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Exploring *Bhavana samskara* using *Tinospora cordifolia* and *Phyllanthus emblica* combination for learning and memory in mice

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ABSTRACT

Background: Current medications for dementia and enhancement of learning and memory are limited hence we need to explore traditional medicinal systems like Ayurveda to investigate agents that can improve learning and enhance memory. **Objective:** The present study was carried out to evaluate effects and mechanisms of Ayurveda drug formulations, *Tinospora cordifolia* (*Tc*) and *Phyllanthus emblica* (*Pe*) with and without *Bhavana samskara* on learning and memory of mice. **Materials and Methods:** After approval of Animal Ethics Committee, Swiss albino mice were divided into seven groups, administered orally: Distilled water, Rivastigmine (2.4 mg/kg), *Tc* (100 mg/kg), *Pe* (300 mg/kg), formulation 1 (*Tc* + *Pe*: 400 mg/kg) and formulation 2 (*Tc* + *Pe* + *Ocimum sanctum*: 400 mg/kg) daily for 15 days. Piracetam (200 mg/kg) was injected daily intraperitoneally for 8 days. The mice underwent a learning session using elevated plus maze. Memory was tested 24 hours later. **Results:** Mice pretreated with all the drugs showed a trend toward reducing transfer latencies but values were comparable to vehicle control. In all drug-treated groups, a significant reduction in transfer latency was observed after 24 h. Improvement in learning and memory by both formulations were comparable to individual plant drugs, *Tc* and *Pe*. **Conclusion:** The plant drugs showed improvements in learning and memory. The fixed-dose formulations with *Bhavana samskara,* showed encouraging results as compared to individual agents but the difference was not statistically significant. Hence, the concept of *Bhavana samskara* could not be explored in the present study. However, these drugs showed comparable or better effects than the modern medicinal agents thus, their therapeutic potential as nootropics needs to be explored further.

Key words: Cognition, dementia, nootropic, *Ocimum sanctum*, *Rasayana*

INTRODUCTION

The acquisition of any new information and skills is termed as learning and its subsequent retention is known as memory, both are the fundamental properties of central nervous system and play a critical role in "process of thought" which is known as cognition.[1] Memory can be broadly divided as short and long-term.[2] Short-term memory only lasts for a short period while long-term memory is intended to store the information for a long period. Information from short-term memory is stored in the long-term memory by rehearsal.[2,3]

As per Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, dementia refers to a group of disorders in which memory and cognition are impaired and includes symptoms like decline in any one of the following: Ability to speak coherently or understand

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spoken or written language or recognize objects or ability to perform motor activities or ability to think abstractly.[4] The cognitive impairment in dementia is severe enough to interfere with the daily activities.[4] The burden of dementia is increasing world-wide particularly in the elderly population. Total number of people with dementia worldwide in 2010 was 35.6 million and is projected to nearly double every 20 years to reach 115.4 million by 2050. A new case of dementia is added every 4 s.[4] The causes of dementia include ageing, Alzheimer's and other neurodegenerative diseases like Parkinsonism, ischemic brain damage, head injuries, alcoholism, drug intoxication, vitamin deficiencies, psychiatric or psychological disorders; Alzheimer's disease being most common.[5] Only drugs available for management of dementia include Piracetam and anticholinesterases, which are inadequate to provide optimum treatment.[5] Hence, research is ongoing to explore the neurobiology of learning and memory to investigate the nootropic agents as well as the agents that can prevent progressive memory loss or improve existing capacity of learning and memory. Scientists are looking for new directions and alternative strategies for managing dementia. This motivated us to explore the Indian traditional system of medicine - Ayurveda.

Ayurveda classifies drugs into multiple groups according to their actions, one of them being *Rasayana,* which provides longevity, memory, intelligence, freedom from disorders, youthful age, optimum strength of physique and sense organs, respectability and brilliance.[6,7] Of these *Rasayana* drugs, "Amla"-*Phyllanthus emblica* (*Pe*), "Guduchi"-*Tinospora cordifolia* (*Tc*) and "Tulsi"-*Ocimum sanctum* (*Os*) have been studied earlier for their effects on learning and memory performances and have shown promising results.[8-11]

Charaka Samhita mentions *Samskara* as transformation of the inherent properties of the formulation. This is possible by heating, cleansing, churning, dilution, storage in specific place and temperature, maturing, flavoring, impregnation, preservation, container, etc., *Bhavana* is one of the various types of *Samskara* mentioned in Ayurveda texts, in which the powder of the drug is washed or coated with liquid media of same drug or drug with similar properties.[12] In Ayurveda clinical practice, *Os* is administered either as fresh juice of leaves or is used to coat other plant drug particles by the process described as "*Bhavana samskara*."[12,13] And it is claimed that *Bhavana samskara* potentiates the efficacy of coated drugs,[12] which is equivalent to the concept of synergism or potentiation in modern pharmacology. *Bhavana samskara* is also postulated to have an impact on physicochemical parameters, increase shelf life, enhance the bioavailability of the coated plant drugs, reduce the dose of plant drugs required.[12,13] We found one study reporting the penetration enhancing capacity of *Tulsi*, which may enhance the bioavailability of the coated drug.[14] Thus, present study tried to evaluate the effects of formulations containing *Tc* and *Pe* with and without the coating of *Os* on learning and memory performance of mice and compared them with those of *Tc* and *Pe* alone to explore the concept of *Bhavana samskara*. We also evaluated the ability of these formulations to reverse the amnesia induced by various amnestic agents (viz. scopolamine, diazepam and cyclosporine) to explore the possible mechanisms and actions on central nervous system.

MATERIALS AND METHODS

Permission from the Institutional Animal Ethics Committee was obtained prior to initiation of study (AEC/20/07 dated 28th December 2007).

Animals

Swiss albino mice of either sex weighing 18–25 g were procured from the center for animal studies at Seth G. S. Medical College and K. E. M. Hospital Parel, Mumbai, Maharashtra, India. Mice were maintained in accordance with laboratory guidelines for the care and use of laboratory animals laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals, India. Nine mice were housed per cage in polypropylene cages. Autoclaved paddy husk was served as the bedding. Cages were fitted with stainless steel grill top having provision to supply food and water. The mice were given commercially available rodent food (Amrut Oil Mills Ltd.) with filtered and ultraviolet purified water *ad libitum*.

Drugs

Formulation 1 (H1) contained dried aqueous extract of combination of *Tc* + *Pe* in a ratio of 1:3 (as mentioned in Ayurveda)[6,7,13] and formulation 2 (H2) contained same *Tc* + *Pe* combination coated with juice of fresh leaves of *Os*. These formulations were supplied by Shri Dhootpapeshwar Ltd., Panvel, Mumbai, Maharashtra, India with authentication certificate (SDL01, SDL02).

Aqueous extract of *Tc* prepared from the dried stem of *Tc* and was administered orally in a dose of 100 mg/kg/day. Aqueous extract of *Pe* prepared from the dried fruit of *Pe* and was administered orally in a dose of 300 mg/kg/day. The doses of these plant drugs used in the present study are as mentioned in Ayurveda literature and have shown the effect on learning and memory in earlier studies.[6-9,12,13] The standardization of the doses was done with a pilot study. These doses were used in the present study after the standardization of the test was done with same doses.

Both the aqueous extracts were obtained along with their authentication certification from Natural Remedies, Bangalore (NR002, NR003). All these drugs were given by gavage feeding. Individual plant drugs (*Tc* and *Pe*) with *Os* coating could not be procured and hence were not used for the comparison.

Method of preparation of test drugs

Dried stem of *Tc*, dried fruits of *Pe* were procured from the suppliers and authenticated. Fresh leaves of *Os* were obtained from the cultivation of Shri Dhootpapeshwar Ltd. The specific procured parts of *Tc* and *Pe* were pulverized. 2 kg of *Tc* and 6 kg of *Pe* were mixed and passed through #2 mesh size. The filtered powdered drugs were soaked in 64 L of water for 8 h. The mixture was then boiled and filtered to get extract (16 L). To the remaining residue 32 L of water was added, and again the mixture was boiled for 8 h. It was filtered to get 8 L of extract. Both the extracts were then mixed to get thick semi-solid mass. To convert this semi-solid formulation into a dry powdered formulation, fine powder (#60 mesh size) of dried stems of *Tc* (0.2 kg) and dried fruits of *Pe* (0.6 kg) were mixed. This mixture was placed in trays and dried at 60–70°C for 24–26 h in a dryer. After 24 h, trays were taken out from the dryer and turned up and down and again kept in the dryer for another 24 h. This hard and dry material was pulverized and passed through #40 mesh. The total yield was 1.96 kg.

Juice obtained from 1 kg fresh leaves of *Os* was added to H1 formulation (*Tc* + *Pe)* prepared as mentioned above and mixed thoroughly. The mixture was dried at 60–70°C for 24–30 h, followed by pulverization and then passed through #40 mesh to get the second (H2) formulation (*Tc* + *Pe* + *Os*). The extractive values of both the formulations were 24.5%. These formulations were stored at room temperature in a desiccator. Whenever required, solutions were made by adding distilled water to it.

Controls

Rivastigmine, a centrally acting anticholinesterase, which enhances concentration of Acetylcholine in the brain synapses by blocking acetylcholinesterase-an enzyme that degrades Acetylcholine. Thus, it was selected as a positive control in normal mice and in Scopolamine induced amnesia model. Commonly used nootropic agent Piracetam has nonspecific memory enhancing actions and acts through multiple mechanisms; hence it was selected as a positive control in all the models to compare the memory enhancing properties. Rivastigmine (Sun Pharmaceutical Industries Ltd., Ahmednagar, Maharashtra, India) in a dose 2.4 mg/kg/day was administered orally for 15 days and Piracetam (UCB India Pvt. Ltd., Vapi, Gujarat, India) was given in a dose of 200 mg/kg/day intraperitoneally for 8 days. The dose of Rivastigmine and Piracetam was selected from the previous studies and was standardized before using in the present study.[8-11] Mice from the control group were given equivalent volume of distilled water as it was used to dissolve the test drugs.

Amnestic agents

Inducing amnesia by amnestic agents like scopolamine or diazepam is a commonly used interoceptive behavioral model.[8,11] Scopolamine (as a hydrobromide salt purchased from Sigma-Aldrich, USA), an antimuscarinic agent, which produces transient memory deficits. It was administered intraperitoneally in a dose of 3 mg/kg. Diazepam known to induce anterograde amnesia and affect the short-term memory, (purchased as Calmpose® tablets Ranbaxy Ltd., India) was used in a dose of 1 mg/kg intraperitoneally. Scopolamine and diazepam were given as a single dose, 90 min after the last dose of drugs/vehicle. Cyclosporine, an immunosuppressant was used to induce neurodegeneration and subsequent memory deficit in earlier animal studies.[9] Five doses of cyclosporine (Novartis India Ltd., Mumbai, Maharashtra, India) were given in the dose of 25 mg/kg/day intraperitoneally on alternate days with distilled water for total period of 10 days before initiating study drugs.

Assessment of learning and memory using elevated plus maze test

Elevated plus maze (EPM) is an "exteroceptive behavioral model" commonly used as a rodent model of anxiety. It is also commonly used for experimental evaluation of drugs acting on learning and memory.[8,11,15-20]

There are various behavioral and pharmacological models available for evaluation of learning and memory, which includes exteroceptive and interoceptive stimuli models. Exteroceptive stimuli models include behavior on mazes (e.g. EPM, Hebb Williams maze, complex maze, radial arm maze, Y-maze, T-maze, Morris water maze etc.), passive avoidance (e.g., two chambered box including dark and light chambers) and active avoidance (e.g. Cook's pole apparatus). Interoceptive stimuli models include electroshock induced amnesia, hypoxic stress induced learning deficits or pharmacological agents induced learning and memory deficits (e.g., scopolamine, diazepam or cyclosporine).[15-17]

Elevated plus maze uses natural anxiety of rodents to light, open environment, and height. The height and open arms of the maze act as an aversive stimulus to the rodents. This is a simple, less time-consuming procedure that does not involve any sophisticated equipment or prior training or noxious stimuli like electric shock and also there is no need to manipulate appetite behaviors.[15-18] Therefore, it was decided to use this apparatus in the present study.

It has two opposite open arms (16 cm × 5 cm) crossed with two closed arms (16 cm × 5 cm × 12 cm). The arms are connected with a central square (5 cm × 5 cm) to give the apparatus a plus sign appearance. While conducting the test, a mouse was placed on the open arm facing opposite to the closed arm. Time taken by mouse for the first entry with all the four limbs inside a closed arm was measured and expressed as transfer latency (TL). First day readings of TL were considered as acquisition of learning and its' subsequent retention (memory) was examined 24 h later.

Behavioral tests have inherent limitations as these tests depend on memory type to be evaluated. Each dimension of learning and memory demands a specific experimental approach. EPM test can be used only to assess the short-term memory, and additional evaluation is needed to understand the other aspects of learning and memory.

Study procedure

A pilot study was carried out for standardization of the study drugs, controls and amnesic agents (including their doses), parameters and establishments of models using the same methodology as mentioned in the present study.

Before selecting for the study, mice were subjected to one time screening for their activity on the EPM. The mice having close range of TL were selected for the study. This was done to reduce the drastic variations in TL that is the parameter of assessment. Total 156 mice were selected for the study, 15 days after the screening. The mice were weighed, and drug doses were given accordingly. Mice were randomly divided for drug treatment as shown in Table 1. These mice were subsequently used in different models and the evaluation of test drugs was done with normal mice (n = 42), mice with Scopolamine induced memory impairment (n = 42), mice with diazepam-induced memory impairment (n = 36) and mice with cyclosporine-induced neurodegeneration (n = 36). Normal mice were tested for learning, 90 minutes after the last dose of test drugs/distilled water. To induce memory impairment, scopolamine (3 mg/kg) or diazepam (1 mg/kg) was given intraperitoneally as a single dose, 45 min after the last dose of the test drugs/distilled water. On day 15, mice were subjected to test for learning 45 mins after the amnesic agent was given and subsequently allowed to explore the EPM. Memory was tested on Day 16. Cyclosporine (25 mg/kg/day) was given intraperitoneally alternating with distilled water injections for total 5 doses in 10 days. From Day 11 onwards, the test drug/distilled water treatment was given orally for next 15 days. On Day 25, 90 min after the last dose of test drug/distilled water EPM test was done to evaluate learning. Memory was tested 24 h later.

Table 1: Drug treatment for the mice

Group	Number of mice (n)	Drugs	Duration (days)
1	24	Distilled water	15
2	24	Distilled water followed by Piracetam	7+8
3	12	Rivastigmine	15
4	24	*Pe*	15
5	24	*Tc*	15
6	24	Formulation 1 (H1: *Pe*+*Tc*)	15
7	24	Formulation 2 (H2: *Pe*+*Tc*+*Os*)	15

Pe=Phyllanthus emblica, Tc=Tinospora cordifolia, Os=Ocimum sanctum

On the day of training, the mice were kept fasting for 18 h and allowed access to food for only 1 h at the end of the day's training. TL was noted on the training day, and the mouse was allowed to explore the maze for 180 s. 24 h later, the mouse was again placed on the edge of open arm, and TL was noted for memory.

Statistical analysis

The data was expressed as mean ± standard deviation. The statistical analysis was done using statistical software GraphPad InStat, manufactured by GraphPad software from United States of America. Results of the training phase were compared with retention phase for each group using paired t-test. The results of memory impaired mice treated with distilled water were compared with distilled water treated normal mice by unpaired t-test. In addition, inter-group comparison in learning and retention phase was done using one-way ANOVA followed by *post-hoc* test. The level of significance was at $P < 0.05$.

RESULTS

In normal mice [Table 2], Rivastigmine and Piracetam have not shown any effect on learning but they were found to have memory-enhancing effects at the doses used in this study.[8-11] Both of them showed decreased TL when tested for memory on day 16 as compared to normal control. This indicated retention of learning about the path for closed arms of the EPM. In normal mice, pretreatment with plant drugs, single as well as combinations showed a trend toward a decrease in TL during the learning phase but the decrease was not statistically significant. Compared to their own readings on day 15, on repeat testing 24 h later they exhibited significantly lower TL. On day 16, TL with plant drugs was significantly less as compared to normal control group [Table 2]. The TL of mice given H1 and H2 were comparable to comparators *Tc*, *Pe* and positive controls Rivastigmine and Piracetam. However, the value for the TL on day 16 for H2 group was the lowest.

In scopolamine-induced memory impaired mice [Table 2], TL of distilled water control group was significantly higher than the distilled water control normal mice on day 15

and 16 showing learning and memory impairment by scopolamine. Significant reduction in TL was found on day 16 with Rivastigmine and Piracetam treatment as compared to the control group. Thus, Rivastigmine and Piracetam could not prevent the learning impairment but prevented memory impairment induced by scopolamine. There was a significant reduction of TL by all plant drugs on day 15 as compared to the control group, showing prevention of scopolamine-induced learning impairment. From the values of TL on day 16, it is evident that they maintained the memory to normal in spite of scopolamine-induced insult [Table 2]. The TL of mice given H1 and H2 was comparable to comparators *Tc*, *Pe* and positive controls Rivastigmine, Piracetam. But on day 16, TL for H2 group was lowest and was significantly less compared to Piracetam group indicating better reversal of amnesia induced by scopolamine.

Diazepam treated mice showed significantly higher TL with distilled water control mice than the distilled water control normal mice on day 15 and 16 proving the learning and memory impairment by diazepam but there was no reduction in TL on day 16 as compared to that on day 15 in distilled water treated mice [Table 3]. Piracetam administration did not prevent learning impairment, however, on day 16 there was a reduction in TL [Table 3]. Thus, Piracetam significantly prevented the memory impairment after diazepam. Mice pretreated with plant drugs showed a trend toward reduction in TL on day 15 but results were comparable to those of control. As against this, they showed better retention of learning on day 16 and prevented the memory impairment induced by diazepam [Table 3]. TL of mice given H1 and H2 was comparable to comparators *Tc*, *Pe* and Piracetam. As in earlier model, amongst all the drugs, effects of H2 formulation on memory were the best but not statistically different than the H1 formulation, *Tc* or *Pe* [Table 3].

Differing from the earlier models, 10 days treatment with cyclosporine was given before initiating study drugs and hence this model tested the ability of test drugs to reverse the changes induced by cyclosporine. It was found that cyclosporine increased TL significantly on day 25 and 26 [Table 3] as compared to distilled water control normal mice. This signifies impaired learning and memory with cyclosporine insult. There was no significant difference between readings of day 25 and 26. Treatment with Piracetam failed to reverse both learning and memory impairment induced by cyclosporine. However, treatment with plant drugs, showed a trend toward reduction in TL when tested on day 25 but these values were not significantly less than cyclosporine control [Table 3]. Thus, none of the plant drugs were found to correct the learning impairment

Table 2: Effect of test drugs on normal mice and scopolamine-induced memory impairment

Normal mice treated with vehicle/drug	Transfer latencies in normal mice		Transfer latencies in mice with scopolamine-induced memory impairment	
	Day 15 (learning)	Day 16 (memory)	Day 15 (learning)	Day 16 (memory)
Distilled water (15 days)	27.33±6.28	23.67±8.76	35.17±3.55$^{@}$	34.17±4.12$^{@}$
Rivastigmine (2.4 mg/kg for 15 days)	28±5.83	10±2.37$^{***\#\#\#}$	29.17±6.43	12.33±3.45$^{*\#\#}$
Distilled water (7 days) followed by piracetam (200 mg/kg for 8 days)	26.33±7.47	11.83±6.85$^{*\#\#}$	28±4.94	17.5±5.17$^{*\#\#}$
Tc (100 mg/kg for 15 days)	20±3.8	11.5±3.21$^{***\#\#}$	26.33±3.5$^{\#}$	13.83±6.4$^{*\#\#}$
Pe (300 mg/kg for 15 days)	21.83±8.68	12.83±4.71$^{*\#}$	25±6.07$^{\#}$	15.5±6.44$^{*\#\#}$
H1 (*Tc*+*Pe*) (400 mg/kg for 15 days)	22.33±4.63	10.33±2.94$^{***\#\#}$	27.17±5.19$^{\#}$	12.33±4.93$^{*\#\#}$
H2 (*Tc*+*Pe*+*Os*) (400 mg/kg for 15 days)	22±4.82	9.5±2.51$^{**\#\#\#}$	22.5±3.33$^{\#}$	8.17±2.48$^{**\#\#\$}$

n=6/group. All values represent mean±SD; paired *t*-test; $^{*}P$<0.01, $^{**}P$<0.001 ANOVA followed by *post-hoc* Tukey's test: $^{\#}P$<0.05, $^{\#\#}P$<0.001 compared to control, $^{\$}P$<0.05 as compared to Piracetam. Unpaired *t*-test; $^{@}P$<0.05 compared to distilled water control normal mice. SD=Standard deviation, *Pe=Phyllanthus emblica, Tc=Tinospora cordifolia, Os=Ocimum sanctum*

Table 3: Effect of test drugs on diazepam and cyclosporine-induced memory impairment

Mice treated with vehicle/drug	Transfer latencies in mice with diazepam-induced memory impairment		Transfer latencies in mice with cyclosporine-induced memory impairment	
	Day 15 (learning)	Day 16 (memory)	Day 25 (learning)	Day 26 (memory)
Distilled water (15 days)	36.83±6.97$^{@}$	34.67±6.41$^{@}$	43.33±6.02$^{@@}$	37.83±7.55$^{@}$
Distilled water (7 days) followed by Piracetam (200 mg/kg for 8 days)	29.83±5.19	13.67±4.37$^{**\#}$	41.33±5.79	32.83±4.79**
Tc (100 mg/kg for 15 days)	29±6.39	13±3.8$^{**\#}$	39.33±6.35	15±4.98$^{**\#\$}$
Pe (300 mg/kg for 15 days)	28.67±4.55	12.67±3.39$^{**\#}$	33.83±8.59	14±3.85$^{*\#\$}$
H1 (*Tc*+*Pe*) (400 mg/kg for 15 days)	27.17±5.64	12±2.61$^{**\#}$	33.17±5.85	13.5±5.79$^{**\#\$}$
H2 (*Tc*+*Pe*+*Os*) (400 mg/kg for 15 days)	26.5±5.75	11.33±4.18$^{*\#}$	32.83±5.19	12.67±2.34$^{**\#\$}$

n=6/group. All values represent mean±SD, paired *t*-test; $^{*}P$<0.01, $^{**}P$<0.001; ANOVA followed by *post-hoc* Tukey's test: $^{\#}P$<0.001 compared to control, unpaired *t*-test; $^{@}P$<0.05, $^{@@}P$<0.01 compared to distilled water control normal mice. $^{\$}P$<0.001 as compared to Piracetam. SD=Standard deviation, *Pe=Phyllanthus emblica, Tc=Tinospora cordifolia, Os=Ocimum sanctum*

induced by cyclosporine. However, on day 26, there was decrease in TL and it was significantly less as compared to cyclosporine control as well as Piracetam treated mice indicating the ability of plant drugs to reverse the memory deficit induced by cyclosporine. The TL of mice treated with *Tc*, *Pe,* H1 and H2 formulations remained comparable with each other like in earlier model. Amongst all, again H2 formulation showed better values but statistically all the plant drugs were comparable [Table 3].

DISCUSSION

The dearth of an ideal drug for dementia in modern medicine prompted us to investigate traditional system of Indian medicine-Ayurveda. Various plants have shown actions on learning and memory. Studies are done on *Bacopa monniera*, *Centella asiatica*, *Rose alba*, *Dacus carota*, *Desmo gagenticum*, *Vitis vinifera*, *Withania somnifera*, *Convolvulus pluricaulis*, *Eclipta alba*, *Glycyrrhiza glabra*, *Myristica fragrance*, *Zingiber officinale*, *Butea frondosa*, *Hypericum perforatum*, *Phyllanthus amarus*, *Ginkgo biloba*, St. John's wort, *Kava kava* and *Valerian* for their actions in brain disorders.[21] Indian traditional system, Ayurveda mentions plants like *Pe, Tc* and *Os* to have memory enhancing properties. Fruits of *Pe*, stems of *Tc* and leaves of *Os* have been evaluated earlier for their effects on learning and memory performances and have shown promising results.[8-10,19,21,22] *Os* also reported to have anti-inflammatory, chemo-preventive, antioxidant, adaptogenic, psychopharmacological activities.[23-25] Additionally *Os* extracts have shown to inhibit acetylcholinesterase and improve cognition in rats and also reversed the ibotenic acid or colchicine induced learning and memory deficits.[25-27] Recently published review by Cohen has highlighted multiple important uses of *Tulsi* including the actions against physical, chemical, metabolic and psychological stress.[26] The Ayurvedic texts also revealed that these plants are often prescribed in combination as per the concept of *Pathsanyojana* or rational combination of drugs.[28] In the Indian market too; most of the Ayurvedic formulations for improving learning and memory are multi-ingredient in nature. *Tc*, *Pe* and *Os* are often the common ingredients of these formulations. Though the efficacy of extracts of individual plants was proven, an extensive literature search revealed that the effects of a combination of these three plant drugs on learning and memory had not been evaluated using modern scientific methodology. It was, therefore, interesting to find out whether combining these plants produces a greater effect on learning and memory. The results of the present study substantiated the memory enhancing properties of *Tc*, *Pe* and *Os* as seen in earlier studies.[8-13,19,20] It has also shown that effects of individual plant drugs as well as in combination were comparable and at times better to those of modern medicinal agents. In addition, the effects of combinations of *Tc* and *Pe* with or without *Os* were not more than those of the individual agents.

Acetylcholine is an important neurotransmitter involved in the regulation of cognitive functions. According to the cholinergic hypothesis, memory impairments in patients with senile dementia are due to a selective and irreversible deficiency of the cholinergic functions in the brain.[29] Hence, Rivastigmine is used in the management of Alzheimer's dementia. Similarly, scopolamine, an antimuscarinic agent has shown memory impairment when given to mice shortly before training.[30] Thus, a model of amnesia induced by scopolamine was selected in the present study. Rivastigmine is a centrally acting acetylcholinesterase inhibitor and increases acetylcholine concentration in the brain.[31] By increasing high-affinity choline uptake, Piracetam facilitates acetylcholine production and turnover with varying actions at both muscarinic and nicotinic receptors.[32] Hence, we used these two positive controls in the present study. The plant drugs have successfully prevented the learning impairment and deficit in short-term memory caused by scopolamine insult. The effects of Rivastigmine and Piracetam on memory were comparable though plant drugs showed a trend toward enhancement. Amongst all the plant drugs, H2 formulation exhibited promising results and was significantly better than Piracetam indicating actions on the cholinergic system that was possibly enhanced by *Bhavana samskara*. This suggests that selected plant drugs may have actions on the cholinergic system or preventing the effects of the cholinergic blocker. But in the present study, acetylcholinesterase activity or concentration of acetylcholine was not measured. Hence, the exact mechanism of action of these plant drugs cannot be commented upon.

Diazepam and several other benzodiazepines have been reported to impair learning and memory in animals and humans.[33-35] Diazepam is known to produce anterograde amnesia and state-dependent learning.[35-37] Thus, it was used as an amnestic agent in the present study. As the mechanism of action of diazepam is mediated through facilitation of gamma-aminobutyric acid (GABA),[37] it was decided not to use Rivastigmine as a positive control, which has a specific action mediated through acetylcholine. Instead, Piracetam was selected due to its nonspecific nootropic actions and multiple mechanisms.[32] As evident from the results, diazepam impaired learning in EPM test. The learning impairment appears to be partly due to anxiolytic effect. This was contrary to the observations made by Itoh *et al.*[33] The plant drugs prevented the amnestic effect of diazepam indicating possible actions on GABA that needs to be evaluated further.

Cyclosporine has shown to cause degeneration of neurons and subsequent memory deficit.[9] The dose and regime of pretreatment with cyclosporine was as described by Agarwal *et al*., 2002.[9] *Tc* has successfully reversed the memory deficit and protected against the neurodegenerative changes produced by cyclosporine.[9] Thus we used cyclosporine to induce amnesia by neurodegeneration and the findings of Agarwal *et al*., 2002 corroborate with ours.[9] In this model too, Piracetam was selected as a positive control based on the claims regarding its neuroprotective effects.[32] Treatment with plant drugs after the use of cyclosporine has shown to reverse the memory impairment indicating possible protective effects of plant drugs against the degeneration of neurons induced by cyclosporine. The results with the plant drugs were significantly better as compared to Piracetam indicating possibly better neuroprotective effects, which can be substantiated further by studying histopathological examination of mice brains.

The present study revealed that both, H1 and H2 formulations have memory-enhancing properties in normal animals. In three models of amnesia, wherein memory impairment was induced by scopolamine, diazepam, and cyclosporine, they showed promising results and these effects were comparable and at times better than currently used modern medicinal agents like Rivastigmine and Piracetam. However, the results with a combination of *Tc* + *Pe* with or without *Os* were comparable. Moreover, their effects were not statistically different than the individual plant drug that is, *Tc* and *Pe*. Hence, the concept of *Bhavana samskara* could not be explored in the present study but results showed a trend of better effects with *Bhavana samskara* that can be evaluated further. These results are consistent with our earlier findings using complex maze, evaluating spatial learning and memory in rats.[38]

Validation of *Bhavana samskara* will need series of studies involving larger sample size and multiple models that was beyond the scope of the present study. It will be interesting to compare the combination of these plant drugs with the *Bhavana samskara* on individual plant drugs. It will also be interesting to compare these plant formulations with only *Os* treated group as well. This offers a good scope for further research, which can help validation of *Bhavana samskara*.

The plant drugs have also shown to have some possible actions on the cholinergic system, GABA and in the prevention of neurodegeneration. This can be further studied using tests like acetyl-cholinesterase assay or histopathological examination of brains in affected animals to see the reversal of neurodegenerative changes. It is important to carry out further studies with both the plant formulations as well as single-ingredient plant drugs using battery of other behavioral tests including tests for effects on long-term memory. Multiple such studies will help us to identify the lead compound, which can be developed further by exploring for its mechanism/s of action using various neurobiological and cellular techniques. And further investigations in human subjects and patients with dementia will make it a valuable and cost-effective addition to the existing armamentarium of the nootropics.

CONCLUSION

Bhavana Samskara on *Tinospora cordifolia* and *Phyllanthus emblica* combination showed promising results as nootropic agent st times it was better than modern medicinal agents. Clinical studies need to be done to evaluate the potential of these plant formulations for the management of dementia and the potential benefits of bhavana samskara needs to be explored further in other therapeutic areas.

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Conflicts of interest

There are no conflicts of interest.

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