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SCREENING OF THE EHANOLIC EXTRACT OF RHIZOMES OF *ACORUS TATARINOWII* SCHOTT ON NOOTROPIC AND ITS INFLUENCE ON BRAIN CHOLINERGIC SYSTEM OF RATS

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ABSTRACT

Dementia is a syndrome of failing memory and other intellectual functions with little or no disturbance in consciousness. Degeneration of the cerebral neurons is one of the commonest and vital causes for dementia with increasing age, thereby leading to deterioration in the 'quality of life' in elderly. Traditionally rhizomes of *Acorus tatarinowii* Schott belonging to family Acraceae. The rhizomes of *Acorus tatarinowii* Schott has been historically used to treat neurodegenerative diseases in china for thousands of years. But its effect on learning and memory is not scientifically proven. The present study was carried out to study the effect of ethanolic extract of *Acorus tatarinowii* Schott on memory deficits caused by Scopolamine in rats. In wistar rats of either sex, amnesia was induced by subjecting Scopolamine (0.3mg/kg i.p) for seven days. Ehanolic extract of *Acorus tatarinowii* Schott (500mg/kg, p.o) was evaluated for its nootropic activity in terms of Transfer Latency (TL) by using elevated plus maze and step down passive avoidance test. Rats were sacrificed at the end of study and Acetyl Cholinesterase (AChE) enzyme activity was estimated. The extract showed significant improvement in 'learning' and 'memory' as compared to control group in all the models and also showed significant reduction in acetylcholine-esterase activity.

Keywords: Amnesia, Transfer Latency, *Acorus tatarinowii* Schott, Acetylcholine esterase, scopolamine, nootropic

INTRODUCTION

The traditional system of medicine, Ayurveda and Unani systems are based on the use of plants **[1]**. Man has been dependent on the higher plants as a source of food and medicine by experience and experimentation. He discovered over time that the different plants possess different chemical composition. The rhizomes of *Acorus tatarinowii* Schott has been historically used to treat neurodegenerative diseases in china for thousands of years **[2]**.

The present study was designed to evaluate the effect of ethanolic extract of *Acorus tatarinowii* Schott on memory disorders, where the experimental amnesia was produced by subjecting Scopolamine. Transfer latency (*using elevated plus maze*), step-down latency (*using step-down apparatus*) were used to assess the memory.

The study was also performed to evaluate the effect of the extract on the Acetyl Cholinesterase Enzyme (AChE) activity in different parts of the rat brain as an additional parameter to establish the correlation between ACh and memory.

MATERIALS AND METHODS

Drugs:

Mentat: A poly herbal preparation containing around 25 different herbs, and is a proven memory enhancing drug available in the market. It was procured from Himalaya Herbal Healthcare, Bangalore **[3]**.

Scopolamine: An antimuscarinic agent for induction of loss of memory. It was purchased from Sigma Chemicals, USA **[4]**.

Collection and extraction of medicinal plants:

The dried rhizomes *Acorus tatarinowii* Schott of was gifted by Dr. K. S. Laddha Sir, Professor of Pharmacognosy, Institute of Chemical Technology, Matunga (E), Mumbai.

The dried rhizomes were later powdered and then used for the extraction process. The ethanolic extract of plant material was obtained and used as the test drugs for the evaluation of memory enhancing activity.

Reagents:

The reagents required for acetylcholine esterase enzyme estimation were:

- Acetylthiocholine iodide – Sigma Aldrich, Germany
- Ellman's Reagent [5,5′-Dithiobis(2-Nitrobenzoic acid)] – Sigma Aldrich, Germany

Chemicals:

- Sodium dihydrogen orthophosphate – Research Lab Fine Chem, Mumbai, India

- ♦ Disodium hydrogen phosphate – Research Lab Fine Chem, Mumbai, India
- ♦ Scopolamine - Sigma Chemicals, USA.
- ♦ CMC - 1%

Instruments used:

- ♦ **E**lectronic Digital Balance
- ♦ Digital pH meter
- ♦ Electroconvulsiometer- Mohit scientific, Ambala, India,
- ♦ High Speed Tissue Homogenizer – Usha instruments and chemicals, Ambala, India.
- ♦ UV Visible Spectrophotometer - Germany
- ♦ Cooke's Pole Climbing response apparatus - Mohit scientific, Ambala, India.
- ♦ Elevated Plus Maze - Mohit scientific, Ambala, India,

Procurement of Experimental Animals

- Healthy wistar rats (Non–Pregnant and nulliparous Females) weighing about 85 - 100 gm were collected from Crystal Biological Solutions, Pune, India.
- The animals were feed with Pelleted Standard feed and distilled water ad libitum. All the animals were housed in well polypropylene cages with stainless steel grill top. The animals were kept under room temperature between 22 ± 3^0 C, relative humidity 55 $\pm$ 5 % and illumination cycle set to 12 hours light and 12 hours dark.
- The animals were acclimatized to the laboratory condition for one week before starting the experiment. For experimental purpose the animals were kept fasting overnight but allowed for access to water.
- All the pharmacological experimental protocols were approved by CPCSEA committee of Crystal biological Solution, Pune in their IAEC meeting. (REGISTRATION NO. 2030/PO/RcBiBt/S/18/CPCSEA)

METHOD

Extraction of Plant material:

The dried rhizomes *Acorus tatarinowii* Schott were later powdered and used for the extraction process. The extraction was carried out by maceration process taking absolute ethanol (99.5%) as the solvent. Later, the extract was condensed on water bath to remove excess of solvent and then dried using flash evaporator to maximum dryness. The extract obtained was in the form of thick paste due to the presence of resinous matter. The extract was labeled and stored in airtight glass container at 4ºC throughout the study.

Preparation of Drugs:

Extract:

The extract was weighed and triturated with CMC (1%) and then was suspended in distilled water quantity sufficient to produce a suspension of the strength: 100mg/ml.

Dose: 500mg/kg b.w. by oral route.

Mentat:

The tablets were crushed and used for preparing the drug suspension. Specified quantity of Mentat powder was weighed and mixed with CMC (1%), triturated well and suspended in distilled water quantity sufficient to produce a suspension of 10mg/ml and was administered orally at a dose of 100mg/kg b.w.

Scopolamine: The scopolamine was administered intraperitoneally. The ampoule containing a solution of 20mg/ml was diluted with distilled water in a volumetric flask to get the final volume of 100ml with a final concentration of 0.2mg/ml and was administered at a dose of 0.3mg/kg b.w **[5]**.

Solutions used in Ellman's Method for Estimation of Acetyl Cholinesterase enzyme activity:

Phosphate buffer: 0.05M Phosphate Buffer (pH – 7.2)

Solution A: 6.85g Sodium dihydrogen orthophosphate dissolved in 100ml distilled water.

Solution B: 13.40g Disodium hydrogen phosphate dissolved in 100ml distilled water.

Solution A was mixed with Solution B until pH reached 7.2 and then was diluted in a ration of 1:10 with distilled water. This diluted solution was used for estimation.

Substrate: Acetyl thiocholine iodide (0.075M Solution)

21.68 mg/ml solution was prepared in 0.05M Phosphate buffer pH 7.2. The solution was used successfully for 10 – 15 days by keeping it in the refrigerator.

Ellman's Reagent: 5, 5′-Dithiobis (2-Nitro benzoic acid) [DTNB] (0.01M Solution)

19.8mg/5ml (3.96mg/ml) solution was prepared in 0.05M Phosphate buffer pH 7.2. At this pH, the reagent was more stable and was used successfully for 2 – 3 days by keeping it in the refrigerator.

Grouping of Animals:

The animals were divided into 6 groups, each consisting of 6 rats, viz;

Group 1 – Normal Control: Treated with vehicle.

Group 2 – Positive Control: Treated with extract of *Acorus tatarinowii* Schott

Group 3 – Positive Control: Treated with Mentat (Standard Drug).

Group 4 – Negative Control: Scopolamine Induced.

Group 5 – Treatment Group: Scopolamine + Treated with extract of *Acorus tatarinowii* Schott

Group 6 – Treatment Group: Scopolamine + Treated with Mentat (Standard Drug).

Experimental Schedule:

All the animals were dosed once in a day with respective drugs for seven consecutive days. Group-1 animals received only the vehicle (1% CMC), Group-2 received test drug only, Group-3 received standard drug only, Group-4 received only Scopolamine (0.3mg/kg b.w.)

Group-5 and Group-6 received both Scopolamine and respective drug treatment.

The animals were trained on the 0 (zero) day and the acquisition of memory was tested on the day 1, later the animals were subjected to induction followed by drug treatment, that was continued for up to day 7. Then, the animals were subjected for the retention test on the day 7. Soon after the passive avoidance task (step-down latency) elevated plus maze (transfer latency) and open field behavioral test the animals were sacrificed for Acetyl Cholinesterase enzyme estimation.

In-vivo method:

Procedure:

Amnesia was induced in animals by administrating Scopolamine (0.3mg/kg b.w.) intraperitoneally.

Drug induced (Scopolamine) amnesia: [6]

Scopolamine is a powerful muscarinic antagonist capable of crossing blood brain barrier, acts both peripherally by blocking the receptors for ACh at the synapse. It impairs memory storage of new information (short term memory) and learning acquisition. The dose of 0.3mg/Kg is approved to produce cognitive and memory changes without causing debilitating peripheral anticholinergic effect. Though the several models for amnesia are available, but the scopolamine induced memory deficits has been proposed to have symptomatological similarities with AD and related disorders.

Models used for Screening of memory: [6-7]

1. **Step-down Apparatus (Step-down Latency):**

Pole climbing apparatus chamber is used with little modification for passive avoidance response where the pole is replaced by a wooden platform fixed on electrified grid floor. When rats stepped off the platform, they receive a continuous foot shock from grid floor. The normal reaction of rat was to jump back to the wooden platform. After about 4-5 trials the animals acquired the passive avoidance response and they refrained from stepping down. The criterion was reached when the animal remained on the platform for at least 60s.

The animals were then subjected for the passive avoidance test on the day-1, and the time was noted down for step down latency taking 60s as the cut-off period. 5min. later, the animals of Group – 4, 6 and 7 received electroshock of 150mA for 0.2s through a pair of ear electrode from an Electroconvulsiometer and then the animals were dosed with respective drug and kept in their home cage. Similarly, animals of Group –5, 8 and 9 received scopolamine i.p. (0.3mg/kg b.w.) and then were dosed with respective drug and returned to their home cage. The electroshock/scopolamine and dosing with drug continued for up to 7 days and on 7th day, the animals were subjected to the retention test 30min. after the last dose, for evaluating the step-down latency keeping the time period of 60s as cut-off criterion.

2. Elevated Plus Maze (Transfer Latency):

An elevated plus maze consists of two open arms (50 x 10cm) and two closed arms (50 x 10 x 40cm) with an open roof. The maze was elevated to a height of 50cm. The animals were individually placed at the end of either of the open arms and the time taken for the animal to move from open to closed arm (Transfer latency, TL) was taken as the criterion of task . If the animal didn't move into the closed arms it was later pushed into one of the closed arm. The animals were allowed to explore the apparatus for 30s.

After 24h of the first exposure; TL was again noted on the day-1 of the study for determining the acquisition. The criterion was reached when the animal moved into the closed arms in very short period keeping the cut-off time of 60s (as maximum time taken for moving from open arms to closed one). Five minutes later the animals of Group – 4, 6 and 7 received electroshock of 150mA for 0.2s through a pair of ear electrode from an Electroconvulsiometer and then the animals were dosed with respective drug and kept in their home cage. Similarly, animals of Group – 5, 8 and 9 received scopolamine i.p. (0.3mg/kg b.w.) and then were dosed with respective drug and returned to their home cage. The electroshock/scopolamine and dosing with drug continued for up to 7 days and on 7th day, the animals were subjected to the retention test 30min. after the last dose, for evaluating the step-down latency keeping the time period of 60s as cut-off criterion.

In-vitro method:

Estimation of Acetyl Cholinesterase Enzyme Activity of Discrete Parts of Brain:

Dissection:

Exactly 60min. after the electroshock and scopolamine treatment the rats were

decapitated by Gillette, and the whole brain were taken out quickly. The cerebral cortex, cerebellum, medulla oblongata and midbrain were dissected out as described by Glowinsky and Iverson 1966 suspended in phosphate buffer and weighed accurately.

Preparation of Brain Homogenate:

Procedure:

The different regions of the brain viz. cortex, cerebellum, medulla oblongata and midbrain were homogenized in a tissue homogenizer. [Approximately 20mg of tissue per ml of phosphate buffer pH 7.2].

A 0.4ml aliquot of this homogenate was added to a cuvette containing 2.6ml phosphate buffer (pH 7.2, 0.05M). To this, 100µl of Ellman's reagent was added and then taken into the photocell. The absorbance was set at 412nm when the fluctuations stopped.

Of the substrate (Acetyl thiocholine iodide) 20µl was added. A change in the absorbance per minute was noted.

The rate of moles of substrate hydrolyzed per minute per gram of tissue was later calculated as per the following equation:

$$R = \frac{\Delta A}{1.36\,(10^4)} \times \frac{1}{(400/3120)\,C_0} = 5.74(10^{-4})\,\frac{\Delta A}{C_0}$$

Where, ΔA = Change in absorbance per minute (mean change in absorbance from the 1st to 7th min. was taken); C_0 = Original concentration of the tissue; R = Rate in moles substrate hydrolyzed per minute per gram of tissue

Statistical Analysis:

The step-down latency and transfer latency were analyzed using the Student's paired't' test (two tailed). A probability level of $P<0.01$ was considered as significant.

The AChE activity of different groups were analyzed using One Way Analysis of Variance (ANOVA), followed by Dunnett's test for individual comparison of groups, viz.;

A probability level of $P<0.0001$ for One way ANOVA was considered as significant, and for post test (Dunnett's test), a probability level of $P<0.01$ was considered as significant.

RESULT AND DISCUSSION

Effect on Transfer Latency (using Elevated Plus maze):

The animals were subjected to transfer latency (TL) to evaluate the retrieval of memory in behavioral paradigm after a

period of 7 days of acquisition trial, to know the effect of extract on the long term memory. TL of day 1 reflects learning behavior of the animals whereas; TL of day 7 reflects the retention of the information or memory.

The Normal Control animals have showed significant retrieval of the memory in this behavioral paradigm. In the Positive Control group, the animals treated with extract of Mentat , Ethanolic extract of *Acorus tatarinowii* schott. Mentat produced significant (**P<0.01, *P<0.05) activity. Ethanolic extract of *Acorus tatarinowii* schott produced highly significant (***P<0.0001, **P<0.01) activity.

In the Negative Control group, the animals exposed to Scopolamine produced highly significant (***P<0.0001) loss of memory in behavioral paradigm, which resulted in increase in TL on day 7 when compared to day 1.

In the Treatment Group, the animals exposed to both Scopolamine and treated with Mentat and Ethanolic extract of *Acorus tatarinowii*, Produced highly significant (***P<0.0001) retrieval of memory in PAT (Passive Avoidance Task).

Effect on Step-down Latency (SDL)

The Normal Control animals showed significant (**P<0.01) retrieval of memory in passive avoidance task (PAT).

In the Positive Control group, the animals treated with Standard (Mentat) and Ethanolic extract of *Acorus tatarinowii*. Mentat showed significant (**P<0.01), Ethanolic extract of *Acorus tatarinowii* showed significant (*P<0.05) retrieval of memory in PAT.

In Negative Control group, scopolamine induced animals produced significant (P<0.05) loss of memory in PAT.

In the Treatment Group, the animals exposed to both Scopolamine and treated with Mentat and Ethanolic extract of *Acorus tatarinowii* schott produced highly significant (***P<0.0001) retrieval of memory in PAT.

Acetyl Cholinesterase (AChE) Enzyme Activity:

The animals were sacrificed at the end of the study period of 7 days after last dosing and evaluating SDL and TL, to dissect and isolate the brain. Then different parts of brain were separated and subjected for the estimation of AChE enzyme activity and the results were expressed as Mean±SD (moles x 10^{-6}/minute/gram of tissue).

The animals of Positive Control group, Negative Control (Scopolamine Exposed) groups were compared with Normal Control group, whereas scopolamine induced + Treatment group was compared to Negative Control Scopolamine induced group.

The animals of Positive Control group treated with Ethanolic extract of *Acorus tatarinowii* (3.28±0.73, 2.87±1.13, 3.14±1.21

and 3.28±1.32) and Mentat (3.76±0.54, 2.87±0.78, 2.80±0.60 and 2.67±0.96) produced significant (P<0.01) reduction of AChE enzyme activity in comparison with Normal Control (3.62±0.80, 3.21±0.48, 3.14±0.50 and 3.28±0.37) in different parts of brain viz., medulla, cortex, cerebellum and midbrain respectively.

The animals of Negative control group exposed to Scopolamine (3.83±0.92, 4.92±1.16, 4.65±1.12 and 4.31±1.15) showed highly significant (***P<0.0001) increase in ACh Enzyme activity compared to the Normal Control group.

In the Treatment group, the animals exposed to Scopolamine and treated with Mentat (3.08±0.77, 3.28±0.64, 2.53±0.73, 2.80±0.40) Ethanolic extract of *Acorus tatarinowii* (2.67±0.50, 3.08±0.77, 2.94±0.80 and 3.08±0.43) produced significant (***P<0.0001) reduction in Acetyl Cholinesterase Enzyme activity in comparison with negative control Scopolamine exposed group.

Table 1: Summary of Transfer Latency And Step-Down Latency of *Acorus Tatarinowii* Schott

Group	Sub Group	Transfer Latency (in Sec.) in Elevated Plus Maze		Step Down Latency (in Sec.) in Step-Down Apparatus	
		Before	After	Before	After
		Day 1	Day 7	Day 1	Day 7
Normal Control	Normal control	21.00±8.43	21.5±4.57	22.00±2.10	25.00±2.28
Positive Control	Treated with Ethanolic extract of *Acorus tatarinowii* schott	29.00±5.22	27.67±2.34	24.83±2.40	29.17±2.40**
	Treated with Standard (Mentat)	41.67±5.82	34.00±3.22*	25.83±2.48	32.00±4.15**
Negative Control	Scopolamine Induced	24.33±10.17	25.50±6.47	24.67±2.34	12.67±1.97***
Treatment Group Scopolamine + Drug Treated	Treated with Ethanolic extract of *Acorus tatarinowii* schott	48.33±8.96	37.5±4.51**	25.33±2.16	44.67±4.80***
	Treated with Standard (Mentat)	21.50±9.85	25.33±6.50	23.83±3.92	36.83±3.31**

N = 6; Values are expressed as Mean±SD; Student's't' Test – Paired, two tailed; ***P<0.0001, **P< 0.01, *P< 0.05

Table 2: Summary of Acetylcholinesterase Enzyme Activity In Different Parts Of Rat Brain

Group	Sub-Group	Acetylcholinesterase Enzyme activity (Mean± SEM) (in moles x 10^{-6}/min/g of tissue)			
		Medulla	Cortex	cerebellum	Midbrain
Normal Control	Normal control	3.85±0.56	3.28±0.44	3.20±0.47	3.28±0.36
Positive Control	Treated with Ethanolic extract of *Acorus tatarinowii* schott	3.62±0.55****	4.17±0.98****	3.14±0.92****	3.83±0.85****
	Treated with Standard (Mentat)	3.76±0.54****	2.87±0.78****	2.80±0.60****	2.67±0.96****
Negative Control	MES Induced	4.99±0.88****	4.92±0.37****	4.65±0.85****	5.54±0.77****
Treatment Group Scopolamine + Drug Treated	Treated with Ethanolic extract of *Acorus tatarinowii* schott	2.67±0.50††††	3.08±0.77††††	2.94±0.80††††	3.08±0.43††††
	Treated with Standard (Mentat)	3.08±0.77††††	3.28±0.64††††	2.53±0.73††††	2.80±0.40††††

N = 6; Values are expressed as Mean± SD; One Way Analysis of Variance (ANOVA) followed by Dunnett's't' Test; **P<0.01 – Compared to Normal Control; †† P<0.01– Compared to Negative Control – Scopolamine Induced.

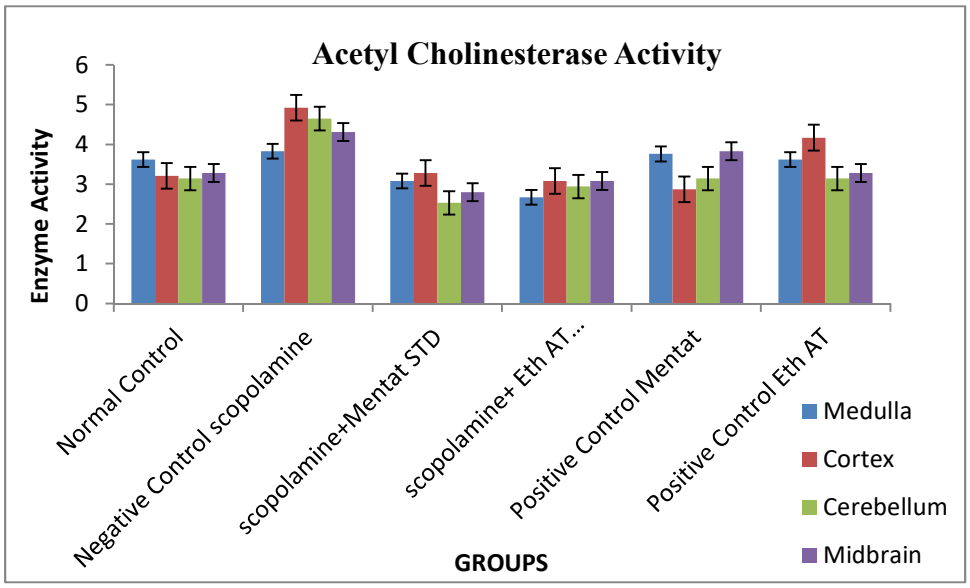

Figure 1: Mean Acetyl Cholinesterase Enzyme activity of *Acorus tatarinowii* schott, in different parts of Rat brain

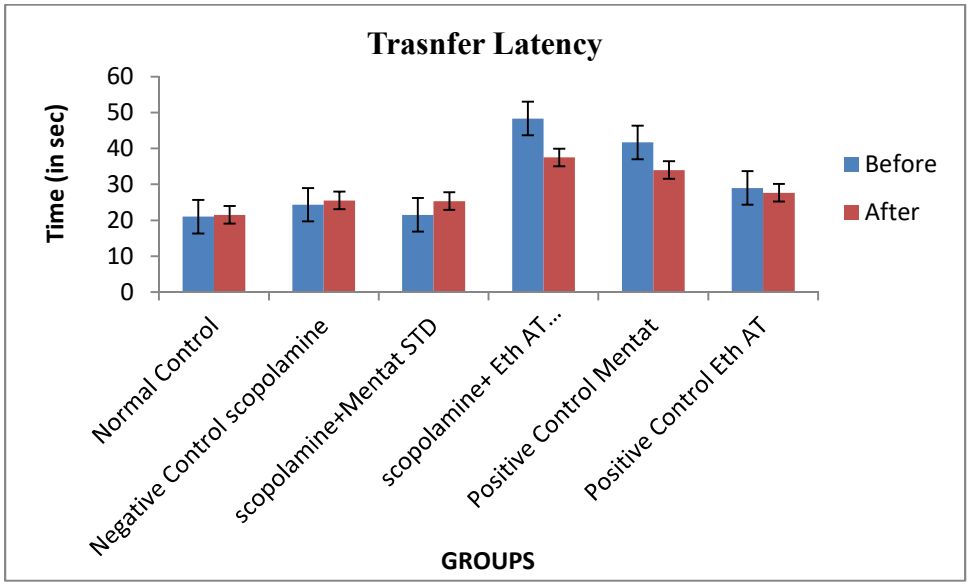

Figure 2: Mean Transfer Latency (*Using Elevated plus Maze*) in different groups of Animals

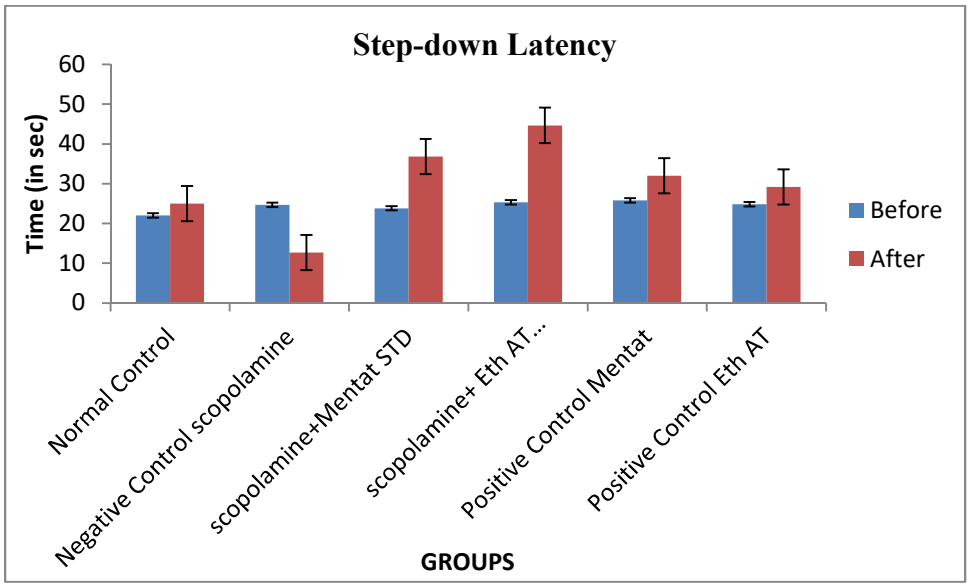

Figure 3: Mean Step-down Latency (*Using Step - Down Apparatus*) in different groups of Animals

CONCLUSION

In the present study, Ethanolic extract of ***Acorus tatarinowii* schott** possess cognitive enhancement activity. The cognitive enhancement activity is further supported by the decrease in AChE enzyme activity in different regions of the brain.

Further studies to evaluate the efficacy of the extract of ***Acorus tatarinowii* schott** in neuro- protection and to establish the therapeutic values in the treatment of dementia, where combination of this extract along with other drugs like Cholinesterase inhibitors might also represent future alternative to the present monotherapy. Extensive preclinical and clinical work is desirable.

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