

Formulation, Characterization and Evaluation of Behavioral Effects of Suspension and Effervescent Granules of *Evolvulus alsinoides* Linn. and *Convolvulus pluricaulis* Choisy

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ABSTRACT

Objectives: The objective of the present study was to formulate, characterize and evaluate herbal formulations of *Evolvulus alsinoides* and *Convolvulus pluricaulis* against scopolamine-induced behavioral disturbances for Alzheimer's disease. **Methods:** The herbal drugs were extracted using ethanol. The herbal suspension and effervescent granules were prepared using the extract and evaluated on rats. Scopolamine was used to induce behavioral and locomotor disturbances. **Results:** Results revealed that both the formulations were stable and effective in enhancing cognition against scopolamine-induced behavioral disturbance. The activity score of suspension of *Evolvulus alsinoides* and *Convolvulus pluricaulis* in the actophotometer were 128.19 and 110.23 while in effervescent granules of *Evolvulus alsinoides* and *Convolvulus pluricaulis* were 124.21 and 120.29 after 60 min, respectively. Suspension of *Evolvulus alsinoides* and *Convolvulus pluricaulis* reduced the fall off time to 94.18 and 102.03 while effervescent granules of *Evolvulus alsinoides* and *Convolvulus pluricaulis* reduced 98.01 and 98.16 after 30 min using the rota-rod, respectively. Suspension of *Evolvulus alsinoides* and *Convolvulus pluricaulis* reduce the activity of acetylcholinesterase as

19.10 and 18.01 Units/L while effervescent granules of *Evolvulus alsinoides* and *Convolvulus pluricaulis* reduce the activity of acetylcholinesterase as 17.69 and 14.36 Units/L. **Conclusion:** The formulations were significantly reduced locomotor activity using actophotometer, rotarod activity and the level of acetylcholinesterase. Thus, herbal suspension and effervescent granules of *Evolvulus alsinoides* and *Convolvulus pluricaulis* may be a safe alternative for Alzheimer's disease against scopolamine-induced behavioral disturbances.

Key words: Scopolamine, Rotarod, Actophotometer, Alzheimer's disease, Memory.

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INTRODUCTION

Dementia is a clinical condition of progressive decline in cognition including memory, behavior and personality that results in loss of abilities to perform a basic activity of daily life and unable to perform proper bodily functions.¹ The behavioral responses associated with dementia are agitation, apathy and rejection of care or aggression.² Other behavior and psychological problems in dementia are motor behavior, hallucination, alteration in sleep and elation.³ These symptoms have seen in around 90% of people with dementia.⁴ Alzheimer's disease (AD) is a neurodegenerative disorder and the most common cause of dementia.^{5,6} Dementia is often associated with other neurodegenerative disorders also including Parkinson's disease and Huntington's disease.⁷ The cluster of behavioral and psychological symptoms led to a deficiency of acetylcholine (ACh) level in the brain.⁸ Acetylcholinesterase (AChE) is an enzyme responsible for the hydrolysis of ACh. Thus, an AChE inhibitor may help to improve cognition and approved for relief in AD.⁹ The researchers are still in a search for an effective and safe medicine for the cure of memory disorders.

Ayurveda is believed to be the oldest alternative system of medicine in the world that is recognized as the comprehensive health-care system and as alternative medicine.¹⁰ Ayurveda is an ancient system of medicine in India including medicinal plants in the treatment of several diseases. *Evolvulus alsinoides* L. (EA) belongs to family Convolvulaceae, it is an ancient and well-known plant containing numerous medicinal

properties.¹¹ EA was adopted as the alien plant of Asia based on its geographical distribution, laboratory analyses and its biological uses; it was also incorporated in pharmacopeias.¹² This plant is popularly known for its therapeutic use in bronchitis, cognition impairment, asthma, etc. It is used as a brain tonic against neurological disorders and amnesia.¹³ *Convolvulus pluricaulis* (CP) is a traditional herbal plant, it is a well-known herb in India and used as a nerving tonic for a long time ago.¹⁴ All parts of this plant possess medicinal and therapeutic properties; it has been used traditionally for its antiepileptic, antioxidant, sleep-inducing, anticancerous, antianxiety and cough relieving properties.^{15,16} Ayurvedic herbal drugs and formulations have been used to enhance memory and slow down the progressive decline in cognition.¹⁷ ACh plays a role in the regulation of behaviors and scopolamine is expected to affect locomotion, avoidance and rearing.¹⁸ Scopolamine increases locomotor activity during a stress condition. Induction of motor activity may help to determine the effect of the drug on animal's behavior; however, it is affected by factors such as the strain of animals, changing environmental conditions, light-dark chamber, social isolation and stressful conditions.^{19,20} It has reported that giving hypnotics to the patients may help a person with dementia for enhancing cognition and also treating anxiety. In the present study, the herbal formulations were prepared to enhance the efficacy of herbal drugs and evaluated against scopolamine-induced behavioral disturbance.

MATERIALS AND METHODS

Plants material

C. pluricaulis and *E. alsinoides* were collected from area adjoining to University campus. It was authenticated in the Department of Botany, Dr. Harisingh Gour Vishwavidyalaya, Sagar (M.P.).

Herbarium no. for *C. pluricaulis*: Bot/H/05/111/04

Herbarium no. for *E. alsinoides*: Bot/H/05/111/01

Extraction of *C. pluricaulis* and *E. alsinoides* using soxhlet assembly

The whole plant of *C. pluricaulis* and *E. alsinoides* were dried, grounded and defatted with petroleum ether for a day then inserted in a soxhlet apparatus with ethanol (90%) individually for 72 hrs at 55°C. The collected extracts were concentrated and proceed for next step.

Estimation of Total alkaloids content

Preparation of drug sample: Small parts of the extracts were dissolved in 2N HCl and filtered separately. 1 ml of both solutions were transferred to two different separating funnel and washed with 10 ml chloroform (3 times). pH of solutions was adjusted to neutral with 0.1 N NaOH solution. 5 ml of Bromocresol green (BCG) solution and 5 ml of phosphate buffer (pH 4.7) were added with shaking. Formed complex were extracted with 5 ml chloroform by vigorous shaking. Then chloroform layer were collected in a 10ml volumetric flask and volume makes upto mark with chloroform.

Preparation of BCG solutions: 69.8 mg BCG was heating with 3 ml of 2N NaOH. 5 ml distilled water was added to dissolve it then diluted upto 1000 ml with distilled water.

Preparation of Standard solution and standard curve: 100 µg/ml atropine solution was prepared. Measured aliquots (0.2, 0.4, 0.6, 0.8, 1 and 1.2 ml) were transferred to different separating funnels. 5 ml pH 4.7 phosphate buffers and 5 ml BCG solution were added and 5 ml of chloroform was added. The chloroform layer was collected in a 10 ml volumetric flask and makeup the volume with chloroform. The absorbance was measured at 470 nm against blank. Total alkaloid content was determined in term of mg atropine equivalent per 100g extract (Figure 1).²¹

Preparation of herbal suspensions

Excipients such as Tween 80 (0.1%), sodium CMC (0.5 g), sodium benzoate (0.5 g), lemon oil (0.5 ml), sweetening agent (0.1 g) and water (100 ml) with ethanolic extracts of EA and CP were mixed individually. All ingredients were triturated separately to form a fine powder. The

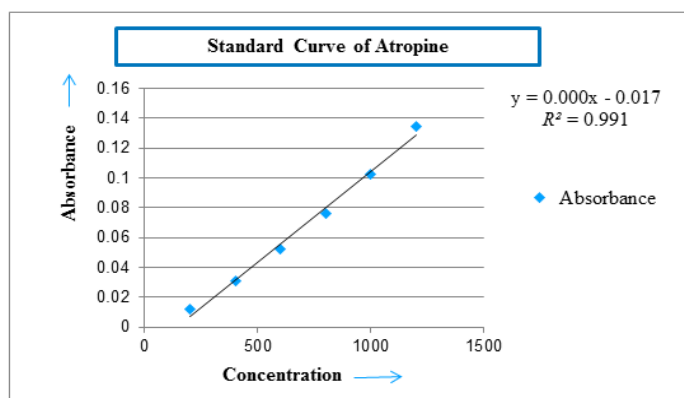


Figure 1: Standard curve of Atropine for determination of Total Alkaloids Content.

extracts were mixed in water and tween-80 and then mixed to triturated substances. CMC, sweetening agent, flavoring agent and sodium benzoate were mixed.²²

Preparation of herbal effervescent granules

Effervescent granules were prepared by a wet granulation technique. The excipients were polyvinyl pyrrolidone (PVP, 24 mg), talc (7.5 mg), magnesium stearate (3.75 mg), sweetening agent (5 mg), polyethylene glycol (PEG, 12 mg), citric acid (79.33 mg), tartaric acid (158.66 mg) and sodium bicarbonate (69.71 mg). Extracts and other ingredients were triturated separately followed by subsequent addition of PVP, talc powder, PEG, sodium bicarbonate, citric acid, tartaric acid, sweetening agent and magnesium stearate then sufficient quantity of alcohol was added to form a lumpy mass which was then passed through sieve no. 18 to form granules. Granules were dried and stored in an airtight container.²³

Characterization of herbal suspension

Physical parameters and ph determination

Nature, color, odor and texture of herbal suspensions were determined by physical evaluation. The pH of herbal suspensions were determined using a pH meter.²⁴

Sedimentation volume

It was determined by placing a measured volume of suspension in a graduated cylinder (as Initial Height) in an undisturbed state for a certain period and note that the volume of the sediment which is expressed as ultimate height under standard condition.²⁵

Redispersibility

It was determined through settled closed mouth measuring cylinder. The cylinder was inverted at 180° and the number of inversions was noted to restore a homogeneous suspension.²⁶

Viscosity and Rheology (F)

The viscosity of the sample was determined using Oswald Viscometer. However, rheology is the required time of the suspension to flow in the measured pipette.

Particle size

The particle size of herbal suspension was determined using an optical microscope.

Stability studies

Stability of suspension decreases due to crystal growth, which was determined at different temperatures i.e. 4°C, 25°C and 47°C (Table 1).²²

Characterization of herbal effervescent granules

Physical parameters

Nature, color, odor and texture of the herbal granules were determined by physical evaluation. The pH of the herbal suspension was determined using a pH meter.

Angle of repose

The funnel method was used to perform the test and calculated as follows:

$$\theta = \tan^{-1} (h/r)$$

Bulk density

It is the ratio of the total mass of powder to the bulk volume of powder.

$$D_b = m / V_o$$

Where, m: Mass of the blend, Vo: Untapped Volume

Tapped density

Tapped density is the ratio of the mass of powder to the tapped volume. Tapped volume is the volume occupied by the same mass of the powder after a standard tapping of a measure.

$$Dt = m / V_t$$

Where, m: Mass of the blend, V_t: Tapped Volume

Carr's index

It is a measure of the propensity of the granules to be compressed and determined from the bulk and tapped densities.

$$\text{Carr's Index} = \frac{Dt - Db}{Dt} \times 100$$

Where, Dt: Tapped density, Db: Bulk density

Hausner's ratio

It is an indirect index of ease of powder flow, calculated by the following formula.

$$\text{Hausner's Ratio} = \text{Tapped density} / \text{Bulk density}$$

Effervescent cessation time

100 ml of distilled water was taken in a beaker and one dose of effervescent granules was poured into the beaker then effervescent cessation time and effervescent production was observed.

Table 1: Effect of temperature on stability of herbal suspensions.

Sn	Sample	Time duration (h)	Temperature (°C)	Crystal formulation		
1.	EA	24	4	No		
			25	No		
			47	No		
		48	4	No		
			25	No		
			47	No		
		72	4	No		
			25	No		
			47	No		
		3 months		RT	No	
				6 months	RT	No
					2.	CP
25	No					
47	No					
48	4	No				
	25	No				
	47	No				
72	4	No				
	25	No				
	47	No				
3 months		RT	No			
		6 months	RT	No		

Determination of memory using behavioral models

Acute toxicity study

The acute toxicity study was performed for both the formulations as per OECD guidelines 423. Three animals were selected for each dose group i.e. 5, 50, 300 and 2000 mg/kg body weight, given p.o. Different activities of animals (change in fur color, behavior, any lethargic sign, etc.) were observed in the first 4 h after 10 h and once a day daily for 14 days.

Experimental animals

Wistar rats of either sex weighing 150-200g were selected for the study. The animals were procured from the animal house of the institute. The experimental protocols were approved by the Institutional animal ethics committee (IAEC No. 379/GO/ReBi/S/01/CPCSEA, Reference no. 379/CPCSEA/IAEC-2018/035) after scrutinization. The animals were fed with standard pelleted diet and water *ad libitum*. The animals were acclimatized to the laboratory condition before experiment. The animals were fasted for at least 24 h before treatment. The animals were divided into nine groups i.e. Group 1: Negative control group (0.2% v/v Tween-80), Group 2: Positive control group (scopolamine, 0.3 mg/kg; i.p), Group 3: Standard group (piracetam, 100 mg/kg; oral), Group 4: Ethanolic extract of EA (200 mg/kg; oral), Group 5: Suspension of EA (200 mg/kg; oral), Group 6: Effervescent granules of EA (200 mg/kg; oral), Group 7: Ethanolic extract of CP (200 mg/kg; oral), Group 8: Suspension of CP (200 mg/kg; oral), Group 9: Effervescent granules of CP (200 mg/kg; oral) each group contained six animals. The animals were treated for 15 days, however, scopolamine was administered to the animals on the 9th day, intraperitoneally.²⁷

Determination of animal's behavior using actophotometer

Actophotometer was used to determine animals' locomotor behavior. The basal activity was evaluated by placing animals for 5 min. The animals were treated with the test drug and scoring was performed after 30 min and 1 h. The index of CNS depression was taken as the decreased activity score (Table 2).²⁸

Rota-rod performance of animals

Animals were placed on a rod rotating at 20-25 rpm speed. The animals that remained stable and showed their ability were on the revolving rod

Table 2: Effect of EA and CP formulations on activity score in actophotometer method.

Groups	Mean score in 5 min		
	Basal	30 min	60 min
Group 1	350.23±12.29	354.22±7.38	352.02±9.30
Group 2	341.48±9.39	397.27±9.04	426.81±11.39
Group 3	340.02±10.27	244.36±8.99***	105.26±12.39***
Group 4	354.18±11.27	329.52±9.01	308.08±8.03**
Group 5	348.83±12.28	252.36±6.49***	128.19±9.30***
Group 6	345.26±11.02	250.88±11.93***	124.21±13.20***
Group 7	344.74±8.38	324.11±10.49	309.14±11.03**
Group 8	343.37±9.38	247.29±8.39***	110.23±9.30***
Group 9	344.32±10.28	253.09±7.39***	120.29±8.38***

Data are represent as mean ± SEM (n=6) and one-way analysis of variance (ANOVA) followed by Dunnett test for multiple column comparison. *P<0.05, **P<0.01 and ***P<0.001 were considered to be less significant, significant and more significant when all groups were compared with control.

for 5 min were selected for the study. The fall-off time was recorded prior and post-drug administration (Table 3).²⁹

Determination of AChE inhibitory activity

The activity was determined through 96-well microplate assay according to the method of Ellman *et al.* 1961.³⁰ In this method, thiocholine produced by AChE reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form a colorimetric (412 nm) product. Acetylthiocholine iodide (15 µM) in 25 µl was added into a microplate well along with the addition of DTNB (125 µl) in 50 mM of Tris/HCl (pH 8) with NaCl (0.1 M) and MgCl₂·6H₂O (0.02 M). Further, 50 µl of bovine albumin fraction V (0.1%), 25 µl of test drugs dissolved in methanol and diluted in Tris/HCl (50 mM, pH 8) at concentrations of 1.25, 12, 20, 40, 80, 160, 320 µg/ml were added into well. The absorbance was measured after 2 min of incubation at room temperature; the initial absorbance was taken at 412 nm and after 10 min, the final measurement was taken. All the analysis was performed in triplicate.³¹ The AChE activity was calculated using the following formula (Table 4):

$$\text{AChE Activity (Units/L)} = \frac{(A_{412})_{\text{Final}} - (A_{412})_{\text{Initial}}}{(A_{412})_{\text{Calibrator}} - (A_{412})_{\text{Blank}}} \times n \times 200$$

200 = Equivalent activity (Units/L) of the calibrator when assayed is read at 2 min and 10 min = dilution factor

(A₄₁₂)_{Calibrator} = Absorbance of the calibrator at 10 min

(A₄₁₂)_{Blank} = Absorbance of the blank at 10 min

Histopathology of Brain Tissues

Histopathological analysis of brain specimens was performed and the obtained tissue sections were de-paraffinized and stained with Hematoxylin and Eosin stain examination (Figure 2).³²

Statistical analysis

The results were presented as the mean ± S.E.M. and one-way analysis of variance (ANOVA) using GraphPad Prism 5 followed by Dunnett test for multiple comparisons.

RESULTS

Percentage yield of ethanolic extract of CP and EA was 8.43% (w/w) and 12.68% (w/w) respectively. Total alkaloids content of ethanolic extract

Table 3: Effect of EA and CP formulations on fall off time in rotarod.

Groups	Mean score in 5 min	
	Basal	30 min
Group 1	223.38±13.39	229.66±10.35
Group 2	226.39±9.03	342.43±11.44
Group 3	224.02±11.38	89.86±9.98***
Group 4	223.77±8.03	219.56±8.77
Group 5	226.48±9.28	94.18±10.71***
Group 6	228.92±11.38	98.01±11.43***
Group 7	224.82±12.99	202.32±12.94
Group 8	221.64±10.30	102.03±13.77***
Group 9	224.31±9.03	98.16±9.61***

Data are represent as mean ± SEM (n=6) and one-way analysis of variance (ANOVA) followed by Dunnett test for multiple column comparison. *P<0.05, **P<0.01 and ***P<0.001 were considered to be less significant, significant and more significant when all groups were compared with control.

of EA and CP were found 75.100 ± 0.265 and 142.267 ± 0.416 in mg AE/100g respectively.

EA and CP suspensions were liquid in nature, characteristics odor and suspension texture. EA appeared olive green and CP was greenish-brown in color. pH, dispersibility, flow rate and particle size of EA were 7.1, 2 Inversion, 2 ml/sec and 0.0431, respectively and CP were 7.3, 2 Inversion, 2 ml/sec and 0.0424, respectively. Table 1 shows the results for stability studies, no crystal formation was seen at different temperatures i.e. 4°C, 25°C and 47°C.

EA and CP effervescent granules were solid, light green in color, characteristics odor and cylindrical texture. The angle of repose, bulk density, tapped density C index, H ratio and effervescent cessation time for EA were 17.223°, 0.33, 0.38, 13.15, 1.15 and 2.50 min, respectively and for CP were 13.495°, 0.34, 0.36, 5.55, 1.05 and 2.27 min, respectively.

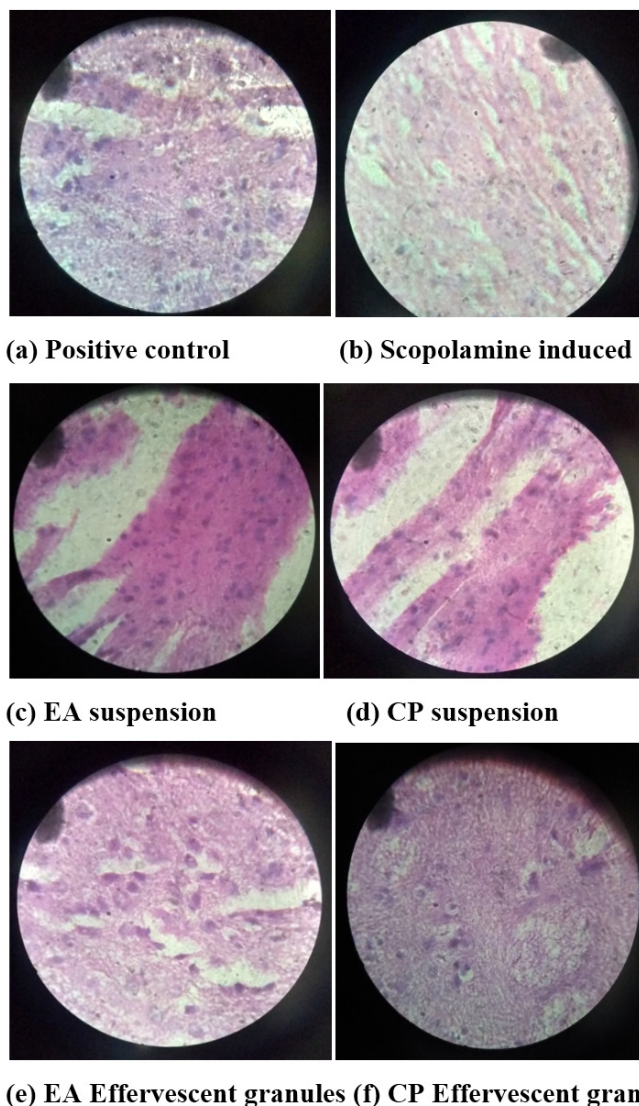


Figure 2: Brain Histological study in rats (magnification 100X). In control group, no neuronal loss was observed (a). In scopolamine induced group, inflammation, ruptured cytoplasm and neurodegeneration were found (b). In EA suspension group, remarkable improvement was observed (c). In CP suspension group, improvement was observed but little degeneration was also found (d). In EA effervescent granules group, cure for inflammation and cytoplasm were found (e). In CP effervescent granules group, cure but slight damage, degeneration of few neurons was found (f).

Table 4: Effect of EA and CP on AChE activity.

Groups	AChE Activity (Units/L)
Group 1	8.55 ± 0.30
Group 2	22.91 ± 0.07
Group 3	15.17 ± 0.29***
Group 4	36.70 ± 1.82
Group 5	19.10 ± 1.51***
Group 6	17.69 ± 0.26***
Group 7	35.27 ± 0.28
Group 8	18.01 ± 0.21***
Group 9	14.36 ± 0.57***

Data are represent as mean ± SEM ($n=6$) and one-way analysis of variance (ANOVA) followed by Dunnett test for multiple column comparison. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ were considered to be less significant, significant and more significant when all groups were compared with control.

No clinical signs of toxicity and mortality were observed in animals. Thus, lower doses after an acute toxicity study were selected for behavioral studies.

Scopolamine raised the activity score of animals in 30 and 60 min. Piracetam and the test drugs showed significant CNS depressant activity when compared with control; however; this depression was less with test drug than the standard group. Results from Table 2 show that herbal suspension and effervescent granules were more effective in depressing CNS activity in comparison to plant extracts of EA and CP. The plant's extracts and formulations were effective in reducing mean fall off time in comparison to the negative control group. The positive control group raised the fall off time, which was reduced by both the formulations of EA and CP significantly. Results from Table 3, both herbal suspension and effervescent granules were found equally effective in reducing CNS activity in comparison to the control group.

AChE activity was raised chronically with the administration of scopolamine. The test formulations were effective in reducing the activity of AChE in comparison to the control group. Piracetam showed significant activity i.e. 15.17 units/L. Results from Table 4, both the formulations of EA and CP reduced the activity of AChE in which effervescent granules of CP showed the highest reducing capability among all treated groups.

The histopathology of brain tissues after treatment with test drugs is shown in Figure 2. It was performed at 100X magnification. Neurons, glial cells and dendrites are shown in figures. White space shows the arachnoid mater. In control group, no neuronal loss was observed. Scopolamine caused lesion, inflammation, erupted cytoplasm and neurodegeneration in the brain which was healed by both EA and CP in comparison to the control group. In EA suspension group, remarkable improvement was observed(c). In CP suspension group, improvement was observed but little degeneration was also found (d). In EA effervescent granules group, cure for inflammation and cytoplasm were found (e). In CP effervescent granules group, cure but slight damage, degeneration of few neurons was found. Both formulations were effective in healing lesion present in brain tissues.

DISCUSSION

ACh play very important role in peripheral and central nervous systems both. In peripheral nervous system, it is responsible for the locomotor activity while in central nervous systems, it help in the formation of

the memory. In brain, deficiency of ACh or depletion of ACh cause various neurodegenerative diseases in which AD is one of them. AD, an irreversible neurodegenerative disorder primarily targeting elderly populations, affects approximately 36 million people worldwide. This illness is characterized by progressive neurodegenerative disorders, collapse of cognitive functions and formations of amyloid plaques and neurofibrillary tangles. The level of ACh is decrease due to AChE which affects the cognition and progressed with locomoter disfunctioning. Dementia is major symptom of AD patients. The inhibition of AChE has been one of the most used strategies for the treatment of AD. The AChE inhibitors produce not only short-term symptomatic effects, but can also play a role in other pathological mechanisms of the disease (eg, formation of amyloid- β plaques), which has renewed interest in the discovery of such inhibitors.

Dopamine controls the locomotor behavioral activity of the body by activating the mesolimbic dopaminergic pathway.³³ The behavioral effects due to excitation of dopaminergic stimulation include enhanced locomotor activity, self-administration and more turning behavior.³⁴ ACh also affects the locomotor activity due to nicotinic acetylcholine receptors present in the mesolimbic dopaminergic system.³⁵ Scopolamine causes memory impairment and disturbed locomotor activity.^{36,37} Piracetam is a standard drug used for cognition enhancement that improves the functioning of ACh and implicated in the memory process.³⁸ Rotarod and actophotometer experimental models are used to determine the motor coordination of rodents. This method is sensitive to evaluate cerebellar dysfunction. It is the due acceleration of dopamine level in the brain that can be easily discriminate with rats having striatal dopamine depletion show no motor incoordination.^{39,40} In the present study, EA and CP both are used which are varieties of shankhpushpi. Both these are traditionally claimed for memory enhancement. Ethanolic extracts of both EA and CP were prepared using Soxhlet apparatus method. Both are alkaloid in nature that's why total alkaloids contents were estimated using atropine as standard. The behavioral effects of EA and CP formulations were determined on rats. In this study locomotor activity using actophotometer and muscle relaxing property using rotarod were determined along with estimation of AChE inhibition by ellman's method. In our study, herbal suspension and effervescent granules both the formulations were significantly effective in actophotometer and rotarod methods of behavioral experimental models. Additionally, the formulations also decreased AChE activity to enhance cognition in animals. Both formulations of EA and CP restored the locomotor activity of rats. It may be due to cognition enhancement by the test drugs.

The efficacy of EA extract has already reported about the enhancement of cognition and behavioral response in animals against amnesia induced by scopolamine.²⁷ However, the formulations of EA showed more significant results in comparison to extract. CP extract has shown efficacy in boosting memory and cognition, which is reported by several researchers.^{15,41} It elicits a significant effect in animal models by interacting with the dopaminergic, adrenergic and serotonergic systems in the body.⁴² The suspension of herbal extracts has been formulated to achieve good results in animal models.^{22,24,43,44} Effervescent granules of EA were also equally effective as a suspension to reduce fall off time in rota rode and activity score in actophotometer.⁴⁵ Similar results were reported by another researcher regarding the efficacy of herbal effervescent granules in experimental animal models for other drugs.^{46,47}

CONCLUSION

EA and CP suspension and effervescent granules showed a protective effect against scopolamine-induced locomotor impairment and degradation of ACh. It may be used as a sedative, mood enhancer and

memory enhancer for neurodegenerative disorders after trials in human beings.

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CONFLICT OF INTEREST

The authors declare no Conflict of interest.

ABBREVIATIONS

CP: *Convolvulus pluricaulis*; EA: *Evolvulus alsinoides*.

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