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SCREENING OF THE EHANOLIC EXTRACT OF ROOTS OF *ASTRAGALUS MEMBRANACEOUS* BUNGE ON NOOTROPIC AND ITS INFLUENCE ON BRAIN CHOLINERGIC SYSTEM OF RATS

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ABSTRACT

Recently memory complaints and memory disorders are becoming more prevalent due to various factors such as natural (ageing, physical and mental stress), environmental (excess levels of carbon monoxide, carbon dioxide, methyl mercury in atmosphere, aluminium in foods), iatrogenic (electroconvulsive shock therapy and use of certain CNS depressants). nootropic drugs, meaning drugs that enhance mental performance. In ancient study roots of *Astragalus membranaceus* Bunge family Fabaceae gives good protection against aging and age related diseases. 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 roots of *Astragalus membranaceus* on memory deficits caused by MES and Scopolamine in rats. In wistar rats of either sex, amnesia was induced by subjecting to MES (150mA for 0.2s) through corneal electrodes for seven days. Ehanolic extract of roots of *Astragalus membranaceus* Bunge (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. The present study indicates that treatment with roots of *Astragalus membranaceus* Bunge extract enhances the memory function and this could be mediated through brain cholinergic system.

Keywords: Amnesia, Transfer Latency, *Astragalus membranaceus* Bunge, Acetylcholine esterase, MES, learning and memory

INTRODUCTION

The modern science has accepted the potential of plant kingdom as a source of new biodynamic constituents and phytochemicals often serve a chemical models or templates for the design and synthesis of new drug entities. The growing concerns in the recent past over the toxic effects of various synthetic drugs have forced pharmaceutical researchers and physicians to use herbal drugs. As a result of modern isolation techniques and pharmacological testing procedures, new plant drugs usually find their way into medicines as purified substances **[1]**.

Cognition in a broad sense means information processing. It denotes a relatively high level of processing of specific information including thinking, memory, perception, motivation, skilled movements and language **[2]**.

The present study was designed to evaluate the effect of ethanolic extract of roots of *Astragalus membranaceus* on cognitive disorders, where the experimental amnesia was produced by Electroshock induced memory disruption. Elevated plus maze and Passive avoidance task (PAT) 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]**.

Collection and extraction of medicinal plants:

The dried roots of Astragalus membranaceous Bunge was gifted by Dr. K. S. Laddha Sir, Professor of Pharmacognosy, Institute of Chemical Technology, Matunga (E), Mumbai.

The dried roots was 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
- ♦ 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
- ♦ Cooke's Pole Climbing response apparatus - Mohit scientific, Ambala, India.
- ♦ Elevated Plus Maze - Mohit scientific, Ambala, India,

TEST SYSTEM AND MANAGEMENT:

(Procurement of Experimental Animals)

Species	**:**	**Rat**
Strain	**:**	**Wistar**
Source	**:**	**CRYSTAL BIOLOGICAL SOLUTIONS**
Age and Sex	**:**	**Young Male of 8 to 12 week.**
Body weight range	**:**	**150-200 gm**
Environmental Conditions	**:**	**Room temperature between 22 ± 3^0 C, relative humidity 55 $\pm$ 5 % and illumination cycle set to 12 hours light and 12 hours dark.**
Accommodation diet and Water	**:**	**Used polypropylene cages with stainless steel grill top.**
	:	**Pelleted Standard feed and distilled water ad libitum.**

METHOD

Extraction of roots of Astragalus membranaceous Bunge:

The dried roots 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 **[4]**.

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 Astragalus membranaceous

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

Group 4 – Negative Control: MES Induced.

Group 5 – Treatment Group: MES + Treated with extract of Astragalus membranaceous Bunge

Group 6 – Treatment Group: MES + 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 electric shock (150mA for 0.2s), Group-5 and Group-6 received both electric shock 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 Electroshock induced amnesia

Electroshock induced amnesia [5, 6]:

1. Amnesia was induced in experimental animals by electroshock stimulation by silver corneal electrodes induced clonic-tonic seizures and impaired memory.
2. The electroshock was applied immediately after the training trials in the task being tested.
3. An electroshock (150mA for 0.2sec) was applied through commercially available electro- stimulators.
4. After the Amnesia induction different group of animals were treated with the different extraction of medicinal plants provided by the sponsors for next 7 days.

The following screening methods will be employed for assessment of memory: [7, 8]

Elevated Plus Maze:

1. 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 were used.
2. 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 was taken.

Step-Down Passive Avoidance:

1. The cage with a grid floor and shock free zone in the center was used.
2. The animals were individually placed in the cage and the time taken for the animal to move from shock grid to shock free zone.

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

1. The whole brain was taken out quickly. The cerebral cortex, cerebellum, medulla oblongata and midbrain were dissected

out and suspended in phosphate buffer and 20 mg were weighed accurately.

2. 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].
3. The homogenate was centrifuged for 5min at 1000 rpm and supernatant was collected for the estimation of acetyl cholinesterase activity.
4. 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 450nm when the fluctuations stopped.
5. Of the substrate (Acetyl thiocholine iodide) 20µl was added.
6. 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:

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

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 Mentat and Ethanolic extract of *Astragalus membranceous Bunge* Mentat produced significant (**P<0.01, *P<0.05) activity. Ethanolic extract of *Astragalus membranceous Bunge* produced Highly significant (***P<0.0001, **P<0.01) activity.

In the Negative Control group, the animals exposed to MES; 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 MES and treated with Mentat produced significant (**P<0.01) activity and animals treated with extract of Ethanolic extract of *Astragalus membranceous Bunge* did produced Highly significant (***P<0.0001) retrieval of memory in behavioral paradigm, but the animals showed reduction of time taken to perform the task in Elevated plus maze.

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 extract of In the Positive Control group, the animals treated with extract of Mentat , Ethanolic extract of *Astragalus membranceous Bunge,* Mentat showed significant (**P<0.01) Ethanolic extract of *Astragalus membranceous Bunge,* showed highly significant (***P<0.0001) retrieval of memory in PAT.

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

In the Treatment Group, the animals exposed to both MES and treated with standard Mentat and Ethanolic extract of *Astragalus membranceous Bunge.* Ethanolic extract of *Astragalus membranceous Bunge,* increased the SDL period and showed highly significance (***P<0.0001) retrieval of memory.

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 (MES Exposed) and Negative Control (Scopolamine Exposed) groups were compared with Normal Control group, whereas the MES induced + Treatment Group was compared to Negative Control (MES induced group)

The animals of Positive Control group treated with Ethanolic extract of *Astragalus membranceous Bunge,* (3.69±1.00, 3.42±1.46, 2.800±.80 and 3.83±1.12) 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 MES (4.99±0.80, 4.92±0.37, 4.65±0.85, 5.54±0.77) 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 MES and treated with Standard (Mentat) (3.21±0.54, 3.35±1.31, 3.55±1.44 and 3.35±1.33) and extracts of Ethanolic extract of *Astragalus membranceous Bunge,* (1.44±0.91, 1.23±0.52, 1.23±0.73 and 1.16±0.80), significantly (***$P<0.0001$) decreased the Acetyl Cholinesterase Enzyme activity in comparison with negative control MES exposed group in different parts of brain viz. medulla, cortex, cerebellum and midbrain respectively.

Table 1: Summary of Transfer Latency And Step-Down Latency

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 *Astragalus membranceous Bunge*,extract	45.5±6.63	35.83±3.43**	26.50±2.07	34.67±3.14***
	Treated with Standard (Mentat)	41.67±5.82	34±3.22*	25.83±2.48	32.00±4.15**
Negative Control	MES Induced	15.5±8.16	17.5±5.09	21.17±2.14	13.00±1.79***
Treatment Group MES + Drug Treated	Treated with *Astragalus membranceous Bunge*,extract	43.00±12.13	16.17±4.62***	25.83±1.94	48.67±3.72****
	Treated with Standard (Mentat)	38.00±8.39	35.00±2.77	25.50±1.87	34.00±2.76**

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 Ache 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 *Astragalus membranceous Bunge*,extract	3.69±1.00****	3.42±1.46****	2.80±0.80****	3.83±1.12****
	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 MES + Drug Treated	Treated with *Astragalus membranceous Bunge*,extract	1.44±0.91$^{\#\#\#\#}$	1.23±0.52$^{\#\#\#\#}$	1.23±0.73$^{\#\#\#\#}$	1.16±0.80$^{\#\#\#\#}$
	Treated with Standard (Mentat)	3.21±0.54$^{\#\#\#\#}$	3.35±1.31$^{\#\#\#\#}$	3.55±1.44$^{\#\#\#\#}$	3.35±1.33$^{\#\#\#\#}$

N = 6; Values are expressed as Mean± SEM; 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 – MES Induced

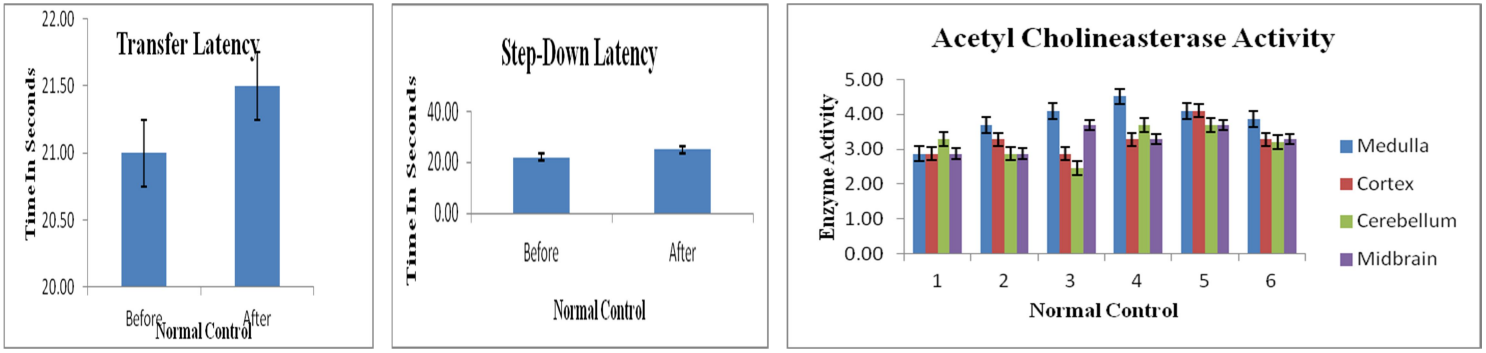


Figure 1: Transfer Latency (Using Elevated Plus Maze), Step-down Latency (Using Step- Down Apparatus) and AChE activity in Normal Control

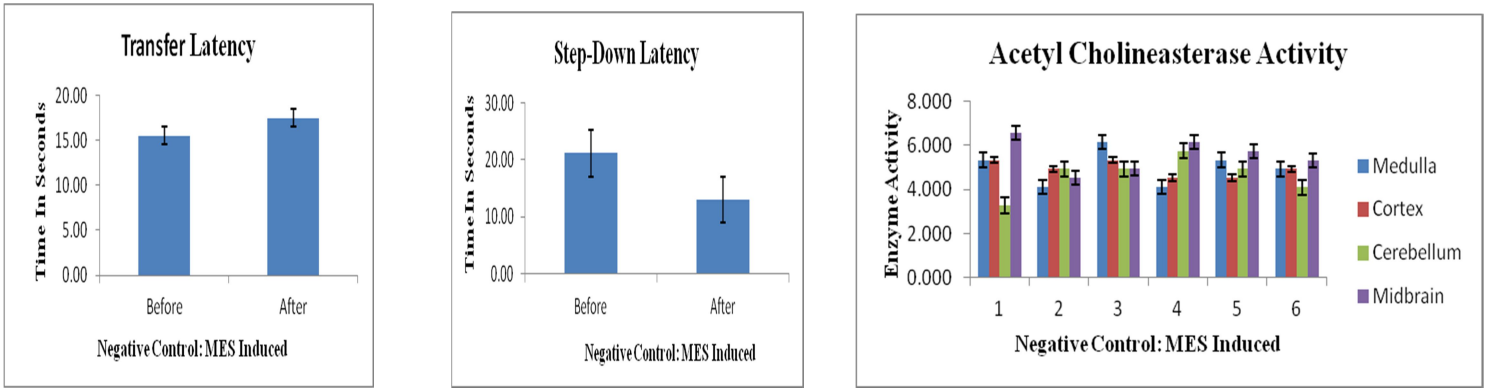
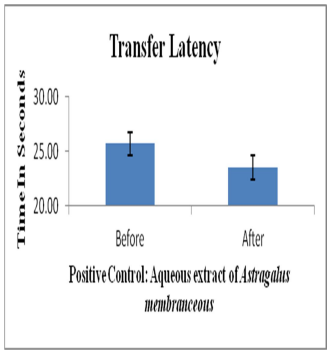
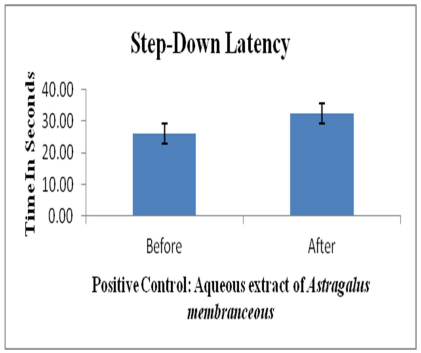


Figure 2: Transfer Latency (Using Elevated Plus Maze), Step-down Latency (Using Step- Down Apparatus) and AChE activity in Negative Control: MES Induced





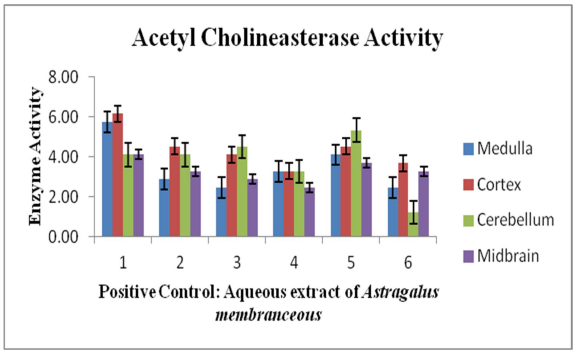
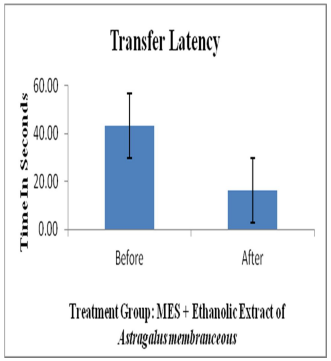
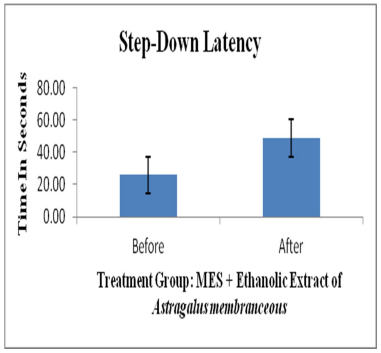


Figure 3: Transfer Latency (Using Elevated Plus Maze), Step-down Latency (Using Step- Down Apparatus) and AChE activity in Positive Control: Ethanolic extract of Astragalus membranceous Bunge





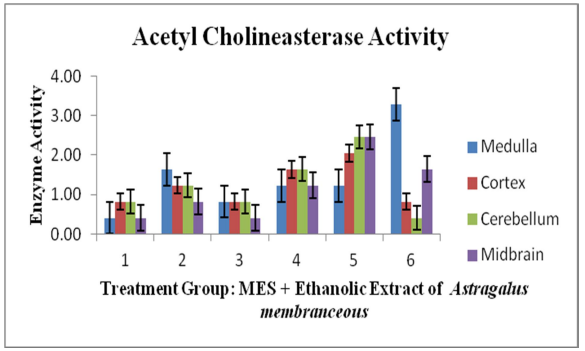


Figure 4: Transfer Latency (Using Elevated Plus Maze), Step-down Latency (Using Step- Down Apparatus) and AChE activity in Treatment Group: MES + Ethanolic extract of Astragalus membranceous Bunge

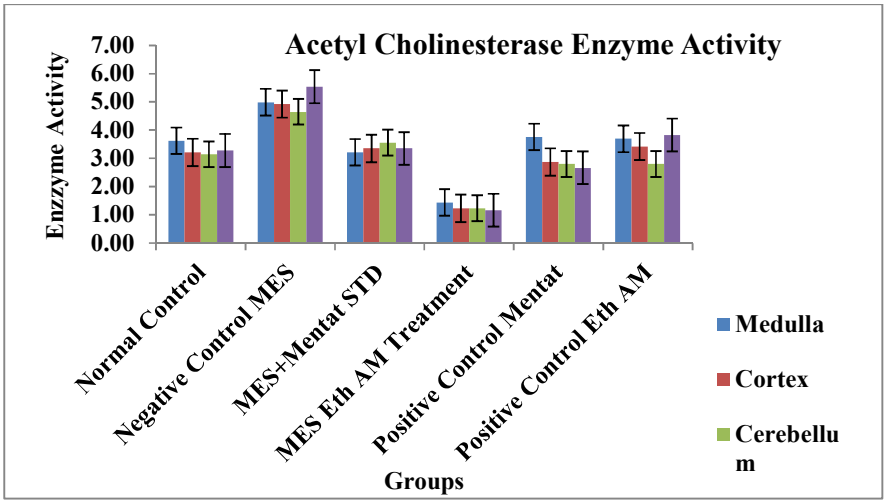


Figure 5: Mean Acetyl Cholinesterase Enzyme activity of *Astragalus membranceous Bunge,* in different parts of Rat brain

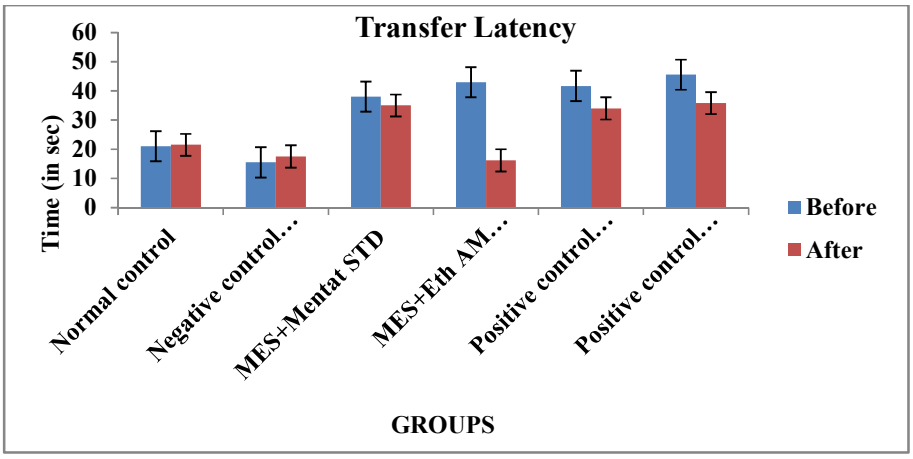


Figure 6: Mean Transfer Latency (*Using Elevated plus Maze*) in different groups of Animals

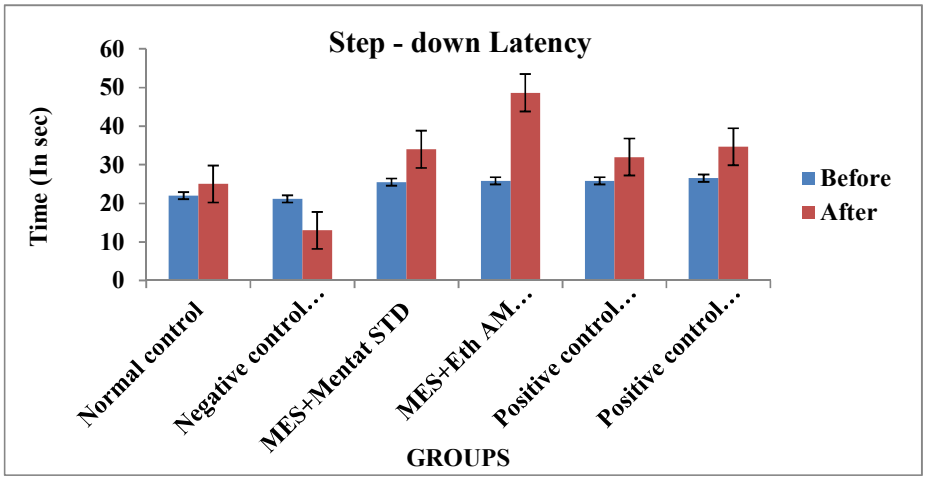


Figure 7: Mean Step-down Latency (*Using Step - down Apparatus*) in different groups of Animals

CONCLUSION

In the present study, Ethanolic extract of *Astragalus membranceous Bunge,* 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 *Astragalus membranceous Bunge,* 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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