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Research Article

Acute Toxicity Test and Nootropic Activity of Ethanolic Extract of *Astragalus membranaceus* Bunge on Scopolamine-Induced Amnesia in Rats

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Abstract

The dried roots of *Astragalus membranaceus* Bunge, belongs to the family fabaceae enhances the memory function and this could be mediated through brain cholinergic system. Historically in China it is used as a medicine for treatment of different types of diseases. It gives good protection against aging and age related diseases. *Astragalus membranaceus* Bunge root extract was investigated to reveal its nootropic effect and acute toxicity study. The powdered roots were tested for the phytochemical constituents. Carbohydrates, proteins, amino acids, glycosides, saponins, volatile oils, alkaloids, sterols and flavonoids were present. The acute toxicity study of ethanolic extract was carried out according to the OECD test guideline 423. There were no signs of toxicity of rats even with a maximum dose of 2000 mg/kg of the extract during 14 days. For evaluation of nootropic activity, ethanolic extract was administered by oral route to scopolamine induced amnesic rat model. The reduction of acetylcholinesterase activity in the rat brain supports the elevated plus maze (EPM) and passive avoidance test by improving memory function by lowering acetylcholinesterase activity. Present study shows *Astragalus membranaceus* Bunge root extract possesses significant nootropic activity in scopolamine induced amnesia rat models.

Keywords: *Astragalus membranaceus* Bunge, EPM, passive avoidance, nootropic, acetyl Cholinesterase and Scopolamine.

INTRODUCTION

Astragalus membranaceus Bunge is a medicinal plant, belongs to family Fabaceae, is one of the important herb from the Chinese material medica. Historically in China it is used as a medicine for treatment of different types of diseases. It gives good protection against aging and age related diseases. [1-7] As per OECD guidelines, toxicological studies are very essential in order to establish the safety and efficiency of a new drug prior to clinical use. [8] Acute toxicity tests are commonly used to determine LD50 of drugs and natural products. Many herbal medicines have been recommended for the treatment of Alzheimer. Traditional plant medicines are used throughout the world for a range of improvement of memory including *Astragalus membranaceus* Bunge.

There is a lack of scientific data regarding the effect of ethanolic extract of *Astragalus membranaceus* Bunge on learning and memory. The present investigation was therefore designed to evaluate the acute toxicity effect and memory enhancing properties of *Astragalus membranaceus* Bunge using two animal models, namely elevated plus maze and passive avoidance (Scopolamine- induced amnesia rat model) followed by determination of acetylcholinesterase enzyme (AChE) activity in rat brain.

MATERIALS AND METHODS

The plant materials were gifted by Dr. K. S. Laddha Sir, Professor of Pharmacognosy, Institute of Chemical Technology, Matunga (E), Mumbai. Authentication was done by Dr. Harshad M. Pandit, Ph.D. (Botany) (Formerly Head and Associate Professor of Botany) Andheri (West), Mumbai. The dried roots 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.

Preliminary Phytochemical investigation of *Astragalus membranaceus* Bunge

The preliminary phytochemical investigation has been undertaken on the roots of *Astragalus membranaceus* Bunge, to determine the presence or absence of organic constituents. The tests have been carried out according to standard technique. [9] The results were shown below.

Acute toxicity study of ethanolic extract of *Astragalus membranaceus* Bunge

Materials:

Test animals - Wistar rats (Non–Pregnant and nulliparous Females) weighing about 85 - 100 gm.

Test agents - Ethanolic extract of roots of *Astragalus membranaceus* Bunge.

Apparatus - Animal balance, Mice cages, oral gavage tube.

Dose schedule - 300mg/kg, 2000mg/kg and 2000mg/kg (body weight) of rats.

Method:

Acute oral toxicity study was performed according to the OECD (Organization for Economic Co-operation and Development) test guideline no. 423-Acute toxic class method. This is a sequential approach using three single-sex animals each stage. Depending on the mortality and morbidity status of the animal. Experiments were performed using healthy young adult female wister rats, nulliparous, non-pregnant and weighing 85 - 100 gm.

The study was performed in a stepwise manner.

Step 1

Three overnight fasting female Rats were administered the diluted extracts at the dose of 300 mg/kg body weight. The Rats were deprived of food overnight and 2 hours after the dosing. Water was allowed ad libitum. After dosing, all of the animals were monitored for 14 days.

Step 2

Three overnight fasting female Rats were administered the diluted extracts at the dose of 2000 mg/kg body weight. The Rats were deprived of food overnight before and 2 hours after the dosing. Water was allowed ad libitum. After dosing, all of the animals were monitored for 14 days.

Step 3

After confirmation of safety at 2000 mg/kg at previous step, 3 overnight fasting female Rats were again administered with the diluted extracts at the dose of 2000 mg/kg body weight. The Rats were deprived of food overnight before and 2 hours after the dosing. Water was allowed ad libitum. All the animals were observed for 14 days after dosing.

Observations

Animals were observed individually first 30, 60, 120, 180 and 240 minutes after dosing, with special attention and once daily thereafter, for a total of 14 days. All observations were systematically recorded with individual record being maintained for each animal. Signs of toxicity and mortality of the animal were recorded. Individual weight of animals was measured shortly before the test substance administered and once weekly. Weight changes were calculated and recorded.

Determination of nootropic activity of ethanolic extract from roots in scopolamine- induced amnesic rat model

Materials:

Test animals -

Young male wister rats of 8 to 12 week weighing 150-200 gm

Drugs and chemicals -

Mentat (100mg/kg b.w. orally), Scopolamine (0.3 mg i.p.), Acetylthiocholine iodide, CMC (1%), Disodium hydrogen phosphate, Ellman's Reagent [5,5′-Dithiobis(2-Nitrobenzoic acid)] and Sodium dihydrogen orthophosphate [10, 11]

Preparation of extract –

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. 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.

Grouping of animals:

Group 1 received distilled water to serve as control, Group 2 received extract of Astragalus membranaceous Bunge, Group 3 received Standard drug mentat (100mg/kg, p.o.), Group 4 received Scopolamine (0.3 mg i.p.), Group 5 received both Scopolamine and extract of Astragalus membranaceous Bunge, and Group 6 received both Scopolamine and mentat (Standard Drug).

Behavioral studies: [12-14]

Elevated plus maze

The plus maze was used to test learning and memory retention. The plus maze is made up of two open arms (50×10cm) intersected by two enclosed arms (40 cm high walls) of the same size. To give the equipment a plus sign look, the arms were linked with a centre square (10×10cm). The rats were split into six groups of six rats each at random. The maze was placed in a dimly lighted area 50 centimeters above the ground level. On day 1, a rat was put at the end of one of the open arms, facing away from the centre, and the time it took the animal to enter one of the closed arms has been recorded (Transfer latency (TL) on day 1). After 10–15 seconds in the enclosed arm, the rat was returned to its home cage. Similarly, after an interval of 7 days drug treatment, on day 7 TL was again recorded.

Passive avoidance test

The passive avoidance apparatus consisted of a Plexiglas box (30×30×40cm) with a steel rod grid floor. A wooden platform was erected in the grid floor's centre. The grid floor was subjected to intermittent electric shocks. Each rat was trained by gently placing it on the platform. Shock was administered for 15 seconds when the animal stepped down from the platform and set all of its paws on the grid floor. Animals had a training session and then 24h later each rat was placed on the platform and the SDL was measured as a passive avoidance behavior. The latencies indicated memory levels. An upper cut-off time of 60 s was set. Similarly after an interval of a fortnight of drug treatment, on day 7 SDL was again recorded.

Biochemical studies

Estimation of acetylcholinesterase enzyme activity in rat brain

Ellman et al. technique was used to determine acetylcholinesterase activity. Following drug treatment, the rats were disected and the brain was taken out. In a Teflon glass homogenizer, the brain tissue was homogenized in 0.1 M-phosphate buffer, pH 7.2. A 0.4mL aliquot of homogenate, 2.6mL phosphate buffer (0.1M, pH 8) and 0.1mL dithiobisnitrobenzoic acid (DTNB, 0.01M) made up the reaction mixture.

The reaction mixture consisted of a 0.4mL aliquot of homogenate, 2.6mL phosphate buffer (0.1M, pH 8) and 0.1mL of dithiobisnitrobenzoic acid (DTNB, 0.01M), incubated at room temperature for 5min. After the addition of the substrate acetylthiocholine iodide (0.075M), the absorbance was measured every min for 5min at 412nm using a spectrophotometer (UV-VIS spectrophotometer)

The rate of moles of substrate hydrolyzed per minute per gram of tissue was calculated by 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 = Initial 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. 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.

RESULTS

Preliminary Phytochemical investigation of *Astragalus membranaceus* Bunge

Sr. No.	Test	Test reagent	Obsevations	Inference
1	Carbohydrates	Molisch's test	Violet ring at the junction	+
		Benedict's test	Orange red ppt.	+
		Fehling's test	Red ppt.	+
2	Proteins and	Millons test	White ppt.	+
	Amino acids	Ninhydrin test	Blue colour	+
3	Phytosterols	Salkowski's test	Red or yellow colour	+
		LibermannBurchard test	Blue green colour	+
4	Cardiac Glycosides	Legals test	Colour changes	+
		Liebermann's test	Blue colour	+
		Keller-Killiani test	Bluish green colour	+
	Anthraquinone Glycosides	Borntrager's test	Colour changes	+
		Modified Borntrager's test	Pink color	+
	Saponins	Distilled water	Appearance of foam	+
5	Flavonoids	Lead acetate test	Yellow colour	+
		Alkaline reagent test	Yellow colour becomes colourless	+
		Shinoda test	Magenta colour	+
6	Alkaloids	Mayer's reagent	Yellow cream ppt.	+
		Dragendorff's reagent	Red ppt.	+
		Wagner's reagent	Brown/reddish brown ppt.	+
		Hager's reagent	Yellow ppt.	+
7	Phenolics and Tannins	Ferric chloride test	No bluish black colour	-
		Gelatin test	No white colour	-
8	Volatile oil			+

Acute oral toxicity study report:-

Step	Dose (mg/kg)	No. of Treated Rat	Terminally Sacrificed	Found Dead(X)
1	300	3	3	0
1	2000	3	3	0
2	2000	3	3	0
TOTAL	-	9	9	0

Necropsy

Not a single female rat shows any alterations in gross pathological study at the dose of 300 mg/kg and 2000 mg/kg.

It is certify that the LD_{50} value of "Acute oral toxicity for ethanolic extract of *Astragalus membranaceus* Bunge uses wistar rats", according to the OECD Guidelines, 423, have been considered to be in GHS Category 5, > 2000 - 5000 mg/kg body weight, with a LD_{50} cut off at 5000 mg/kg body weight.

As the oral LD_{50} cut off value of the "Ethanolic Extract of *Astragalus membranaceus* Bunge" have been found to be at 5000 mg/kg, and considered to be SAFE for use.

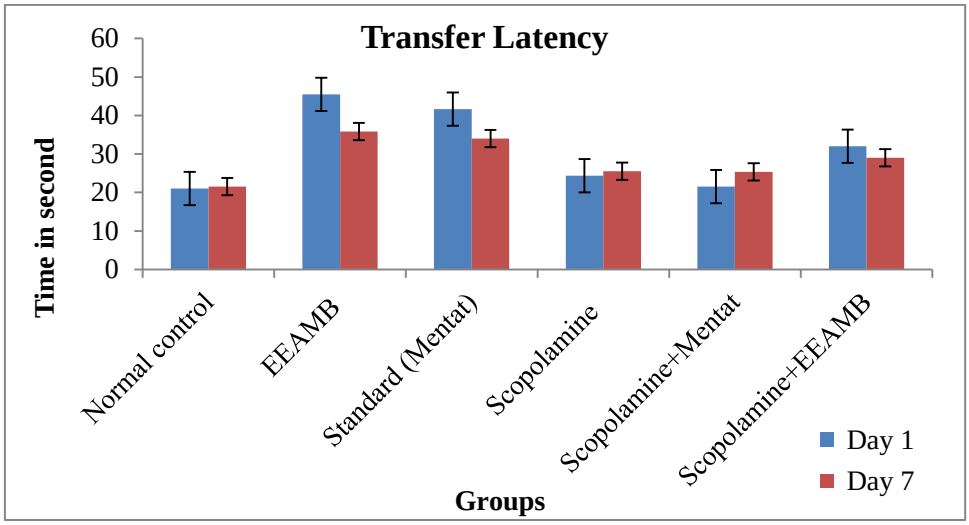
Effect on Transfer Latency by using Elevated Plus maze:

In the elevated plus maze paradigm, the rats treated with the standard drug mentat and the Ethanolic extract of *Astragalus membranaceus* Bunge (EEAMB) showed a decrease in transfer latency (TL) on day 7. Thus the significant decrease in TL of extract indicates the enhancement of cognitive function in rats.

Group	Treatment	TL Day 1 (sec)	TL Day 7 (sec)
1	Normal control	21.00±8.43	21.5±4.57**
2	EEAMB	45.5±6.63	35.83±3.43**
3	Standard (Mentat)	41.67±5.82	34.00±3.22*
4	Scopolamine Induced	24.33±10.17	25.50±6.47
5	Scopolamine + Standard (Mentat)	21.50±9.85	25.33±6.50
6	Scopolamine + EEAMB	32.00±5.55	29.00±4.00

N = 6 Values are expressed as Mean± SD. **P<0.01 – Compared to Normal Control

Mean Transfer Latency in different groups of Animals



Effect on Step-down Latency (SDL) by using Passive Avoidance Test:

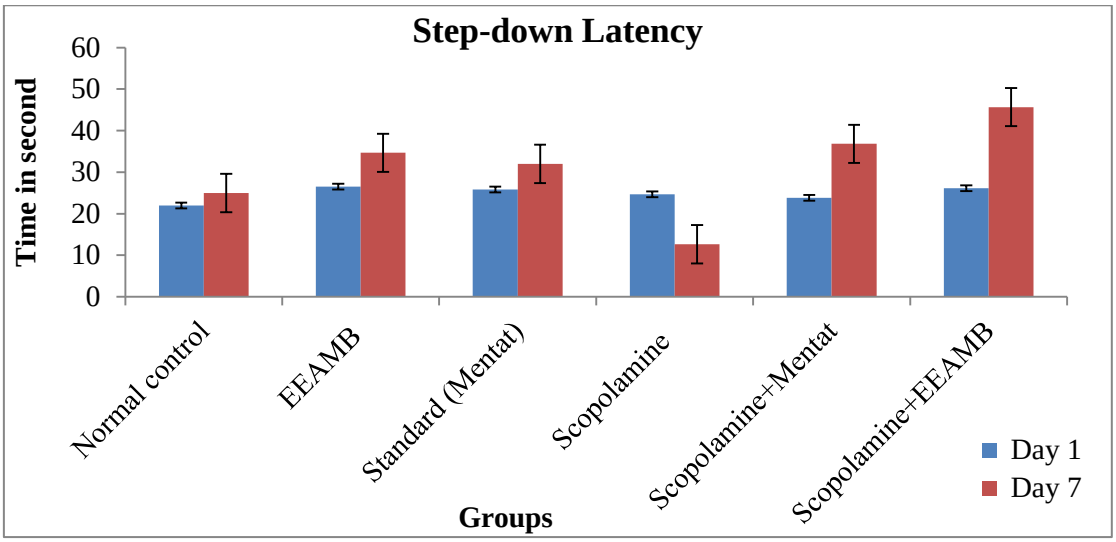
Scopolamine-induced cognitive deficit in rats was indicated by a decrease in SDL during retention (at 24h and on day 7) trials.

The rat treated with the standard drug mentat and the extract showed an increase in SDL on day 7 when compared to control (Scopolamine - treated rat). Mentat and ethanolic extract of *Astragalus membranaceus* Bunge (EEAMB) significantly reversed the hypoxic deficits of retention on day 7.

Group	Treatment	SDL Day 1	SDL Day 7
1	Normal control	22.00±2.10	25.00±2.28**
2	EEAMB	26.50±2.07	34.67±3.14***
3	Standard (Mentat)	25.83±2.48	32.00±4.15**
4	Scopolamine Induced	24.67±2.34	12.67±1.97***
5	Scopolamine + Standard (Mentat)	23.83±3.92	36.83±3.31††
6	Scopolamine + EEAMB	26.17±2.40	45.67±4.68†††

N = 6 Values are expressed as Mean± SD. **P<0.01 – Compared to Normal Control †† P<0.01– Compared to– Scopolamine Induced.

Mean Step-down Latency in different groups of Animals



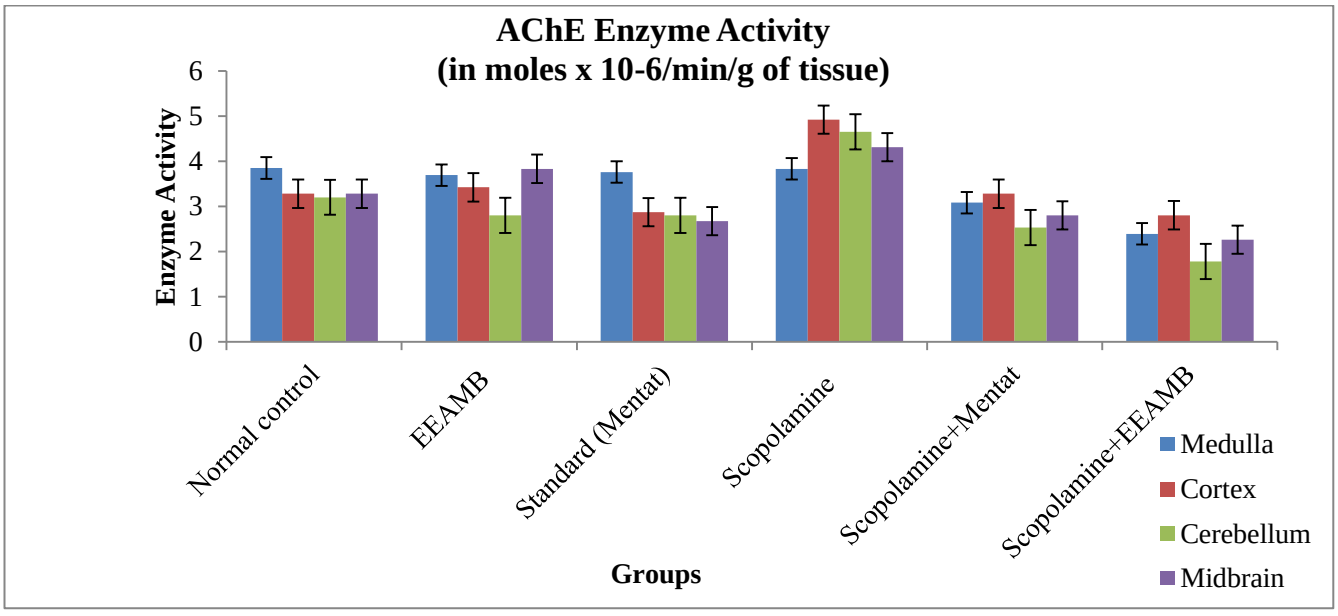
Acetyl Cholinesterase (AChE) Enzyme Activity:

Mentat and extract of *Astragalus membranaceus* Bunge significantly decreased acetylcholinesterase activity. Therefore, these drugs improved the memory of rats by inhibiting acetylcholinesterase enzyme, thereby increasing acetylcholine level in rat brain.

Group	Treatment	Acetylcholinesterase Enzyme activity (Mean± SEM) (in moles x 10^{-6}/min/g of tissue)			
		Medulla	Cortex	Cerebellum	Midbrain
1	Normal control	3.85±0.56	3.28±0.44	3.20±0.47	3.28±0.36
2	EEAMB	3.69±1.00****	3.42±1.46****	2.80±0.80****	3.83±1.12****
3	Standard (Mentat)	3.76±0.54****	2.87±0.78****	2.80±0.60****	2.67±0.96****
4	Scopolamine Induced	3.83±0.92****	4.92±1.16****	4.65±1.12****	4.31±1.15****
5	Scopolamine+Standard (Mentat)	3.08±0.77††††	3.28±0.64††††	2.53±0.73††††	2.80±0.40††††
6	Scopolamine+EEAMB	2.39±0.48††††	2.80±0.48††††	1.78±0.42††††	2.26±0.72††††

Values are expressed as Mean± SEM. **P<0.01 – Compared to Normal Control #P<0.01 – Compared to Negative Control – Scopolamine Induced

Mean AChE Enzyme activity in different parts of Rat brain



Histopathology report:

Microscopic examination of Brain specimens from 6 groups was carried out. All animals from Negative Control Scopolamine group showed abnormalities in less or more severe scale.

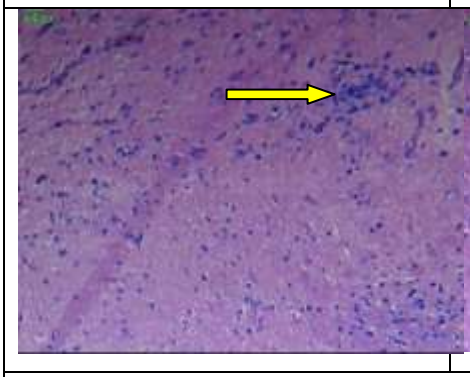
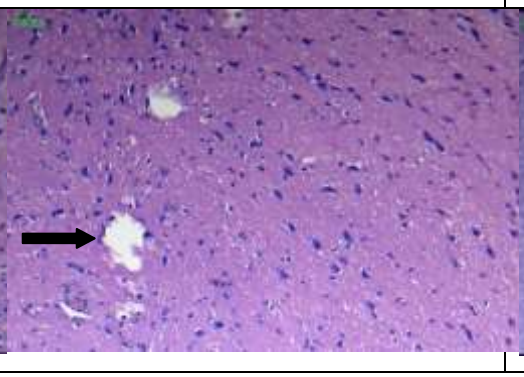
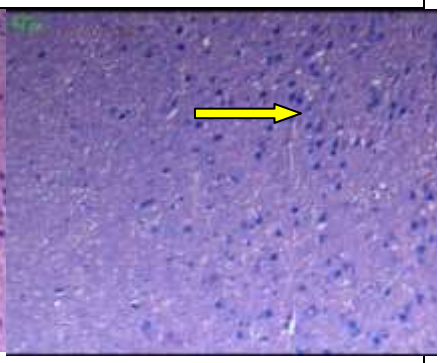
In the histopathology study of normal control group, trace amount of neuronal degeneration was observed, whereas the Negative control group showed vascular degeneration, neuronal degeneration and glial cell infiltration.

Ethanolic extract of *Astragalus membranaceus* Bunge received group without disease induction and standard drug mentat showed trace amount of neuronal degeneration.

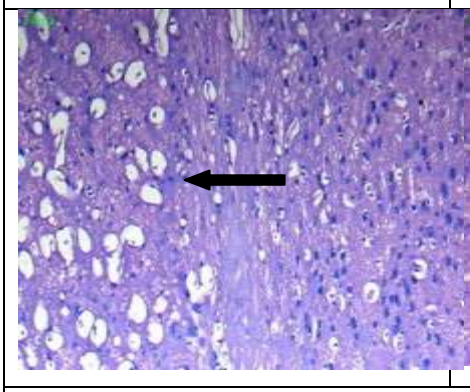
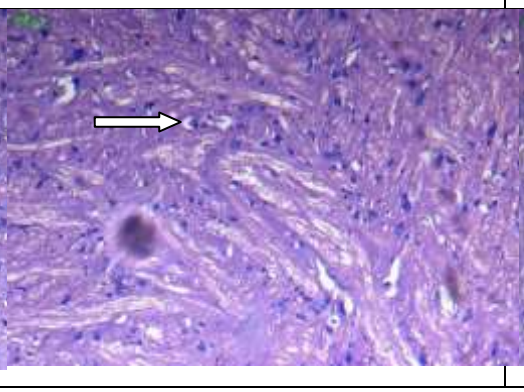
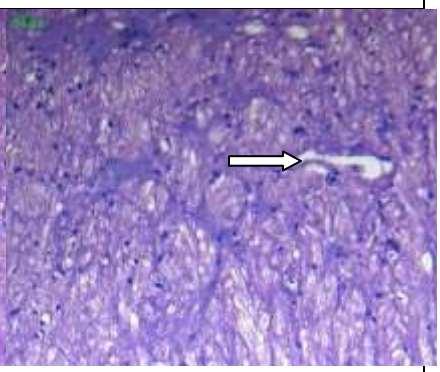
Vascular degeneration, neuronal degeneration, Glial cell infiltration was observed in all the groups with disease induced with Scopolamine, and treated with Standard drug (Mentat) and extract, but observations were mild as compared with Scopolamine-induced group.

Histopathology Pictures:

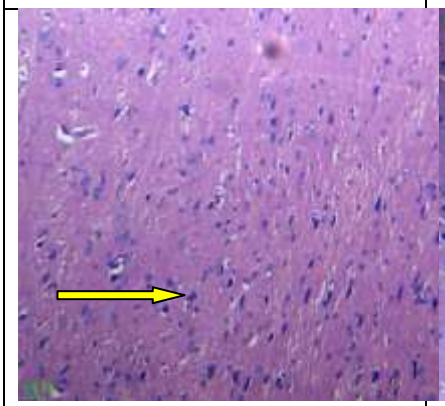
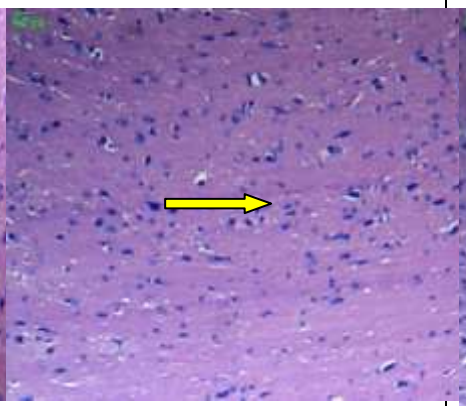
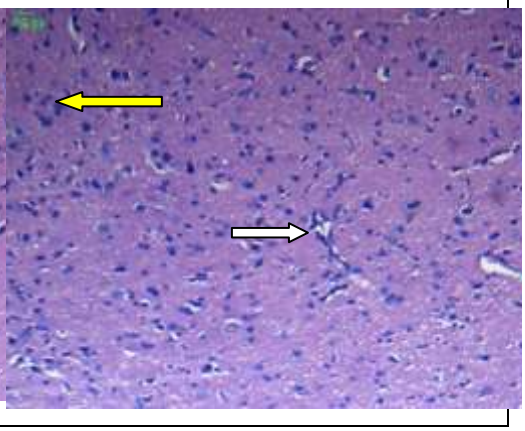
Group 1 - Normal Control

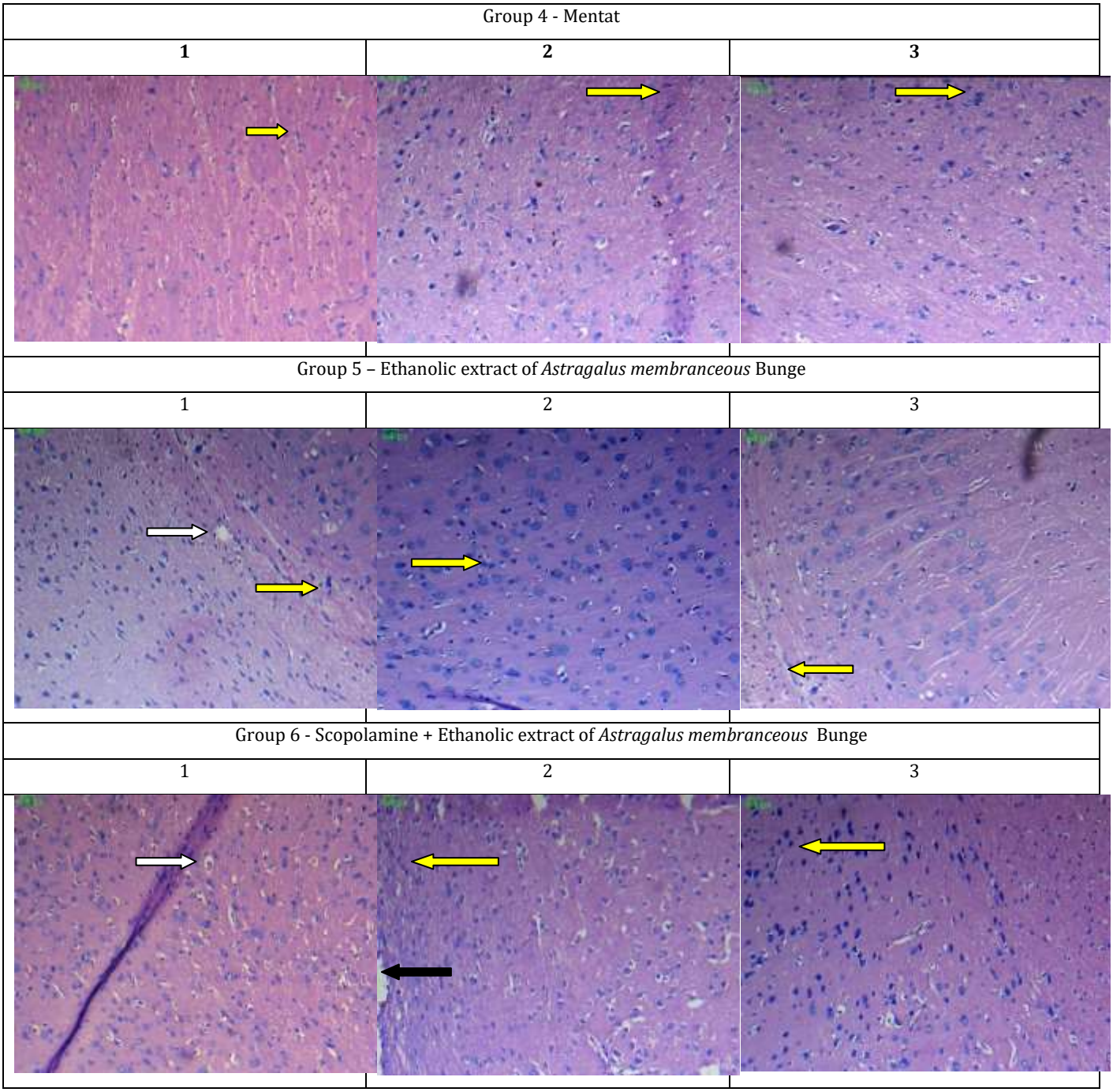
1	2	3
		

Group 2 - Scopolamine - induced

1	2	3
		

Group 3 - Scopolamine + Mentat

1	2	3
		



DISCUSSION

The elevated plus maze is used to measure the nootropic activity in animals, however, transfer latency, i.e. the time elapsed between the movement of the animal from an open arm to an enclosed arm, was markedly shortened if the animal had previously experienced entering open and closed arms, and this shortened transfer latency has been shown to be related to memory processes. This model a widely accepted paradigm to study learning and memory processes in rodents. In EPM, acquisition (learning) can be considered as transfer latency on first day trials and the retention/consolidation (memory) is examined 1 day later and on day 7. The animals treated with ethanolic extract of *Astragalus membranceous* Bunge showed a significant decrease in transfer latency as compared with the control group, which is an indication of the cognitive enhancer effect of extract in rats.

The term "passive avoidance" is usually employed to describe experiments in which the animal learns to avoid a noxious event by suppressing a particular behavior. The step-down latency, i.e. the time taken to step down from the shock free zone to steel-rod grid floor, was markedly increased if the animal had previously experienced the electric shock grid floor, and this increased step-down latency has been shown to be related to memory processes. In the passive avoidance test (Scopolamine-induced amnesia model), acquisition (learning) can be considered as step-down latency on first day trials and the retention/consolidation (memory) is examined 1 day later and on day 7. The animals treated with ethanolic extract of

Astragalus membranceous Bunge showed a significant increase in step-down latency as compared with the control group, which is an indication of the cognitive enhancer effect of extract in rats.

AChE activity was measured in rat brain as a marker enzyme for cholinergic function. The estimation of AChE enzyme supports the plus maze and passive avoidance (Scopolamine-induced amnesia) test by statistically reducing AChE activity, which leads to increased acetylcholine (ACh) level in brain, which helps in memory performance.

The present study therefore demonstrates that the ethanolic extract of *Astragalus membranceous* Bunge improves memory performance by increasing ACh level in rat brain.

CONCLUSION

It is concluded that, the ethanol extracts of *Astragalus membranceous* Bunge might be a potential alternative agent for nootropic activity. The cognitive enhancement activity is further supported by the decrease in AChE enzyme activity in different regions of the brain. It may be due to abundant presence of flavonoid compounds. This investigation may focus on research fields to develop clinical studies which might be of great scientific contribution for the society.

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