Research Article

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Synergistic Effect of the Combination of Ethanolic Extract of *Piper cubeba* and Polyherbal Formulation Triphala on Learning and Memory Enhancement against Scopolamine Induced Amnesia in Mice

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Abstract

Alzheimer's disease is the fourth most common cause of death which estimated that, by 2030 more than 65 million people of world will be living with dementia. Cognition enhancers attenuate some aspects of brain dysfunction by acting on cholinergic system but having limited use. About 75-80% of world population still mainstay on use of traditional medicine. In the present study, ethanolic extract of Piper cubeba L. (Piperaceae) and its combination with Ayurvedic preparation triphala was evaluated for learning and memory enhancement activity against scopolamine induced amnesia. The passive avoidance behavioral model showed significant ($P<0.001$) decrease in step down latency for scopolamine injected group whereas extract treated group significantly ($P<0.01$) apposed the action of scopolamine. However, combination of extract and triphala exhibited more significant ($P<0.001$) action. Similarly, in Y-maze task, there was significant ($P<0.001$) increase in percentage same arm returns, decreased alternate arm returns and spontaneous alternations in scopolamine treated group, indicating memory impairment and these changes were significantly ($P<0.001$) reversed in extract as well as its combination with triphala treated group, suggesting improvement in learning and short term memory function. The extract also exhibited significant reduction in total cholesterol level against Triton-WR 1339 induced acute hypercholesterolemia which represents inhibition of deposition of cellular Aβ- protein and thus reduces the risk of Alzheimer's disease. Thus, it concludes that, combination of ethanolic extract of Piper cubeba and triphala exhibited synergistic effect on improvement of learning and memory function by modulating cholinergic function in hippocampus region of brain.

Keywords: Cholinergic, Dementia, Spontaneous Alternation, Step Down Latency.

Introduction

Learning and memory are two fundamental cognitive functions that confer acquisition of information and its subsequent retention [1]. Any disturbance in these aspects leads to dementia which is largely a hidden problem in the developing countries has been accompanied by enhanced life expectancy. The most common cause of dementia is Alzheimer's disease (AD) which is a neurodegenerative disorder characterized with loss of neuron in distinct area of brain, accumulation of intraneuronal neurofibrillary tangles and a decline in cognitive abilities [2]. Cognition enhancers attenuate some aspects of brain dysfunction by involving interactions between neurohumoral signaling responses and the cholinergic system. Currently, the allopathic system of medicine principally relies on nootropic agents such as piracetam, fosracetam, nefiracetam and cholinesterase inhibitors such as donepezil, revastigmine and galantamine have been approved for the treatment of AD [3,4]. About 75-80% of world population still mainstay on use of traditional medicine. Many medicinal plants provide relief of symptoms comparable to that obtained by allopathic medicines. The Indian traditional system mentioned enormous medicinal plants and their formulations in the management of various psycho behavioral diseases.

The genus Piper belongs to the family Piperaceae, having more than 1000 species throughout the tropical and subtropical regions of the world. *Piper cubeba* L., commonly known as 'Java pepper' is a popular medicinal plant which has been extensively used in many countries including India [5]. The fruit are used for the treatment of rheumatism, abdominal pain, asthma, diarrhea, dysentery, gonorrhea, enteritis and syphilis and has also been proved to have an inhibitory action on hepatitis C virus protease [6,7]. In addition, the extract has been reported to potential antiestrogenic, anti-inflammatory, nephroprotective, cytotoxic, antiparasitic and antimicrobial action [8]. Similarly triphala is a polyherbal preparation contains mixture of the dried powders of three fruits mainly Emblica officinalis, Terminalia belerica and Terminalia chebula in equal proportions. In

Ayurveda it is described as 'tridoshic rasayana' having rejuvenating effects on the three constitutional elements i.e vata, pitta and kapha which governs human life. Traditionally been used as laxative in chronic constipation, colon cleansing, digestion problems and poor food assimilation [9]. It has widely proved for various cardiovascular diseases, high blood pressure, hyperlipidemia, hepatic dysfunction, intestinal inflammation, antibacterial, antitumor, antioxidants and ulcerative colitis [10,11]. The individual plants of triphala Emblica officinalis, Terminalia belerica and Terminalia chebula have been already proved for nootropic potential [12-14]. As, Ayurvedic medicinal plants had successfully attenuated memory dysfunction induced by scopolamine, the present study was undertaken to evaluate antiamnesic potential of ethanolic extract of *Piper cubeba* (EPC) and its combination with Ayurvedic formulation triphala against scopolamine induced amnesia in animal model [15].

Materials and Methods

Chemicals and Drugs

Scopolamine (German Remedies, India), Simvastatin (Krebs Biochemicals, India), Cholesterol reagent kit (Bio-Lab Diagnostics, India), Triton WR-1339 (Sigma-Aldrcih, USA, Triphala churna (Baidyanath, India). All other reagents and solvents used in the experiment were of standard analytical grade.

Plant material and preparation of crude extract

The dry fruits of *Piper cubeba* herbarium authenticated (Auth.15-118) at Agharkar Research Institute Pune, India and the specimen was deposited in Department of Biodiversity & Palaeobiology. The dry powder (500 g) was subjected to ethanolic extraction by using continuous Soxhlet's apparatus. The extract was filtered through Whatman filter paper and the residue obtained was carried to rotary evaporator under reduced pressure for dryness.

Experimental animals and dose selection

All the experiments were carried out using Swiss albino mice (20-25 g) of either sex and are housed under standard husbandry conditions with 12 h light/dark cycle. The animals had free access to standard pellet (Hindustan Lever Ltd., India) and water ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee (DYPIPSR/IAEC/14/P04).

The dose 400 mg/kg BW p.o. of ethanolic extract of *Piper cubeba* (EPC) was selected from previously reported article [16]. Similarly to study synergistic effect, suspension of the combination of EPC and of triphala churna (1:1) was prepared in normal saline for oral gavage. Triphala was selected as reference standard as well for combination with EPC to study synergistic action.

Passive avoidance behavioral task

The animals were divided into following groups containing 6 animals each. The study protocol includes 7 days pretreatment with EPC before administration of scopolamine.
Group I -Vehicle treated (normal saline): 10 ml/kg, p.o.
Group II -Scopolamine: 1.4 mg/kg, i.p. + normal saline
Group III -Triphala 400 mg/kg, p.o. + scopolamine
Group IV -EPC: 400 mg/kg, p.o. + scopolamine
Group V -Triphala + EPC; 400 mg/kg, p.o. + scopolamine

The animals were exposed to training session (on 7th day, considered as day 1) after 45 min of scopolamine administration. The retention was measured after 24 h (on 8th day, considered as day 2).

The apparatus consist of a chamber (29x30x29 cm), featuring a grid floor made up of stainless steel with a wooden platform (10x8x1.7 cm) in the center of the grid floor. Electric shock (20 V, A.C.) was supplied to the grid floor. On the 7th day of drug treatment training was carried out in two similar sessions. Each mouse was gently placed on the wooden platform at the centre and foot shocks were delivered for 15 s to descent animal. The step-down latency (SDL) was defined as the time taken by mouse to step down from the wooden platform to grid floor with all its paws, was recorded. Retention (memory) was tested after 24 hr (i.e. 8th day, 24 hr after last dose) in a similar manner, except that the electric shocks were not applied to the grid floor observing an upper cut-off time of 300 s. Significant increase in SDL value indicated improvement in memory [17].

Y-maze task

The mice were divided into following groups for various treatment schedules. The 7 days pretreatment is given before giving the scopolamine injection.
Group I -Vehicle treated (normal saline): 10 ml/kg, p.o.
Group II -Scopolamine: 1.4 mg/kg, i.p. + normal saline
Group III -Triphala 400 mg/kg, p.o. + scopolamine
Group IV -EPC: 400 mg/kg, p.o. + scopolamine
Group V -Triphala + EPC; 400 mg/kg, p.o. + scopolamine

On the 7th day scopolamine was injected after 45 min of last dose treatment and assessment of learning and memory was performed after 24 h. During the period of treatment, on the day 1, 2 and 5 all the animals were allowed to explore the Y maze apparatus for a 5 minute session.

Y-maze consists of three arms of equal size, labeled as A, B, and C respectively with an angle of 120° between each of the two arms. Each arm was 19 cm long, 5 cm wide, and 14.5 cm height. Mice were placed within one arm and the total number of arm entries (NAE), percentage same arm returns (SAR) and alternate arm returns (AAR) was recorded manually for the period of five minutes each. An arm entry was confirmed when the hind paws of the mouse were completely placed in an arm. The consecutive entry by a mouse into the three different arms was considered as alternation [18]. Percentage of spontaneous alternation performance (% SAP) was determined using the formula,

$$\% \text{ SAP} = \frac{\text{Total alternations}}{\text{Total number of arm entries}-2} \times 100$$

Estimation of total serum cholesterol

The overnight fasted mice were randomly divided into following groups for estimation of serum cholesterol level.
Group I -Normal: standard food pellet and water, 4% DMSO, i.p.
Group II – Hyperlipidemic: received Triton WR-1339 in 4% DMSO, 400 mg/kg, i.p.
Group III -Triphala 400 mg/kg, p.o. + Triton WR-1339
Group IV -EPC: 400 mg/kg, p.o. + Triton WR-1339
Group V -Triphala + EPC; 400 mg/kg, p.o. + Triton WR-1339

All the groups where pretreated for seven successive days before injecting Triton WR-1339 a single dose. After 24 h of treatment, animals were mild anesthetized with diethyl ether. The blood was withdrawn from retro-orbital sinus and immediately centrifuged (3000 rpm for 10 min). The serum was used to estimate the level of

total cholesterol by CHOD-PAP method [19]. The blank, standard and test sample were prepared according to standard procedure as mentioned in the cholesterol diagnostic kit. The absorbance was read out at 510 nm by using Autoanalyzer (Erba, Chem-5). The concentration of total cholesterol was calculated by using formula,

$$\text{Total cholesterol (mg/dL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

Statistical Analysis

Data are expressed as mean ± S.E.M. Statistical analysis was performed with one-way ANOVA followed by Dunnett's multiple comparison tests (n=6). P<0.05 was considered as statistically significant.

Results

Effect of EPC and triphala on passive avoidance behavioral task

The time taken (in seconds) by the mouse to step down from the wooden platform to grid floor with all its four paws was considered step-down latency (SDL). The scopolamine (1.4 mg/kg body weight, i.p.) injected group showed significant (P<0.001) decrease in SDL. The standard drug triphala (400 mg/kg body weight, p.o.) and EPC (400 mg/kg body weight, p.o.) significantly reversed the action of scopolamine. However, combination of EPC and Triphala (400 mg/kg body weight, p.o.; 1:1) exhibited more significant (P<0.001) increase in SDL which is an indication of synergistic action (Figure 1).

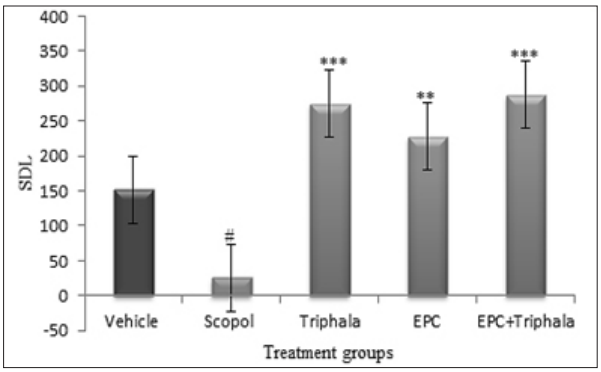


Figure 1: Effect of EPC and its combination with triphala on passive avoidance behavioral task. Data are represented as mean ± S.E.M (n=6). * indicates P<0.05 and refers to the SDL between non-treated and treated group. # indicates P<0.001 refers to comparison between normal and scopolamine induced amnesia group. Scopol= scopolamine.

Effect of EPC and triphala on Y-maze task

The results obtained with the Y-maze task are shown in Figure 2. The total NAEs were not significantly different among all groups (P>0.05); except triphala (400 mg/kg) (P<0.05) and the combination of EPC with triphala (1:1) (P<0.01) treated group showed significant decrease in number of entries (Figure 2.a). There was significant (P<0.001) increase in the percentage of same arm returns (% SAR) in scopolamine induced amnesia group as compared to vehicle treated group. Whereas triphala and EPC treated group showed significant decrease in percentage SAR when compared with scopolamine treated group [20]. However, highly significant (P<0.001) reduced percentage SAR was noted in animals treated with combination of EPC and triphala (Figure 2.b). Similarly, significant (P<0.001) reduced percentage alternate arm returns (% AAR) was observed in scopolamine treated animals as compared to normal group animals. The significant (P<0.01) increase in % AAR was noted among the triphala and EPC treated animals as compared to scopolamine treated animals. As expected highly significant (P<0.001) increase in % AAR was reported in animals treated with combination of EPC and triphala (Figure 2.c). In addition, significant (P<0.001) reduction in percentage spontaneous alternations was observed in scopolamine treated group as compared to normal animals whereas significant (P<0.01) increase in percentage spontaneous alternations was seen among the triphala and EPC treated group. However, combination of EPC and triphala exhibited more significant (P<0.001) improvement in percentage spontaneous alternations as compared to scopolamine treated group (Figure 2.d).

Effect of EPC and triphala on total cholesterol level

A triton WR-1339 induced significant (P<0.001) hypercholesterolemia in mice as compared to vehicle treated animals. The treatment group animals with individual doses of triphala and EPC as well as combination of EPC and triphala showed significant (P<0.001) decreased levels of total cholesterol when compared to control group (Triton WR-1339 treated) (Table 1).

Discussion

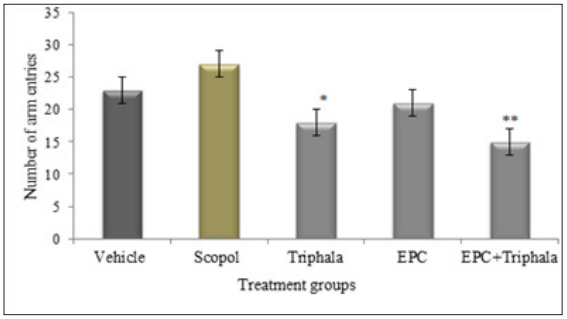
The clinical features of AD are an amnesic type of memory impairment, deterioration of language and visuospatial deficit; whereas, in laboratory animals, cognitive functions must be accessed through behavioral models. Passive avoidance behavior is based on negative reinforcement and was used to observe the long term memory. The time taken (in seconds) by the mouse to step down from the wooden platform to grid floor with all its four paws was considered step-down latency. Significant increase in SDL value represented improvement in memory [21]. Scopolamine induces decreased SDL in young mice, indicating impairment of memory (amnesia). The Ayurvedic polyherbal formulation triphala and EPC reversed the amnesia induced by scopolamine. The highly significant increase in SDL value for combination of triphala and EPC (1:1) is an indication of synergistic effect.

The spontaneous alternation performance (SAP) task based on effects of the hippocampus region of the brain which is also associated with neurological impairment [18,22]. Moreover, the scopolamine induced amnesia has a high selectivity for the M1 muscarinic receptors found in the hippocampus. Extensive evidences also revealed that hippocampal dependent learning is associated with an increase in acetylcholine (ACh) levels [23]. Spontaneous alternation of Y-maze task is a measure of exploration behavior in mice and is a reliable screening model to study effects of drugs against scopolamine induced memory impairments [24]. In the present study a mouse with an impaired short-term memory (STM) cannot recall which arm of Y-maze it has just visited and thus tends to exhibit decreased spontaneous alternation; which was observed in scopolamine induced amnesia animals. The administration of EPC and its combination with triphala significantly increased spontaneous alternation performance indicates memory-enhancing activity.

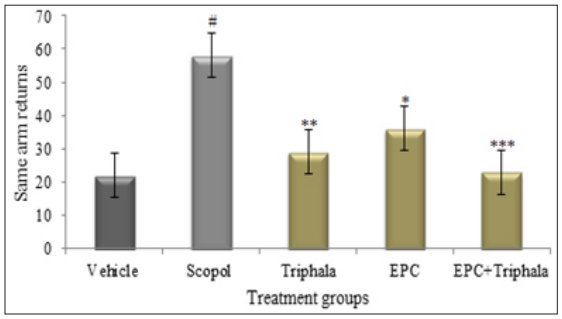
Similarly, the most of animal models showed that increase in blood cholesterol level increases incidence of deposition of cellular Aβ-protein; and drugs that prevent cholesterol synthesis lower Aβ-protein in these models [25]. Most of clinical studies suggested that the net concentration of cholesterol in brain is controlled by

peripheral cholesterol level and there is cross-talk between central and peripheral serum cholesterol pools [26]. The risk of AD is also higher in peoples of countries with high-fat and high-calorie diets [27]. Interestingly, in the present study Triton WR-1339 induced acute hypercholesterolemia was significantly antagonized by standard drug triphala as well as EPC and synergistic action was observed for the combination.

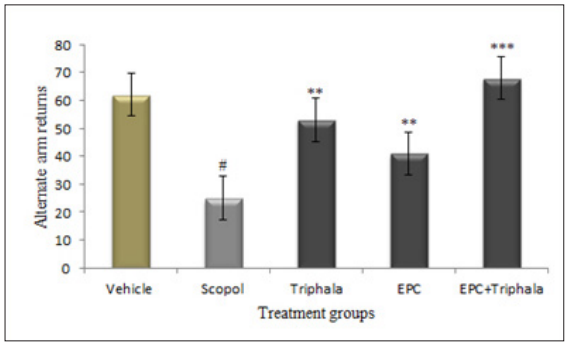
Thus, in the present study the combination of ethanolic extract of *Piper cubeba* and polyherbal preparation triphala exhibited synergistic action on learning and memory improvement against scopolamine induced amnesia in mice. This may be due to modulatory action of combination on cholinergic pathway in hippocampus region of brain.



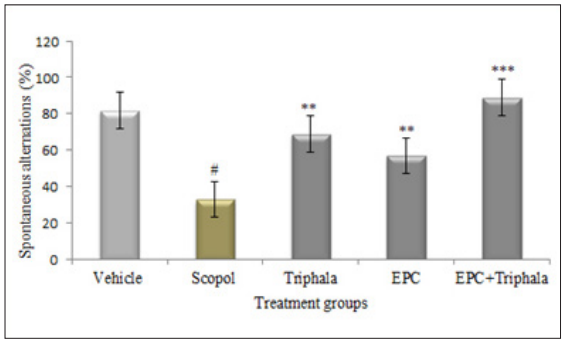
a. Number of arm entries (NAE)



b. Same arm returns (SAR %)



c. Alternate arm returns (AAR %)



d. Percentage spontaneous alternations

Figure 2: Effect of EPC and its combination with triphala on Y-maze task. (a) Total number of arm entries (b) same armreturns (c) alternate arm returns and (d) spontaneous alternation performance.Data are represented as mean ± S.E.M (n=6). * indicates P<0.05 and refers to the SDL between non-treated and treated group.# indicates P<0.001refers comparison between normal and scopolamine induced amnesia group. Scopol= scopolamine.

SDL between non-treated and treated group. # indicates P<0.001 refers comparison between normal and scopolamine induced amnesia group. Scopol= scopolamine.

Table 1: Effect of EPC and Triphala on total cholesterol level

Treatment groups	Total cholesterol level (mg/dL)
Vehicle	158.23 ± 1.07
Triton WR-1339	381.56 ± 2.31#
Triphala	172.38 ± 1.29***
EPC	189.25 ± 1.35***
EPC+Triphala	167.54 ± 1.09***

Values are mean ± S.E.M (n=6). * indicates P<0.05 and refers to the total cholesterol level between non-treated and treated group. # indicates P<0.001 refers comparison between vehicle and triton WR-1339treated group. Scopol= scopolamine.

Conclusion

Piper cubeba L. would be a promising medicinal plant for novel combination with polyherbal preparation triphala for treatment of learning and memory impairments. However, isolation and characterization of active constituents of *Piper cubeba* is necessary to detail study of underlying mechanism.

Acknowledgement

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