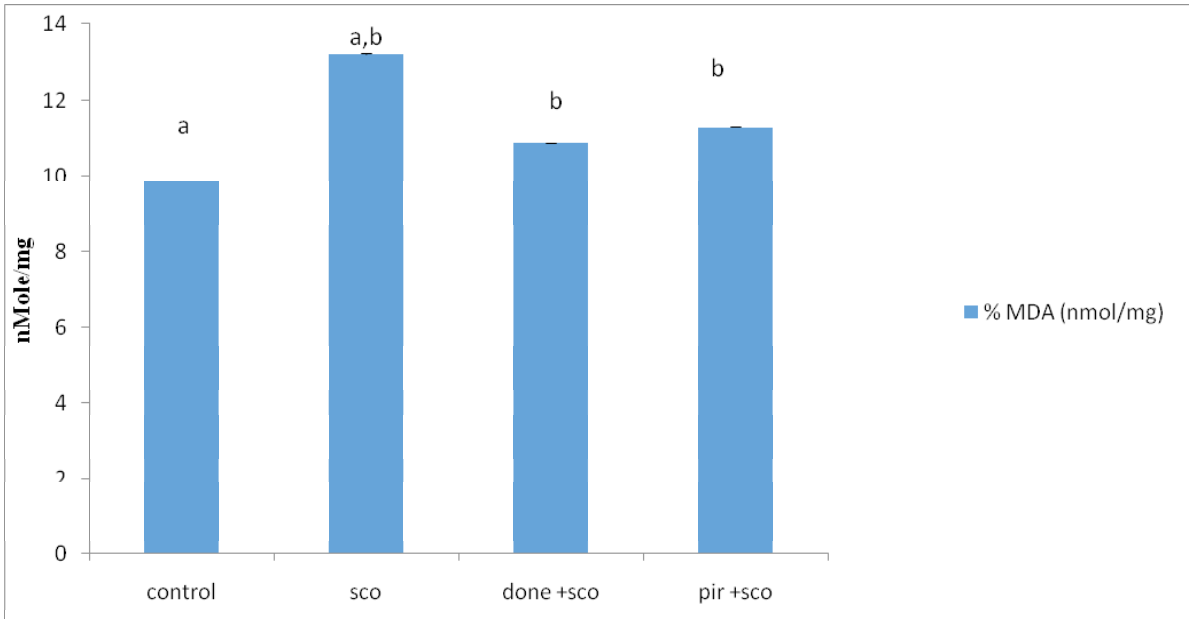


Fig. 8 : Showing effect of TBARS on mice brain.
Eect of various drugs on malondialdehyde levels compared to the disease control group. (Mean±SEM, n=5). Graph showing Mean± SEM of malondialdehyde levels.
[a]P>0.05: scopolamine versus control group.
[b]P>0.05: scopolamine versus don and pir group.

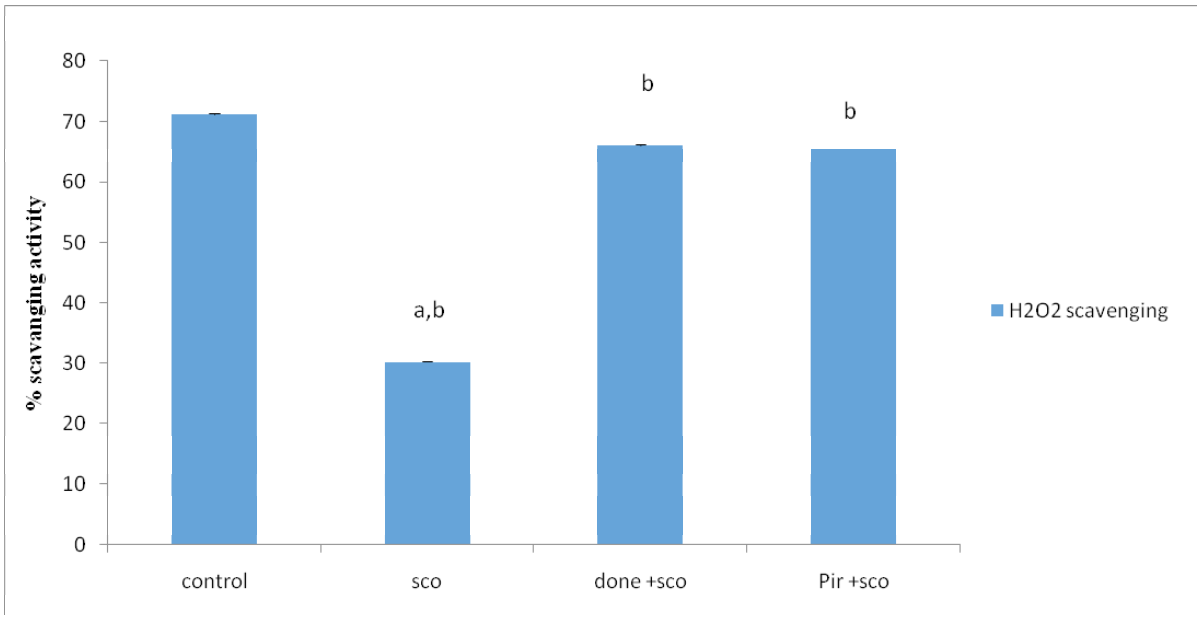
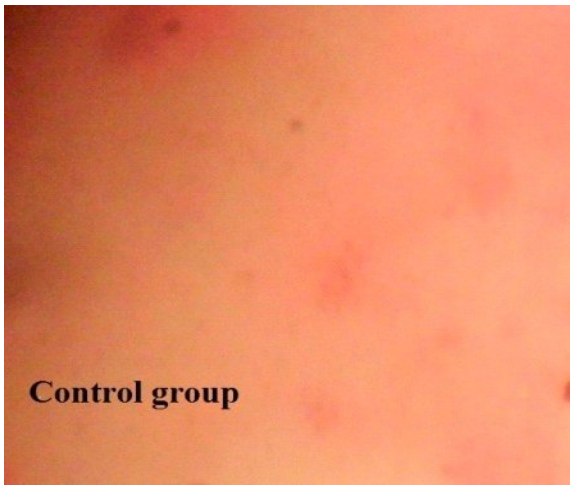
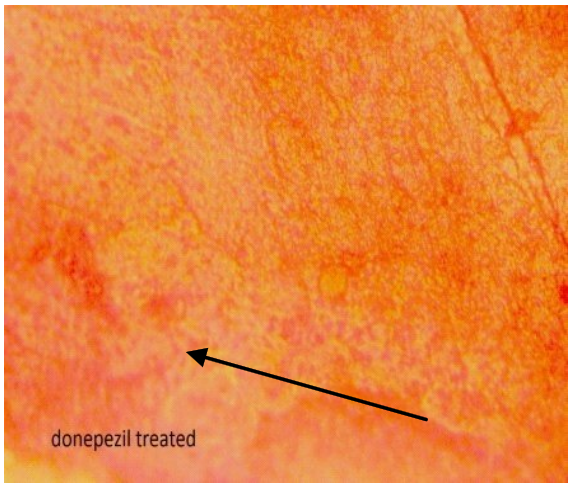


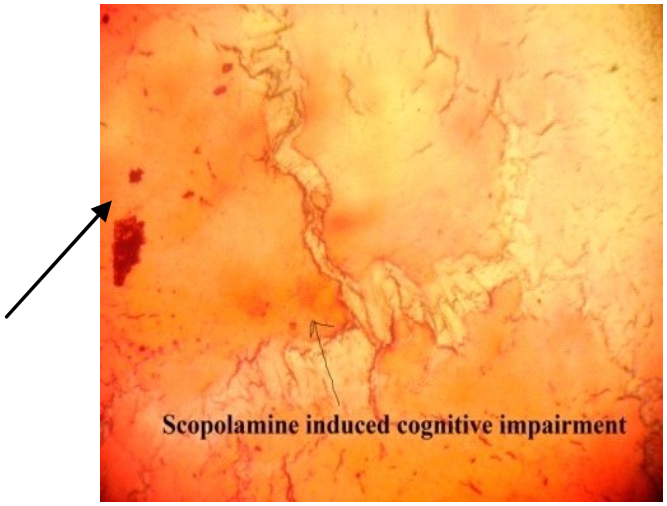
Fig. 9 : Showing effect of Catalase on mice brain.
Eect of Pirfenidone on catalase activity compared to the disease control group. (Mean±SEM, n=5). Graph showing Mean±SEM of % H_2O_2 scavenging activity. a=P<0.05 compared with corresponding values of disease control.
[a]P>0.05: scopolamine versus control group.
[b]P>0.05:scopolamine versus don and pir group.



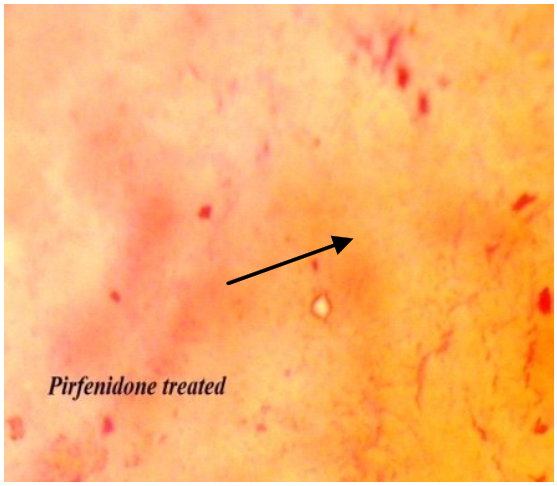
10 CONTROL GROUP



11 DONEPEZIL+SCO GROUP



12 SCOPOLAMINE GROUP



13 PIRFENIDONE + SCO GROUP

Figs. 10,11,12,13 : Showing Histopathological studies of vehicle-control, Scopolamine treated, Donepezil+scopolamine treated, Pirfenidone+Scopolamine treated.

Discussion

Alzheimer disease a genetically heterogeneous, neurodegenerative disease occurs progressively slow with the symptoms related to impaired neurotransmission and disintegration of neuronal circuits in the affected brain areas (20). Patients with cognitive defects in AD are related with a progressive loss of cholinergic neurons and a subsequent decline in the levels of ACh in the brain particularly in the temporal and parietal neocortex and hippocampus. The acetylcholine is said to have an effect on the memory, sleep, concentration abilities, and also implicated in some severe diseases such as Alzheimer, Parkinson and epilepsy (21).

In present study, pirfenidone treatment significantly decreased mean escape latency in Morris water maze from day 1 to day 4 and showed improved search accuracy as indicated by longer time spent

in target quadrants as compared to other non target quadrants on day 5. Pre-treatment with pirfenidone also decreased transfer latency on day 4 and on 5 day, on exposure to elevated plus maze, as compared to scopolamine treated group thereby indicating improvement in learning and memory. Also, Pirfenidone treatment prevented scopolamine induced elevation of TBARS and Catalase activity in mice brain.

In histopathological studies, it was clearly visible that scopolamine treatment caused generation of various cellular mediators which leads to cell death in the mice brain. The degenerated cells were seen in the form of lesions, these lesions were seen as the main markers of cell death or apoptosis. In pirfenidone treated group the lesion were less as compared to scopolamine which is due to the decrease in various cellular mediators, which are responsible for cell death.

There was no significant change in locomotor activity among different groups which excludes the possibility that the change in locomotor activity may have contributed to the observed behavioral effects following scopolamine and/or Pirfenidone treatment.

Conclusion

Pirfenidone an antifibrotic drug with anti-inflammatory and antioxidant effect altered the memory impairment held by scopolamine injection. In animal models, pirfenidone displays a systemic antifibrotic activity and has been shown to reduce biochemical and histopathological indices of fibrosis of the lung, liver, heart and kidney.

From the above discussion it may be concluded that the, pirfenidone, provides beneficial effects by improving learning, memory and it also decreased brain TBARS levels and increased catalase activity in scopolamine induced memory impairment in mice. Pirfenidone also decreased lesion in the brain which were the main markers of apoptosis. Nevertheless further studies are needed to establish the full potential and mechanism of this drug in dementia of AD type.

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