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Memory protective effect of *Phyllanthus emblica* against scopolamine induced spatial memory loss in male Long Evan Rats tested by Radial Arm Maze

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Abstract

Background: Dementia is the commonest problem of middle aged and elderly subjects due to age related changes and oxidative stress. Many traditional herbal agents including Emblica Officinalis (*Phyllantus Emblica)* demonstrated its benefit against memory loss. **Objective:** To investigate the memory protective effect of Ethanolic extract of *Phyllantus Emblica* (EEPE) or Emblica Officinalis in scopolamine induced memory impaired male Long Evan rats. **Methods:** This animal behavioral study was carried out on total 18 male long Evan rats weighing 300-400gm. According to treatment they were divided into NS (control) group (n=6, normal saline 5ml/kg) Sco(n=6; Scopolamine 2mg/kg) and PE(n=6; EEPE 400mg/kg+ Scopolamine 2mg/kg).This experiment was conducted for total 33 days. After 7 days of acclimatization, NS group and PE rats were treated with normal saline and EEPE respectively for consecutive 26 days. Scopolamine was given to sco group and PE group from day 22 to 26 (5 days during acquisition phase. Spatial memory (working and reference memory error) was assessed by 8 arm radial arm maze test (RAM). Statistical analysis was done by ANOVA followed by Bonferroni's post hoc test. **Result:** Working memory error (WME**)** as well as reference memory error (RME) was found significantly higher in scopolamine treated rats compared to normal memory (NS) as

well as PE pretreated rats whereas no significant difference was found in WME and RME between normal memory rats and PE pre- treated rats. **Conclusion:** *Phyllanthus Emblica* can prevent memory impairment in scopolamine induced memory loss in Long Evan rats.

Key words: Memory impairment, scopolamine, working memory error, reference memory error, *Phyllanthus emblica*

Introduction

In Alzheimer's disease (AD) dementia is the commonest presentation of middle aged and elderly subjects.[1] The pathogenesis involves formation of senile plaques, amyloid-â deposits and neurofibrillary tangles in hippocampus and cerebral cortex. Impaired activity of the central cholinergic neuron is the most important neurological feature of this condition.[2-3]

Oxidative stress plays very important role in the pathogenesis of AD. High level of AChE in brain has been reported in previous studies causing reduced cholinergic activity in AD resulting in impaired memory and cognitive loss.[3]

Till date there is no highly effective treatment for AD. Very limited use of some anticholinesterase drug is available to treat AD but it is discouraged due to its untoward side effects.[3] Therefore, efficacy of natural antioxidants is under investigation for treatment of AD. Among the antioxidant rich natural source, *Phyllanthus emblica*(PE) is well known for its antioxidant content. Antioxidants available in PE are vitamin C, vitamin E, gallic acid, tannic acid etc.[4]

Memory refers the ability of the brain to store and retrieve information which is a necessary prerequisite for all learning.[5] All kinds of memory can be short term based working memory or long term retention reference memory. These two components of spatial memory can be assessed by a standard method of radial arm maze test commonly used in behavioral assessment of animal.[5]

Few previous studies investigated combined effect of PE, Tinospara and Ocimum sanctum and found its good impact on learning, memory there by useful in the treatment of dementia & AD. [3,6]

Though few earlier studies investigated the effect of *Phyllanthus emblica* on memory but they used passive avoidance test to assess emotional memory, rewarding alternation test for spatial working memory.[3]

Spatial memory is an important component of learning and animal behavior for day to day survival.AD is characterized by substantial memory loss with deficit of spatial memory.[2] Radial arm maze (RAM) test is widely used in animal experiment to assess spatial memory.[7]

Very limited number of previous studies investigated memory protective effect of PE compared to other effects of PE, In view of this, our study was designed to investigate the effects of PE on spatial memory performance in male Long Evan rats using radial arm maze test.

Methods

Study design & setting

This experimental animal model study was conducted in KM Fariduddin animal research lab of the Department of Physiology of Bangabandhu sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh from 2020 to 2021

Animals

This study involved 18 healthy adult male Long-Evans rats weighing 200-300 gm purchased from central animal house of BSMMU. The rats were housed in 6 per animal case and placed under standard environmental conditions (25± -2°C temperature, 60.5% relative humidity with a half day light and dark cycle. Standard laboratory food and water were administered properly. The care

and use of the animals were monitored according to the guideline of animal experimentation ethics committee of International Center of Diarrheal Disease of Bangladesh. The protocol of the experiment was approved by the Institutional Review Board of BSMMU, Dhaka, Bangladesh.

Drugs and chemicals

Scopolamine hydrobromide (Sanofi Aventis Limited) at dose 2 mg/kg injected *i.p* .[8]Normal saline (Beximco Pharma Ltd) at 5 ml/kg and EEPE at dose of 400mg/kg was given orally.[2] Desired strength of EEPE solution was made by mixing EEPE with normal saline at the ratio of 0.5:400 which was kept as stock solution and was given orally[3] for several days. Ethanol (MERCK Germany) was used for preparation of ethanolic extract of PE.

Preparation of ethanolic extract of *Phyllanthus emblica*(PE)

Fresh leaves of PE were collected and identified by taxonomist from the Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. A voucher specimen was preserved in the herbarium for future reference. Accession number: DACB-65751 for PE.

The fresh unripe fruits of PE were sun dried initially then dried in an oven for 24h and grinded into coarse powder. Five hundred gram (500g) powdered PE was then soaked in 2.5 liter of 98% ethanol for 7 days and then filtered. The liquid filtrates were concentrated and evaporated to dry at 50°C temperature by using a rotary evaporator under reduced pressure to get the crude extract (10.62 g for PE fruit). Finally, dried crude ethanolic extracts were stored at 4°C for further tests.[3]

Acute Toxicity Study[3]

EEPE was under gone oral acute toxicity test as recommended by Organization for Economic Cooperation and Development in healthy male Swiss albino rats. Before test dose of EEPE was given rats were deprived for food for 3-4 hours with only access to water prior to several oral dosing. The extract of PE was given orally through a nasogastric tube at different doses (i.e., 25, 50, 100, 500, 1000, and 2000 mg/kg b.w.) However, effect of EEPE extract on Rat behavior for next 24 hour deeply watched. Rat behavior or any adverse change was observed and subsequently 14 days for any lethality.[3]

Experimental Design

Animals are randomly divided into 3 treatment groups (n= 6 rats/ group).

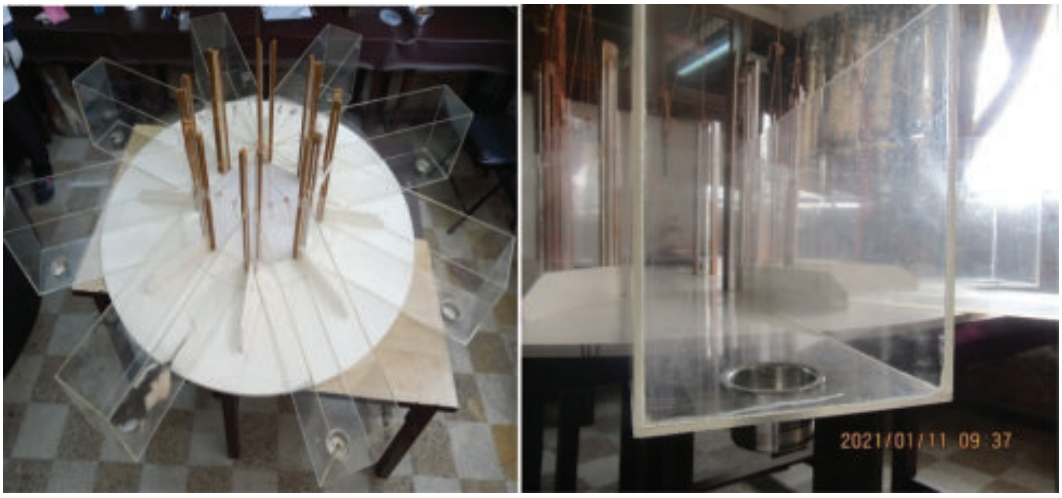
1) NS group (Normal saline 5 ml/kg for 26 consecutive days) served as control
2) Sco group (Scopolamine 0.2 mg/kg for 5 consecutive days).
3) PE group(Rats were pre-treated with Phyllanthus emblica extract 400 mg/kg for 26 days + scopolamine for 5 consecutive days at a dose of 0.2 mg/kg)

Radial Arm Maze (RAM) test

One week training was performed in rats in order to prepare them for behavioral study. During the training period only food and water were administered to rats. The fully trained rats were selected for the study. Studies were done between 10.00 am and 3.00 pm in a soundproof room.

Apparatus

In the experiment, an 8-arm standard radial maze was used which was placed in a well-lighted room with distinct extra maze visual cues such as shelves, desktop, computer, air-conditioner, doorway etc.[9] During the course of all experiments, the maze remained in fixed position with respect to the distal cues.[10] It was built of plexiglass and kept 70 cm above the floor.[11] The maze consisted of a central octagonal platform with a diameter of 42 cm which was surrounded by eight arms. Length of each arm was 60 cm from the centre, width 17 cm and height 25 cm. [10] Each arm radiated outward from the central platform at equal angles and contained a recessed food cup (2 cm deep x 3 cm across) at 4 cm proximal to the distal end of the arm.[12] Each arm was separated from the central platform by a transparent plexiglass guillotine door which can be raised or lowered to allow or prevent entry of the rat by a pulley system that allowed the researcher to open any door from a fixed position within the rat lab.



Procedure

Tests were done according to previous studies.[9,12-13] All rats (total 18) underwent room acclimatization for 7 daysat animal lab for RAM test.During all the phases, every day every rat was brought into the memory lab for 2 trials (trial 1 and trial 2) separated by 3 hours. In this test, a fasting rat had to search food. For this, before the beginning of trial 1, each rat was deprived of only food, not water for approximately 10 hours to motivate it. Trial 1 was started 30 minutes after administering the prefixed treatment based on group assignment. After each trial, the maze was thoroughly cleaned with 70% alcohol to minimize residual odor.

Three days before starting habituation, the rat was introduced to the bait (jilapi in small pellets) in the rat cage, once every day.This test was done in 3 phases

Habituation (day 16 to day21 days)

Rats were brought to maze for habituation for consecutive 6 days,

On the first day (Day 16), 2 rats at a time was taken to the maze with baits being scattered on the floor (platform, 8 arms and foodcups) for a 10 minutes trial. This paired access was given to reduce the reluctance of individual rat for exploration. For the next day (Day 17), individual access (1 rat at a time) was given with baiting as on the previous day. These 2 days were considered as for instrumental and procedural acclimatization for RAM test. During the 3rd and 4th day (Day 18 and 19), only the 8 food cups in 8 arms of the maze were baited. However, on the last 2 days (Day 20 and 21) randomly selected (by lottery) any 4 arms were baited. The arm numbers for baiting were kept fixed in every trial for each rat for consecutive 2 days. However, these arm numbers for baiting varied between rats. During this period, all the gates of all the arms were kept open.

Acquisition phase (from day 22 to day 26)

The performance was tested for 5 days. Randomly chosen any 4 of the 8 arms were baited by jilapi only in food cup. Each trial was started by placing a rat at the center of the platform with all gates closed. Then all gates were opened at a time. When the rat entered any one arm, the 7 other gates remained closed. After exploration, the rat came out and its gate was closed. Just 5 seconds later, all gates were opened again and the whole process was repeated. The trial was continued for, either 10 minutes or all jilapis of the 4 baited arms were eaten by the rat, whichever occurred first. Two trials were given per day with 3 hours interval, for each rat.

Retention phase (from day 27 to day 33)

Retention test was done after 7 days of interval of last acquisition day with 2 trial in single session in similar manner as in acquisition phase

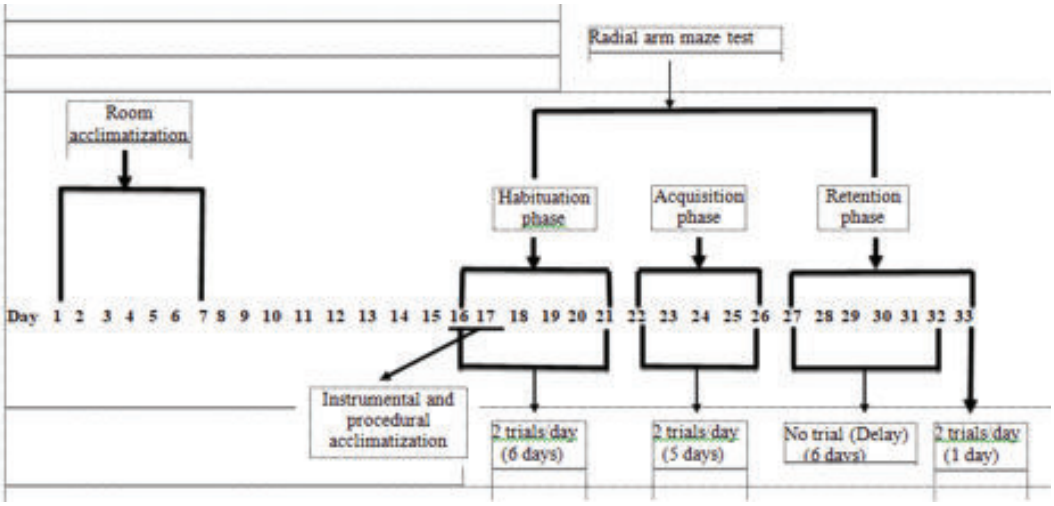


Figure 1: Work plan in different days of radial arm maze (RAm)

Table I: Treatment plan in Radial arm maze

Phase	Duration (days)	Days of experiment	Treatment schedule	Baiting(Jilapi)
Room acclimatization	7	1-7	No treatment	No
Treatment	8	8-15	NS, *EEPE*	No
Habituation	6	16-17	NS, *EEPE*	scattered all over maze
		18-19	NS,*EEPE*	Jilapi in all food-cups
		20-21	NS,-*EEPE*	Jilapi in randomly selected 4 food-cups
Acquisition	5	22-26	NS, *Sco, EEPE*+ Sco	Jilapi in randomly selected 4 food-cups
Delay	6	27-32	NS, *EEPE*	No
Retention testing	1	33	I-NS, III-*EEPE*	Jilapi in randomly selected 4 food-cups

Evaluation of memory

Visit of rat to an arm is considered when it's all four paws are inside as watched by a video. Working memory error (WME) was calculated by the number of re-visit of a rat in a baited or never baited arm and number of first time entries into unbaited arms as reference memory errors (RME)

Statistical analysis

Results were expressed as mean± SEM (Standard error of mean). Statistical analysis was done by using SPSS (version 16.0). Statistical tests carried out by ANOVA followed by Bonferroni's post hoc test. $p \leq 0.05$ was considered as statistically significant.

Results

Effect of EEPE on working memory

The mean WME in scopolamine treated rat was significantly ($p \leq 0.05; 0.001$) higher when compared NS and pretreated PE rat at both trial during most of acquisition and retention days except at trial 2 on day 23,24 & 33 indicating impaired memory in this group and reduction of memory impairment in PE pretreated. Again, lack of significant difference in WME between the NS and PE pretreated rat in all acquisition and retention days at both trial demonstrated PE provides protection against working memory impairment induced by scopolamine. (Table II)

Table II: Working memory error (frequency/trial) in Radial arm maze test in different groups of rats (Effect of Emblica on scopolamine induced impairment of working memory in RAM test)

Phases	Experimental Days	Trials/day	Groups		
			NS(n=6)	Sco(n=6)	PE(n=6)
Acquisition	Day	T 1	2.50±0.43	4.50±0.56**	3.16±0.31
			(1 to 4)	(3 to 6)	(2 to 4)
	22	T 2	1.67±0.56	4.33±0.61**	1.50±0.34##
			(0 to 4)	(3 to 7)	(0 to 2)
	Day23	T 1	1.83±0.31	3.67±0.42**	2.50±0.34
			(1 to 3)	(2 to 5)	(2 to 4)
		T 2	1.33±0.33	2.67±0.42	1.67±0.31#
			(0 to 2)	(1 to 4)	(0 to 2)
	Day24	T 1	1.67±0.33	3.33±0.33**	1.67±0.33##
			(1 to 3)	(2 to 4)	(1 to 3)
		T 2	1.00±0.26	2.83±0.75	1.00±0.37
			(0 to 2)	(1 to 6)	(0 to 2)
	Day25	T 1	1.00±0.37	3.33±0.76**	1.17±0.31##
			(0 to 2)	(1 to 6)	(0 to 2)
		T 2	0.83±0.31	3.17±0.48***	0.50±0.22###
			(0 to 2)	(2 to 5)	(0 to 1)
	Day26	T 1	0.83±0.17	3.50±0.34***	0.50±0.22###
			(0 to 1)	(2 to 4)	(0 to 1)
		T 2	0.67±0.33	3.33±0.49***	0.00###
			(0 to 2)	(2 to 5)	(0 to 0)
Retention	Day33	T 1	1.50±0.22	4.00±0.89**	1.33±0.33##
			(1 to 2)	(1 to 7)	(0 to 2)
		T 2	1.00±0.26	2.17±0.60	0.33±0.21##
			(0 to 2)	(0 to 4)	(0 to 1)

Each column symbolizes mean± SEM for 6 rats. Values in parenthesis indicate ranges;T1: mean trial 1 on that day; T2:mean trial 2 on that day;NS: rats with oral normal saline (5ml/kg) for consecutive 26 days (Day 8 to day 33); Sco: rats with intraperitoneal (i.p.) Scopolamine (2 mg/kg) for consecutive 5 days of acquisition phase (Day 22 to Day 26); PE: rats with oral *Phyllanthus emblica* (PE) (400 mg/kg) for consecutive 26 days (Day 8 to day 33) and i.p. Scopolamine (2 mg/kg) for consecutive 5 days of acquisition phase (Day 22 to Day 26). Statistical analysis was done by ANOVA (among groups) followed by Bonferroni's Post Hoc test (between groups); * = NSvsSco, # = Scoa vsPE , $= NSvsPE; p≤0.05 was considered as significant; p≤0.05 was expressed as */#/$; p≤0.01 as **/##/$$; p≤0.001 as ***/###/$$$.

When WME was compared between trial 2 and trial 1 during acquisition phase, observed WME was reduced in both NS and PE pretreated rats but similar WME scopolamine treated rats indicates gradual reduction of memory error in NS and PE and no change in memory impairment in scopolamine. (Figure 2) This result suggests PE helps recovery from memory impairment and retain learning enhancement.

Again, In this study , number of errors in T1 of next days were not significantly different from those of T2s of previous days in all groups. (Figure 3) This suggests no difference in residual memory for a very short period among all group of rats. Emblica did not affect short term residual memory. In addition, the mean WME difference at T1 between day 33 and day 26 were significant in NS but not in PE treated & scopolamine rats (Table III). This suggests emblica did not affect residual memory.

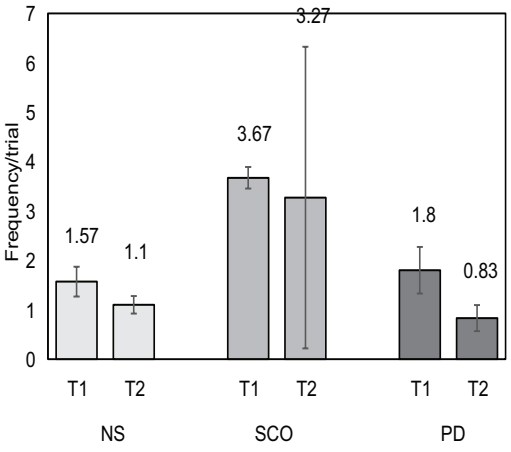


Figure 2: Working memory error of T1s and T2s (with 3 hours interval) in acquisition phase of Radial arm maze test in different groups of rats (N=18).

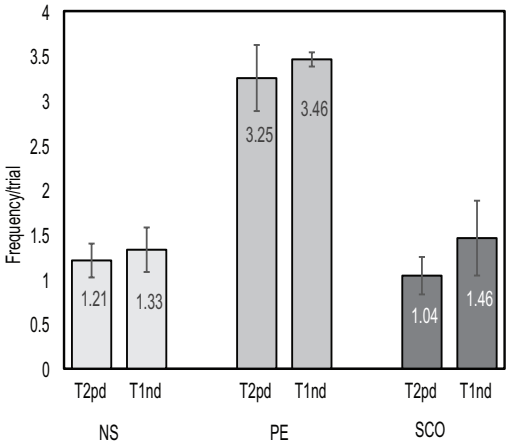


Figure 3: Working memory error of T2s and T1s (with 21 hours interval) in acquisition phase of Radial arm maze test in different groups of rats (N=18). T2pd: mean working memory error of trial 2 of previous days (Day 22, 23, 24, 25); T1nd: mean working memory error of trial 1 of next days (Day 23, 24, 25, 26);

Table III: Working memory error (frequency/trial) of T1s of day 26 and day 33 (with 7 days interval) in Radial arm maze test in different groups of rats (N=18)

Groups	Days ofT1s		p value
	Day 26	Day 33	
NS	0.83±0.17	1.50±0.22	0.025¥
n=6	(0 to 1)	(1 to 2)	
Sco	3.50±0.34	4.00±0.89	0.562
n=6	(2 to 4)	(1 to 7)	
IPE	0.5±0.22	1.33±0.33	0.141
n=6	(0 to 1)	(0 to 2)	

Each column symbolizes mean± SEMtrials of 6 rats. Values in parenthesis indicate ranges; T1: Trial 1;NS: rats with oral normal saline (5ml/kg) for consecutive 26 days (Day 8 to day 33); Sco: rats with intraperitoneal (i.p.) Scopolamine (2 mg/kg) for consecutive 5 days of acquisition phase (Day 22 to Day 26); PE: rats with oral *Phyllanthus emblica* (PE) (400 mg/kg) for consecutive 26 days

Effect of EEPE on reference memory error (RME)

Higher RME of the scopolamine treated rats ($p \leq 0.05$; $p \leq 0.01$; $p \leq 0.001$) in comparison to NS and PE pretreated rats at both trial in acquisition and retention days except trial 1 at 22 day 24 & T1& 2 of day 23and no significant difference in RME between the NS and PE pretreated rats (Table 4) suggests scopolamine increased the error and emblica reversed the error induced by scopolamine. Thus emblica protected the memory loss induced by scopolamine. It has overall reference memory protective effect. Furthermore, lower RME l in NS ($p \leq 0.05$) and PE pretreated

Table IV: Reference memory error (frequency/trial) in Radial arm maze test in different groups of rats (N=18) (Effect of Emblica on scopolamine induced impairment of reference memory in RAM test).

Phases	Experimental Days	Trials/day	Groups		
			NS(n=6)	Scopolamine(n=6)	PE(n=6)
Acquisitionphase	Day22	T 1	3.00±0.45	3.50±0.34*	2.83±0.40
			(1 to 4)	(2 to 4)	(2 to 4)
		T 2	2.67±0.33	3.67±0.21*	2.33±0.21##
			(2 to 4)	(3 to 4)	(2 to 3)
	Day23	T 1	2.83±0.31	3.33±0.21	2.5±0.22
			(2 to 4)	(3 to 4)	(2 to 3)
		T 2	2.33±0.21	3.16±0.30	2.00±0.26#
			(2 to 3)	(2 to 4)	(1 to 3)
	Day24	T 1	2.50±0.43(1 to 4)	3.17±0.31(1 to 4)	2.33±0.33(1 to 3)
		T 2	1.67±0.21	3.33±0.33**	1.83±0.31##
			(1 to 2)	(2 to 4)	(1 to 3)
	Day25	T 1	2.00±0.25	3.67±0.21***	1.67±0.21###
			(1 to 3)	(3 to 4)	(1 to 2)
		T 2	1.33±0.21	3.50±0.22***	1.33±0.33###
			(1 to 2)	(3 to 4)	(0 to 2)
	Day26	T 1	1.50±0.22	3.67±0.33***	1.33±0.33###
			(1 to 2)	(2 to 4)	(0 to 2)
		T 2	1.17±0.60	3.00±0.26**	0.67±0.21##
			(0 to 4)	(2 to 4)	(0 to 1)
RetentionDay	Day33	T 1	2.33±0.21	3.83±0.17**	2.17±0.31##
			(2 to 3)	(3 to 4)	(1 to 3)
		T 2	2.17±0.31	3.50±0.22**	1.67±0.21###
			(1 to 3)	(3 to 4)	(1 to 2)

Each column symbolizes mean±SEM for 6 rats. Values in parenthesis indicate ranges;T1: mean trial 1 on that day; T2:mean trial 2 on that day;NS: rats with oral normal saline (5ml/kg) for consecutive 26 days (Day 8 to day 33); Sco: rats with intraperitoneal (i.p.) Scopolamine (0.2 mg/kg) for consecutive 5 days of acquisition phase (Day 22 to Day 26); PE: rats with oral *Phyllanthus emblica* (PE) (400 mg/kg) for consecutive 26 days (Day 8 to day 33) and i.p. Scopolamine (0.2 mg/kg) for consecutive 5 days of acquisition phase (Day 22 to Day 26). Statistical analysis was done by ANOVA (among groups) followed by Bonferroni's Post Hoc test (between groups); * = NS vs Sco, # = Sco vs PE, $= NS vs PE; $p \leq 0.05$ was considered as significant; $p \leq 0.05$ was expressed as */#/$; $p \leq 0.01$ as **/##/$$; pd"0.001 as ***/###/$$$.

group (p≤0.001)but no significant difference in error in scopolamine group when compared between T1&T2 during acquisition phase (Figure IV) suggests memory error was gradually reduced in normal and pretreated rats but not in scopolamine treated rat.

Furthermore, the significantly higher mean RME in NS whereas non-significant difference in RME in PE pretreated and scopolamine rats on day 33compared to day 26) suggests Amloki did not affect residual memory of rat. (Figure 5**)**

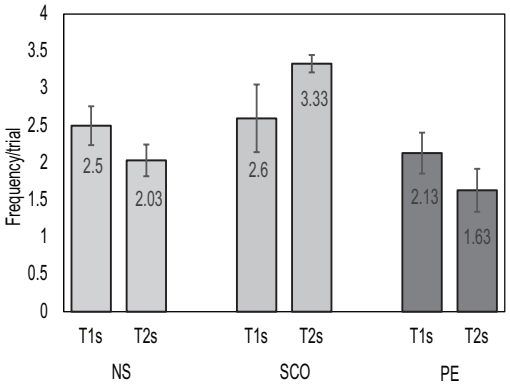


Figure 4: Reference memory error of T1s and T2s (with 3 hours interval) in acquisition phase of Radial arm maze test in different groups of rats (N=18).

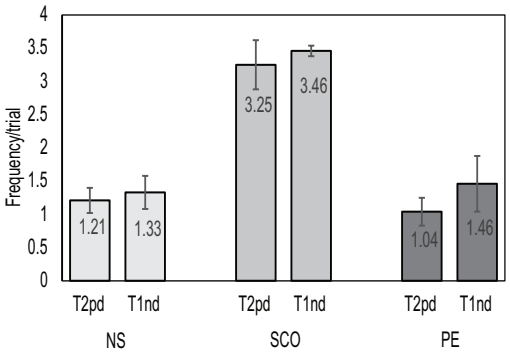


Figure 5: Reference memory error of T1s of day 26 and day 33 (with 7 days interval) in Radial arm maze test in different groups of rats (N=18).

Discussion

Various medicinal herbs are extensively studied for its health promoting and disease preventive or protective effect. [3-4, 6-7] Plant sources having natural active chemicals with biological effects are less harmful for human health and it constitute the alternate for pharmacological agent.**[3]** PE has been used traditionally in various illnesses.

The present study investigated the memory protective effect of EEPE on memory impaired male Long Evan rats by RAM test. In general, the spatial memory task was not much affected in PE pre-treated rats supported by almost similar RME and WME as in normal memory rats as well as lesser value compared to scopolamine treated rats.

In our study, scopolamine (2 mg/kg) caused both working and reference memory impairment in rats.[3, 11] due to its ability to reduce central cholinergic transmission.[14] It is well known that normal cholinergic activity is essential for some cognitive action such as attention, learning and memory.[15] So, either damage of these neuron or deficiency of ACh would cause impairment of memory. In this study scopolamine caused memory loss in rats due to its cholinergic receptor blocking effect by competitive antagonism in cerebral cortex which in turn reduce ACh mediated neuronal activity in hippocampus causing learning and memory deficit.

The lower value of WME in EEPE pretreated rats compared to scopolamine treated rats showed that Emlica was effective to prevent or counteract the impairment of working memory induced by scopolamine.The results of the RME in EEPE pretreated rats also indicates that the reference memory reversal by PE is almost near to intact reference memory in normal rat.

One previous study on isolation of bioactive compounds of PE and their various biological effects showed that among the various chemical ingredients of PE, biphenyl dicarboxylic acid has been found possessing memory enhancing effect. This effect is probably attributed to its cholinergic restorative capacity and protective capacity against hypoxia.[16]

Conclusion

This study concluded that ethanolic extract of Phyllanthus Emblica possess excellent memory protecting effect against memory loss.

Conflict of interest

Authors declare no conflict of interest.

Ethical aspects

This study was approved by institutional Review Board of BSMMU

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