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NEUROPROTECTIVE ACTIVITY OF HIBISCUS ROSA-SINESIS FLOWERS IN SCOPOLAMINE INDUCED COGNITIVE DECLINE RATS

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ABSTRACT

Aim of the present study is Petroleum ether extract of *Hibiscus rosa-sinesis* (PETECHB) was evaluated in scopolamine induced cognitive decline rat model for its nootropic and neuroprotective activity. Effect on spatial working memory, spatial reference memory and spatial working-reference memory was evaluated by Rectangular maze, Morris water maze, Pole climbing apparatus, and locomotor activity respectively. Neuroprotective effects of PETEHB was studied by assaying acetylcholinesterase, TBARS, DPPH and CAT levels in the brain of nootropic rats. The PETEHB (150 and 300 mg/kg) was found to cause significant increase in spatial working memory ($P < 0.05$), spatial reference memory ($P < 0.001$) and spatial working-reference ($P < 0.001$) in retention trials on Rectangular maze, Morris water maze and Pole climbing apparatus respectively. Whereas significant decrease in acetylcholinesterase activity ($P < 0.05$), MDA levels ($P < 0.001$), and significant increase in DPPH, CAT levels was observed in animals treated with PETECP (150 and 300 mg/kg) compared to nootropic control group. The present data indicates that *Hibiscus rosa-sinesis* flowers tenders protection against Scopolamine induced cognitive decline rats and merits the need for further studies to elucidate its mode of action.

Key words: *Hibiscus rosa-sinesis*, Neuroprotectivity, Nootropic, Alzheimer's, Morris water maze, Rectangular maze, Pole climbing apparatus, Locomotor activity.

INTRODUCTION

Alzheimer disease (AD) is disease characterized by progressive cognitive decline usually beginning with impairment in the ability to form recent memories, but inevitably affecting all intellectual functions and leading to complete dependence for basic functions of daily life, and premature death [1]. Alzheimer's disease (AD) is the most common cause of dementia in the elderly, characterized by amyloid plaques, neurofibrillary tangles. However, as for other neurodegenerative disease, a local inflammatory reaction is sustained by activated microglia and reactive astrocytes, a indicated by the presence of antigens associated with microglia/macrophage activation and inflammatory mediators, such as elements of the complement system, cytokines, and free radicals Acetylcholine is a major neurotransmitter which is playing an important role in learning and memory is affected in Alzheimer's disease The disease is associated with plaques and tangles [2-4]. AD was determined by a gradual decline in cognitive function by history. It has been more than 10 years since it was first proposed that the neurodegeneration in Alzheimer's disease (AD) may be caused by deposition of amyloid β-peptide (Aβ) in plaques in the brain tissue. According to the amyloid hypothesis, accumulation of Aβ in the brain is the primary influence driving AD pathogensis [5-7]. The rest of the disease process, in following stage

STAGES OF ALZHEMIER'S DISEASE

Stage 1

Also known as the stage of "asymptomatic cerebral amyloidosis". These individuals have biomarker evidence of Aβ accumulation with elevated tracer retention of low Aβ42 in CSF assay, but no detectable evidence of additional brain alterations suggestive of neurodegeneration. The standards for determining.

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"amyloid-positivity" are still evolving. Although recent work suggests there may be a CSF Aβ42 cutoff value that is predictive of progression to AD dementia [8].

Stage 2

Also known as the stage of "amyloid positivity and early neurodegeneration". These individuals have evidence of amyloid positivity and presence of one or more markers of downstream AD-P-related neuronal injury.

Stage 3

Also known as the stage of "amyloid positivity, evidence of neurodegeneration and subtle cognitive decline". The individuals with biomarker evidence of amyloid accumulation, early neurodegeneration, and evidence of subtle cognitive decline are in the last stage of preclinical Alzheimer's disease [9].

MATERIALS AND METHODS

Animals

Wistar albino rats (150-175gms) are used to study Alzheimer's disease activity. The animals were procured from Sanazyme Scientifics Ltd, Hyderabad. They are housed into group of six rats per cage and maintained at 24ºC ± 1ºc with relative humidity 45-55% and 12:12 hour's dark/light cycle. The animals had free access to food (standard chew pellets) and water *ad libitum*. The Institutional Ethics Committee approved all the experimental procedures. St.John college of Pharmacy, Warangal, Telangana, India.

Plant Material :

The plant material *Hibiscus rosa sinesis* was collected from Warangal District, Telangana, India during th month of April 2014 and authenticated by Dr. Ajmeera Ragan, Fellow of India professor and former Head,- Department of Botany, School of Life Sciences, University of Hyderabad.

Preparation of Plant Extract

The flowers were shade dried at room temperature and finely powdered with help of a hand-grinding mill in such a way that the powdered material passed through sieve no 40. The powered of flower of *Hibiscus rosa sinesis* was extracted separately by continuous hot extraction process using soxhlet apparatus with different solvents successively in increasing order of polarity from petroleum ether, chloroform, alcohol and finally fresh aqueous extract (chloroform: water)

Each extract was concentrated in rotary flash evaporator under vaccum and dried over anhydrous sodium sulphate. The extracts were subjected to preliminary qualitative chemical analysis

Drugs

Donepezil was purchased from Alkem Laboratories Ltd, Hyderabad and Scopolamine purchased from Cadila Health Care Ltd., Hyderabad, Pet ether extract of *Hibiscus rosa sinesis* flowers.

Chemicals

(5,5dithiobis(2-nitrobenzoicacid))DTNB,(1,1-diphenyl-2-Picrylhydrazyl)DPPH reagent, Acetyl thiocholineiodide from (Himedia India Ltd). Hydrogen peroxide (Ethylene Diamine Tetra Acetic Acid) EDTA, Perchloric acid 10% Formalin were purchased from CPC diagnostics Pvt Ltd., Hyderabad. All other used chemicals were of the high analytical grade. Memory impairment was induced by Scopolamine (1.4 mg/kg) i.p and 90 min after administration. Donepezil were dissolved in 0.1% (w/v) solutions. All the drugs and extracts are freshly prepared daily. Doses are given accordingly to the respective rat weight [10-15].

Experimental design

Group-I	Control	Vehicle(0.1% CMC)
Group-II	Disease control	Scopolamine(1.4 mg/kg) *i.p.*
Group-III	Standard	Donepezil(5mg/kg)*P.o*+ Scopolamine(1.4 mg/kg) *i.p.*
Group-IV	Treatment 1	Petetherextract of *Hibiscus-rosa-sinesis*(150mg/kg)*P.o*+ Scopolamine (1.4 mg/kg) *i.p.*
Group-V	Treatment 2	Petether extract of *Hibiscus-rosa-sinesis*(300mg/kg)*P.o*+ Scopolamine(1.4 mg/kg) I p.

Invivo Methods

Rectangular Maze Test

The maze consists of completely closed rectangular box with an entry and reward chamber partitioned with wooden slats into blind passages leaving just twisting corridor leading from the entry to the reward chamber. On the first day all the mice were familiarized with rectangular maze for a period of 10 min. This was known as training session. On the 3rd day the rats was placed in the entry chamber and the timer was activated as soon as the mouse leaves the entry chamber. The time taken for the rats to reach the reward chamber was taken as the latency time. 4readings are taken and average of reading gives learning score. Lower scores indicate efficient learning and higher scored indicates poor learning in animals.

Morris Water Maze Test

Method was carried out in a circular pool (90 cm in diameter and 50cm in height) of water with a featureless inner surface (Morris R., 1984). The 1st day of the experiment was dedicated to swimming training for 60sec in the absence of the platform. During the 4 consecutive days the rats were given the trial session with the platform in place. Once the rats located on the platform, it was permitted to remain on it for 10sec, if the rats did not locate the platform within 120sec; it was placed on the platform for 10sec and then removed from the pool. One day after the final training trial sessions (on day 5). Rats were individually subjected to a probe trial session in which the platform was removed from the pool, and rats were allowed to swim for 120sec to search for it and the latency time was determined [16-20].

Pole Climbing Test in Rodents

Training and testing was conducted in a 25x25x40 cm chamber that was enclosed in a dimly lit, attenuated box, Scrambled shock was delivered to grid floor of the chamber. A 2.8 KHZ speaker and a 28 v light were situated on the top of the chamber. A wooden pole 2.5 cm in diameter was suspended by a counter balance weight through a hole in the upper layer centre of the chamber. The response was recorded when a mice jumps on the pole and activates micro switch, the activation of light and speaker together were used as conditioned stimulus

Measurement of Locomotor Activity

Most of the CNS drugs influence the locomotor activities in man & animals. The locomotor activity of drug can be studied using actophotometer which operates on photoelectric cells which were connected in circuit whit a counter when the bean of light falling on photocell was cut off by the animal a count was recorded. Animals were placed individually in the activity cage for 10min and the activity was monitored. The test was done after 30min of drug administration. The photo cell count was noted and decrease or increase in locomotor activity was calculated

***Invitro* Methods**

On day 9^{th} following the behavioral testing, animals were sacrificed and the brain tissues were quickly removed cleaned with ice-cold saline and stored at -20ºC for bio chemical estimation [21].

Preparation of Brain Homogenate

For preparation of homogenate, the fresh whole brain was weighed and transferred to a glass homogenizer and homogenized in an ice bath after adding10 volumes of 0. 9% sodium chloride solution and phosphate buffer (0.1M, p^H 7.4).The homogenate was centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant liquid was used for estimation of cholinesterase level and other antioxidants

Estimation of Cholinergic Status in the Mice Brain

The cholinergic marker, cholinesterase was estimated in the whole brain according to Ellman method (Ellman *et al*., 1961). The end point was formation of yellow color due to reaction of thiocholine from acetylthiocholine iodide in presence of dithiobis nitrobenzoate ions. The rate of formation of thiocholine from acetylcholine iodide in the presence of tissue cholinesterase was measured using spectrophotometer. The sample was first treated with 5, 5'-dinitrobenzoic acid (DTNB) and the optical density (OD) of yellow color compound formed during the reaction at 412 nm every minute was measured.

Biochemical Estimation of Markers in Oxidative Stress
TBARS (Thiobarbituric Acid) Assay

The estimation of per oxidation of lipids has been carried out by a number of methods of which TBA-reactive substance is selected because of its high sensitivity and simplicity in operation. The TBA test is often said to measure malondialdehyde (MDA) formed in peroxidizing lipid systems. So the results are frequently expressed as micro mate malondialdehyde equivalents.
Calculation:

$$\text{LPO} = \frac{\text{Test O.D.} \times \text{Total Volume} \times 100}{1.56 \times 105 \times 10\text{-}9 \times \text{Sample Volume} \times \text{mg protein per ml}}$$

Unit: nmol MDA / min × mg protein (Kishore *et al*., 2012).

DPPH Radical Scavenging Assay

The free radical scavenging activity of the rest drug was measured in vitro by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. About 0.3mM solution of DPPH was dissolved in 100ml ethanol and 1 ml of this solution was added to 3ml of the brain tissue homogenate was dissolved in ethanol. The mixture was shaken and allowed to keep at room temperature for 30 min and the absorbance was measured at 517nm using a spectrophotometer. The percentage of scavenging activity was determined. Hydrogen peroxide solution (2mm/L) was prepared with standard phosphate buffer (p^H 7.4). The brain tissue homogenate supernatant was added to 0.6 ml of H_2O_2 solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity was determined.

Calculation

$$\text{Cat (U)/100 µl of Sample} \quad \frac{(dy/dx)\times 0.003}{38.3956 \times 10^{-6}}$$

Where dy/dx - Change in absorbance per minute
38.3956x10 - molar extinction coefficient of H2O2 at 240nm (Rama *et al*., 2010).

RESULTS AND DISCUSSIONS

***Invivo* screening methods**

Rectangular Maze

The test extract memory enhancing activity was evaluated by using rectangular maze and scopolamine induced amnesic model. The transfer latency was measured for all the animals day1 to day 9 compared against disease control group of that day. Results showed that there is significant (P<0.001) decreases the transfer latency time in 9 day, PETEHB given groups in comparison with disease control using Neumans keul's test.

Morris Water Maze

The activity of PETEHB was evaluated using Morris water maze. The rats treatment groups except scopolamine-treated group showed significant transfer latency on 7 day with platform and on 9days. This indicates memory enhancing activity of *Hibiscus-rosa-sinesis*. Donepezil (5mg/kg) treated for successive 8 days acts as positive control, possessed significant (P<0.001) decrease in transfer latency compared to disease control (scopolamine) [22].

Locomotor Activity

The test compounds memory enhancing activity was evaluated by using locomotor activity and scopolamine induced amnesic model. The transfer latency was measured for all the animals day 1 to day 9 compared against disease control group of that day. Results showed that there is significant (P<0.01) decrease in transfer latency time on 9day, PETEHB given groups in comparison with disease control using Neumans keul's test.

Pole climbing test

The test compounds memory enhancing activity was evaluated by using pole climbing test and scopolamine induced amnesic model. The escape latency was measured for all the animals day1 to day 9 compared against disease control group of that day. Results showed that there is significant (P<0.01) decrease in escape latency time on 9 day, PETEHB given groups in comparison with disease control using Neumans keul's test [23].

Evaluation of antioxidant activity

Ach Estimation

Scopolamine treatment significantly increased the brain AchE level. Standard drug (Donepezil) and PETEHB treatment significantly inhibited the brain AchE levels compared to scopolamine treated group (disease control) P<0.001.

***Invitro* screening methods**

TBARS Assay

Scopolamine treatment significantly increased the brain MDA level compared to control group. Standard drug (Donepezil) and PETEHB treatment significantly (P<0.001) decreased the brain MDA level compared to scopolamine treated group (Disease control).

DPPH Assay

Anti oxidant levels were decreased in scopolamine treated group compared to the control group. Standard drug (Donepezil) and PETEHB treated groups showed significant (P<0.001***) difference compared to the disease control group[24-25].

Catalase Activity

Anti oxidant levels were decreased in scopolamine treated group compared to the control group. Standard drug (Donepezil) and PETEHB treated groups showed significant (P<0.001) difference compared to the disease control group.

Table 1. Effect of pet ether extract of *Hibiscus-rosa-sinesis* on Rectangular maze test

Days	Normal	Scopolamine	Donepezil	PETEHB-1+Scopolamine	PETEHB-2+Scopolamine
1	62.50±2.88	107.270±3.34	71.00±2.54	106.00±6.23	103.13±3.34
3	61.89± 3.92	110.480±3.90	70.00±3.68	101.12±3.52	98.65±3.90
5	60.22 ±4.32	116.270±3.93	69.00±3.93*	99.20±7.5	93.15±1.12
7	59.65 ±4.62	120.530±2.36	68.13±2.13**	90.94±3.25*	82.13±1.28*
9	58.76 ±3.68	133.160±3.86	67.53±2.45**	95.00±3.6**	79.19±1.33***

Values were expressed as mean ± SD of transfer time in seconds; (P<0.05*, P<0.01**, P<0.001***) as compared to scopolamine treated group. Data were analyzed by one way ANOVA followed by Neuman kaul's test for multiple comparisons (n=6 in each group), Where PETEHB 1&2are150mg/kg &300mg/kg.

Table 2. Effect of pet ether extract of *Hibiscus-rosa-sinesis*on Morris water maze test

Days	Normal	Scopolamine	Donepezil	PETEHB-1+Scopolamine	PETEHB-2+Scopolamine
1	52.00±4.27	94.00±2.28	79.80±3.65	91.65±2.14	90.00±6.23
3	50.00± 2.16	95.13±2.14	77.16±1.77	87.83±2.36	84.00±3.52
5	49.68± 4.49	98.95±6.38	72.00±4.8*	81.66±1.36	78.00±1.72
7	49.00± 4.19	101.84±5.68	67.96±5.87**	75.31±2.65*	71.00±1.52*
9	47.5 ±4.32	105.13±4.23	58.26±6.32***	69.66±2.13**	64.00±3.33***

Values were expressed as mean ± SD of transfer time in seconds; (P<0.05*, P<0.01**, P<0.001***) as compared to scopolamine treated group. Data were analyzed by one way ANOVA followed by Neuman kaul's test for multiple comparisons (n=6 in each group), Where PETEHB 1&2 are 150mg/kg &300mg/kg.

Table 3. Effect of pet ether extract of *Hibiscus-rosa-sinesis* on Locomotor activity

Days	Normal	Scopolamine	Donepezil	PETEHB-1+Scopolamine	PETEHB-2+Scopolamine
1	84.83±2.63	79.00±3.00	124.00±3.00	90.58±3.98	98.50±3.16
3	83.66± 1.36	78.67±2.96	125.67±2.96	10931±3.25	113.12±3.52
5	82.30 ±2.65	72.36±2.30	132.36±2.30*	119.00±3.03	128.09±1.12
7	80.00 ±4.6	69.15±4.27	148.15±4.27**	139.13±2.28*	143.34±1.28*
9	80.35 ±3.65	65.00±5.02	156.00±5.02**	148.12±2.13**	150.19±2.88**

Values were expressed as mean ± SD of transfer time in seconds; (P<0.05*, P<0.01**, P<0.001***) as compared to scopolamine treated group. Data were analyzed by one way ANOVA followed by Neuman kaul's test for multiple comparisons (n=6 in each group), Where PETEHB 1&2 are 150mg/kg &300mg/kg.

Table 4. Effect of pet ether extract of *Hibiscus-rosa-sinesis* on pole climbing activity

Days	Normal	Scopolamine	Donepezil	PETEHB-1+Scopolamine	PETEHB-2+Scopolamine
1	12.12±3.12	45.85±2.73	33.50±3.5	43.50±3.50	40.09±10.62
3	11.2±3.60	52.09±4.36	30.05±3.5	40.50±3.5	38.12±10.92
5	10.01±0.21	50.02±4.49	25.02±0.68*	28.02±3.38	24.09±0.36
7	10.1±0.28	5613±3.38	18.34±0.50**	22.00±3.95*	19.34±0.39*
9	9.53±0.33	58.85±2.72	13.59±0.42**	16.25±4.52**	14.06±0.48**

Values were expressed as mean ± SD of transfer time in seconds; (*P*<0.05*, *P*<0.01**, *P*<0.001***) as compared to scopolamine treated group. Data were analyzed by one way ANOVA followed by Neuman kaul's test for multiple comparisons (n=6 in each group), Where PETEHB 1&2 are 150mg/kg &300mg/kg.

Fig1. (Stage-1)

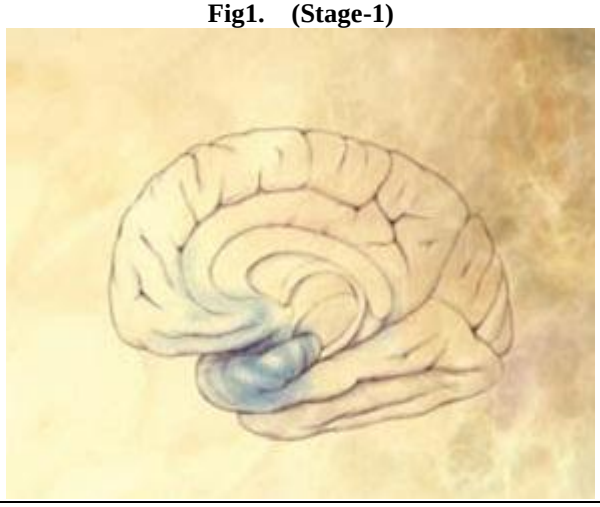


Fig 2. (Stage-2)

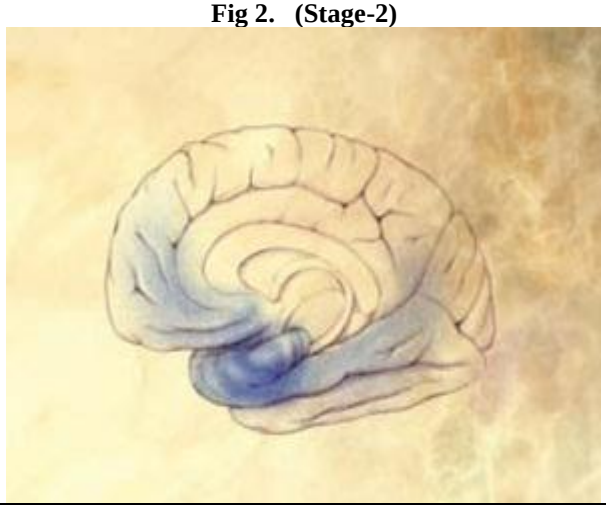


Fig 3. (stage-3)

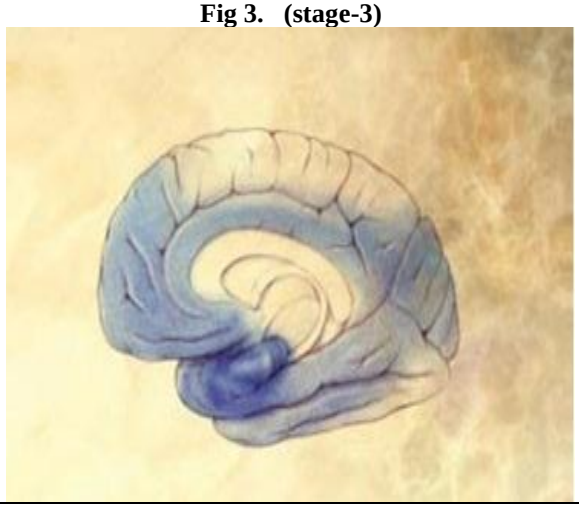


Fig 4. Effect of pet ether extract of *Hibiscus-rosa-sinesis* on Rectangular maze

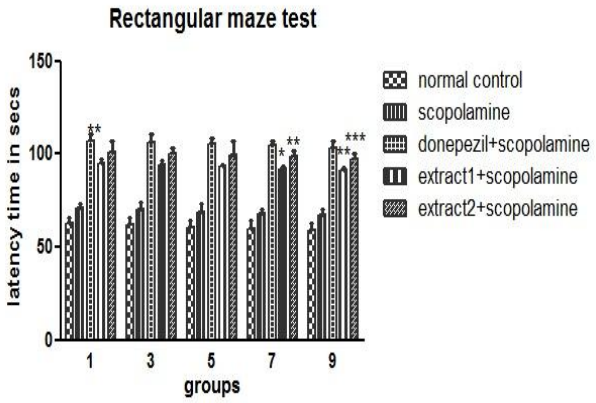


Fig 5. Effect of pet ether extract of *Hibiscus-rosa-sinesis* on Morris water maze

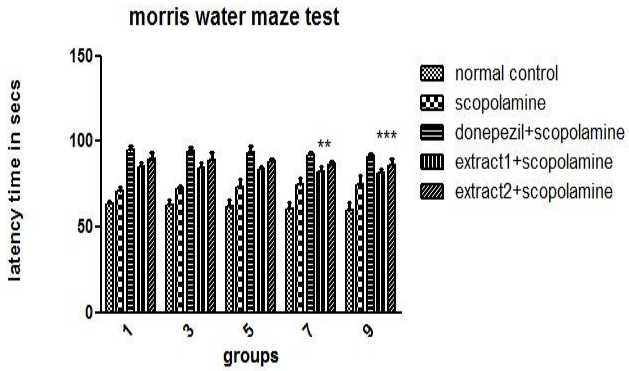


Fig 6. Effect of pet ether extract of *Hibiscus rosa sinesis* on Locomotor activity

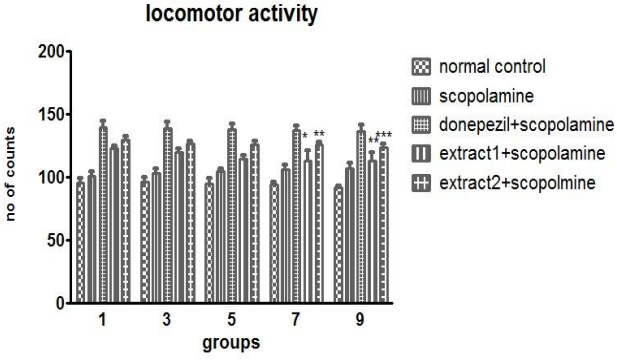
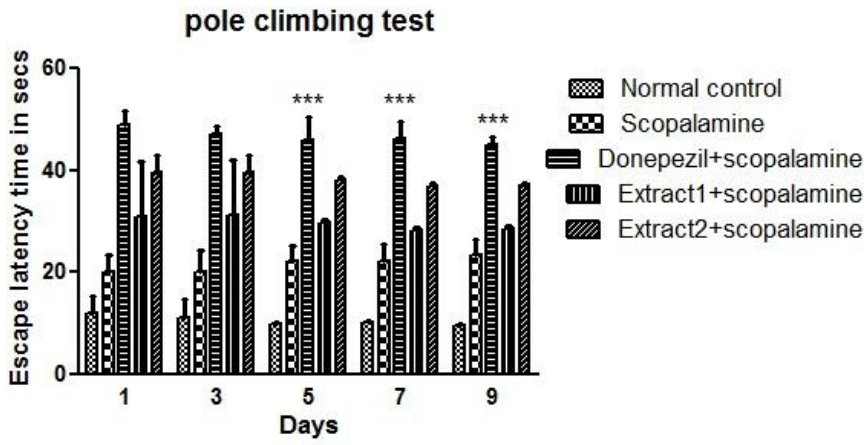


Fig 7. Effect of pet ether extract of *Hibiscus-rosa-sinesis* on Pole climbing



CONCLUSION

In the present study it was demonstrated that Pet extract of *hibiscus rosa sinesis* had a potential therapeutic effect in improving the memory in mice by a decrease in transfer latency time in case of Morris water maze, Rectangular maze, Pole climbing, Locomotor activity and Neuroprotective action through inhibition of Acetylcholinestrease, lipid peroxidation and elevating endogenous antioxidant enzymes such as Catalase which shows Antioxidant and Neuroprotective activity. The present data indicates that hibiscus rosa-sinesis flowers tenders protection against Scopolamine induced cognitive decline rats.

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None.

CONFLICT OF INTEREST

Authors declared no conflict of interest

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