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Nootropic Activity of leaves of *Prunella vulgaris*

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ABSTRACT

Nootropic activity of leaves of *Prunella vulgaris* was studied in mice. Elevated plus maze and passive avoidance paradigm were employed to evaluate learning and memory parameters. Scopolamine (0.4 mg/kg, i.p.) was used to induce amnesia in mice. The ethanolic extracts of leaves of *Prunella vulgaris* PV (50 and 100 mg/kg, p.o.) significantly attenuated amnesic deficits induced by scopolamine (0.4 mg/kg, i.p.) and natural aging. PV (50 and 100 mg/kg) decreased the transfer latencies and in-creased step down latencies significantly in the aged mice and scopolamine induced amnesic mice as compared with Piracetam (100 mg/kg, i.p.). To delineate the possible mechanism through which *P.Vulgaris* elicits the anti-amnesic effects, we studied its influence on central cholinergic activity by estimating the whole brain acetylcholinesterase activity. *P.vulgaris* significantly decreased acetyl cholinesterase activity in mice. The results indicate that, the ethanolic extract of leaves of *Prunella vulgaris* might prove to be a useful memory restorative agent in the treatment of dementia seen in elderly. The underlying mechanism of action can be attributed to its anti acetyl cholinesterase property.

Keywords: *Nootropic activity, Prunella vulgaris, Dementia, Acetylcholine esterase*

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INTRODUCTION

Alzheimer's disease is a progressive neurodegenerative brain disorder that occurs gradually and results in memory loss, unusual behavior, personality changes and ultimately death [1]. It is a chronic, progressive disabling organic brain disorder characterized by disturbance of multiple cortical functions, including memory, judgment, orientation, comprehension, learning capacity and language [2]. Nootropic agents such as piracetam [3], pramiracetam, aniracetam [4] and choline esterase inhibitors like Donepezil® are presently used for improving memory, mood and behavior. However, the resulting adverse effects associated with these agents have limited their use [5] and it is worthwhile to explore the utility of traditional medicines in the treatment of various cognitive disorders. *Prunella vulgaris* (Family:Lamiaceae) also called as self heal or heart of the Earth grow along streams, around ponds and lakes, in road side ditches, wet prairies, as well as in drier habitats[6]. It is a traditional Chinese drug and has hypotensive, anti bacterial, anti-viral, anti inflammatory, anti-tumor and hypoglycemic activities [7]. Traditionally the leaves of the plant *Prunella vulgaris* was used for swelling of lymph glands, hepatic disorder, reduces excitement, nervousness and irritation. A cold water infusion of the freshly chopped or dried and powdered leaves is a very tasty and refreshing beverage, weak infusion of the plant is an excellent medicinal eye wash for sties and pinkeye. It is taken internally as a medicinal tea in the treatment of fevers, diarrhoea, sore mouth and throat, internal bleeding, and weaknesses of the liver and heart. Clinical analysis shows it to have an antibacterial action, inhibiting the growth of pseudomonas, Bacillus typhi, E. coli, Mycobacterium tuberculi, which supports its use as an alternative medicine internally and externally as an antibiotic and for hard to heal wounds and diseases (8,9). It is showing promise in research for cancer, AIDS, diabetes, and many other maladies. Stems are used in teas, tinctures and infusions taken internally to stop bleeding and ease inflammation. Flower spikes are used as a cooling remedy for the liver, and for fevers, hyperactivity, dizziness, and vertigo [10].The plants most useful constituents are Betulinic - acid, D-Camphor, Delphinidin, Hyperoside, Manganese, Oleanolic - acid, Rosmarinic -acid, Rutin, Ursolic-acid, and Tannins [11]. In the present study, we investigated the nootropic effects of leaves of *P.Vulgaris* by employing exteroceptive and interoceptive behavioral models in mice. Elevated plus maze is a neutral exteroceptive model used to assess short-term memory whereas, passive avoidance apparatus is a punishment based exteroceptive model used to test long-term memory [12]. Interoceptive behavioral models such scopolamine and natural aging amnesia are widely cited as models simulating human dementia in general and Alzheimer's disease in particular. To understand the possible mechanism of action by which *Prunella vulgaris* exerts nootropic activity, whole brain acetylcholine esterase activity was determined.

MATERIALS AND METHODS

Plant Materials

The leaves of *Prunella vulgaris* was collected from local area of Hyderabad and authenticated by Dr. Najma, Botanist, S.U.C.P College, Hyderabad. The leaves were shade dried and powdered and extracted with 95 % ethanol for 48 hrs in soxhlet apparatus. The extracts were filtered and concentrated in vacuum under reduced pressure using rotary flash evaporator. A suspension was prepared using distilled water containing 1% (w/v) carboxymethyl cellulose (CMC).

Drugs and Chemicals

Scopolamine hydrobromide (Sigma Aldrich, USA) and piracetam (Nootropil®, UCB India Pvt. Ltd., Vapi, Gujarat) were diluted in normal saline and administered peritoneally. Phenytoin (Dilantin® suspension, Parke Davis) was administered orally. Volume of administration was administration was 1ml/ 100 g/body weight. All the drugs were administered in the morning session i.e. 8 AM- 9 AM on each day. 5, 5'-dithiobis nitrobenzoic acid (DTNB, Ellman's reagent, Sigma, USA) and acetyl thiocholine (Sigma USA) were used.

Acute Toxicity Studies

P.vulgaris ethanolic extract (PV) at different doses (50-2000 mg/kg) was administered orally to mice with the help of a specially designed oral needle connected to a polythene tube. PV was administered at the same time on each day (i.e. 8 AM- 9 AM). During the first four hours after the drug administration, the animals were observed for gross behavioral changes if any, for 7 days. Parameters such as hyperactivity, grooming, convulsions, hypothermia, sedation and mortality were observed. The doses selected were 50 and 100 mg/kg.

Animals

Swiss mice of either sex weighing around 18 g (younger ones, aged 8 weeks) and 25 g (older ones, aged 28 weeks) were used in the present study. The mice were maintained under standard conditions of temperature (25^{o}C±5^{o}C), relative humidity (55±10%) and a 12/12h light / dark cycle. The rats were fed with commercial rat pellet diet and water ad libitum. The study was approved by the Institutional Animal Ethics committee.

Exteroceptive Behavioral Models

Elevated plus Maze (EPM)

The elevated plus maze served as the exteroceptive behavioral model (wherein the stimulus existed outside the body) to evaluate learning and memory in mice. The apparatus consisted of two open arms (16 cm × 5 cm) and two covered arms (16 cm × 5 cm × 12 cm). The arms ex-tended from a central platform (5 cm × 5 cm) and maze was elevated to a height of 25 cm from the floor. On the first day, each rat was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was taken as the time taken by the rats to move into any one of the covered arms with all its four legs. TL was recorded on the first day. If the animal did not enter into one of the covered arms within 90 sec., it was gently pushed into one of the two covered arms and the TL was assigned as 90 sec. The mouse was allowed to explore the maze for 10 sec and then re-turned to its home cage. Memory retention was examined 24 hr after first day trial on the second day [13-15].

Passive shock avoidance paradigm

Passive avoidance behavior based on negative reinforcement was recorded to examine long-term memory. The apparatus consisted of a box (27 × 27 × 27 cm) having three walls of wood and one wall of Plexiglas, featuring a grid floor (3 mm stainless steel rods set 8 mm apart), with a wooden platform (10 × 7 × 1.7 cm) in the center of the grid floor. The box was illuminated with a 15 W bulb during the experimental period. Electric shock (20V AC) was delivered to the grid floor. Training was carried out in two similar sessions. Each mice was gently placed on the wooden platform set in the center of the grid floor. When the mouse stepped down and placed all its paws on the grid floor, shocks were delivered for 15 sec and the step-down latency (SDL) was recorded. SDL was defined as the time taken by the mouse to step down from wooden platform to grid floor with its entire paw on the grid floor. Animals showing SDL in the range (2-15 sec) during the first test were used for the second session and the retention test. The second-session was carried out 90 min after the first test. When the animals stepped down before 60 sec, electric shocks were delivered for 15 sec. During the second test, animals were removed from shock free zone if they did not step down for a period of 60 sec. Retention was tested after 24 h in a similar manner, except that the electric shocks were not applied to the grid floor. Each mouse was again placed on the platform, and the SDL was recorded with an upper cut-off time of 300sec [16-18].

Experimental Protocol

The animals were divided into 29 groups and each group consisted of a minimum of six animals. Separate animals were used for each experiment.

Group I: Represented Control group for young mice (n=6). Distilled water (DW), was administered orally for 8 days. TL was noted after 45 min of administration on 8th day and again after 24 h i.e on 9th day. **Group II & X**: Piracetam 200 mg/kg, i.p. was injected to both young and aged mice respectively. TL was noted after 45 min of injection and again after 24 h.
Group III: Scopolamine (0.4 mg/kg, i.p.) was administered to young mice and TL was noted after 45 min of injection on 8th day and again after 24 h i.e. on 9th day. **Group IV & V**: PV (50 mg/kg & 100 mg/kg) was administered orally to young mice for 8 days. The last dose was given 45 min before subjecting the animals to elevated plus maze test. TL was noted on 8th day and again after 24 h. **Group VI & VII**: PV (50 mg/kg & 100 mg/kg, p.o.) was administered to young mice for 8 days. After 45min of administration of the last dose on the 8th day, scopolamine hydrobromide (0.4 mg/kg, i.p.) was administered. TL was noted after 45 min of administration of scopolamine and again after 24 h i.e. on the 9th day. **Group VIII**: Piracetam (200 mg/kg, p.o.) was administered to young mice for 8 days. After 45min of ad-ministration of the last dose on

the 8th day, scopolamine hydrobromide (0.4 mg/kg, i.p.) was administered. TL was noted after 45 min of administration of scopolamine and again after 24 h i.e. on the 9th day. **Group IX**: Served as the control group for aged mice. Distilled water (DW), was administered orally for 8 days. TL was noted after 45 min of administration on the 8th day and again after 24 h i. e on the 9th day.

Group XI & XII: PV (50 mg/kg & 100 mg/kg) was administered orally to aged mice for 8 days. The last dose was given 45 min before noting TL on the 8th day. **GroupXIII**: Control group for young mice. Distilled water (1 mL/100g) was administered p.o. for 8 days. After 90 min of administration on the 8th day, SDL was recorded. Retention was examined after 24 h. **Group XIV**: Piracetam (200 mg/kg, i.p.) was administered for 8 days to young mice. SDL was recorded after 45 min of administration on the 8th day and again after 24 h. **Group XV**: Scopolamine hydro bromide (0.4 mg/kg) was administered i.p. to young mice after training on the 8th day and SDL was recorded at 45 min after injection. **Group XVI**: 50mg/kg PV was administered orally for 8 days to young mice. SDL was recorded after 90 min of administration on the 8th day and again after 24 h. **Group XVII**: 100 mg/kg PV was administered orally for & days to young mice. SDL was recorded after 90 min of administration on the 8th day and again after 24h. **Group XVIII & XIX**: PV (100 mg/kg & 200 mg/kg, p.o.) was administered to young mice for 8 days. After 45 min of administration of the last dose on the 8th day, scopolamine hydrobromide (0.4 mg/kg, i.p.) was administered. SDL was recorded after 90 min of administration on the 8th day and again after 24 h. **Group XX**: Piracetam (200 mg/kg, i.p.) was administered for 8 days to young mice. After 45 min of administration of the last dose on the 8th day, scopolamine hydrobromide (0.4 mg/kg, i.p.) was administered. SDL was recorded after 60 min of administration on the 8th day and again after 24 h. **Group XXI**: Served as control group for aged mice. Distilled water (1 mL/100g) was administered p.o. for 8 days. After 90 min of administration on the 8th day, SDL was recorded. Retention was examined after 24 h. **Group XXII**: Piracetam (200 mg/kg, i.p.) was ad-ministered for 8 days to aged mice. SDL was recorded after 45 min of administration on the 8th day and again after 24 h.

Group XXIII & XXIV: PV (50 and 100 mg/kg respectively) orally for 8 days to aged mice. SDL was recorded after 90 min of administration on the 8th day and again after 24 h.

Group XXV: served as control and treated with saline water, **Group XXVI**: was treated with Phenytoin (12 mg/kg, p.o.) and **Group XXVII**: was treated with piracetam (200 mg/kg, p.o.). **Group XXVIII and Group XXIX**: were treated with PV (50mg/kg and 100 mg/kg, p.o.) respectively for 8 days and acetyl cholinesterase levels were determined.

Estimation of Brain Acetyl Cholinesterase (AChE) Activity

The time frame of cholinesterase activity estimation was similar to behavioral tests i.e. 8 AM- 11 AM on each day. On the 9th day the animals were killed by cervical dislocation carefully to avoid any injuries to the tissue. The whole brain AChE activity was measured using the Ellman method [19]. The end point was the formation of yellow color due to the reaction of thiocholine with dithiobisnitrobenzoate ions. The rate of formation of thiocholine from acetylcholine iodide in the presence of tissue cholinesterase was measured using a Jasco 530 UV VIS spectrophotometer. The sample was first treated with 5, 5'-dithionitrobenzoic acid (DTNB) and the optical density (OD) of the yellow color compound formed during the reaction at 412 nm every min-ute for a period of three minutes was measured. Protein estimation was done using Folin's method. AChE activity was calculated using the following formula:

$$R = \frac{\delta \text{ O.D X Volume of Assay (3 ml)}}{\text{E X mg of protein}}$$

Where R = rate of enzyme activity in 'n' mole of acetylcholine iodide hydrolyzed / min / mg protein; δ O.D. = Change in absorbance / min; E = Extinction co-efficient = 13600 / M / cm.

Statistical Analysis

All the results were expressed as mean ± Standard error. The data was analyzed using ANOVA and Stu-dent's (Unpaired)'t' test. Kruskal Wallis one-way ANOVA followed by multiple range tests was used for the analysis of non-normally distributed data. p <0.05 was considered as significant.

RESULTS

Acute Toxicity Study

No mortality was observed following oral administration of PV even with the highest dose (2000 mg/kg). However PV at doses more than 1000 mg/kg produced profuse watery stools in animals. Both the doses of PV had no toxic effect on the normal behavior of the rats.

Effect on Transfer Latency Using EPM

Aged mice showed higher transfer latency (TL) values on first day and on second day (after 24 h) as compared to young mice, indicating impairment in learning and memory (i.e. ageing-induced amnesia). Piracetam (200 mg/kg, i.p.) pretreatment for 8 days decreased transfer latency of the 8th day 9th days as compared to distilled water treated group, indicating improvement in both learning and memory. Scopolamine (0.4 mg/kg) increased TL significantly (p < 0.05) in young mice on first and second day as compared to control, indicating impairment of both learning and memory (Fig 1 and Fig 2). PV (50 mg/kg, p.o.) decreased TL on the 8th and

9th days in both young and aged mice ($p < 0.05$) when compared to respective control groups. Higher dose of PV (100 mg/kg, p.o.) improved learning and memory of aged animals rather than the young mice as reflected by marked decrease in TL on the 8th and 9th days, when subjected to elevated plus maze tests (Fig 1 and Fig 2). PV pretreatment for 8 days protected the young as well as the old mice ($p < 0.05$) against scopolamine induced amnesia.

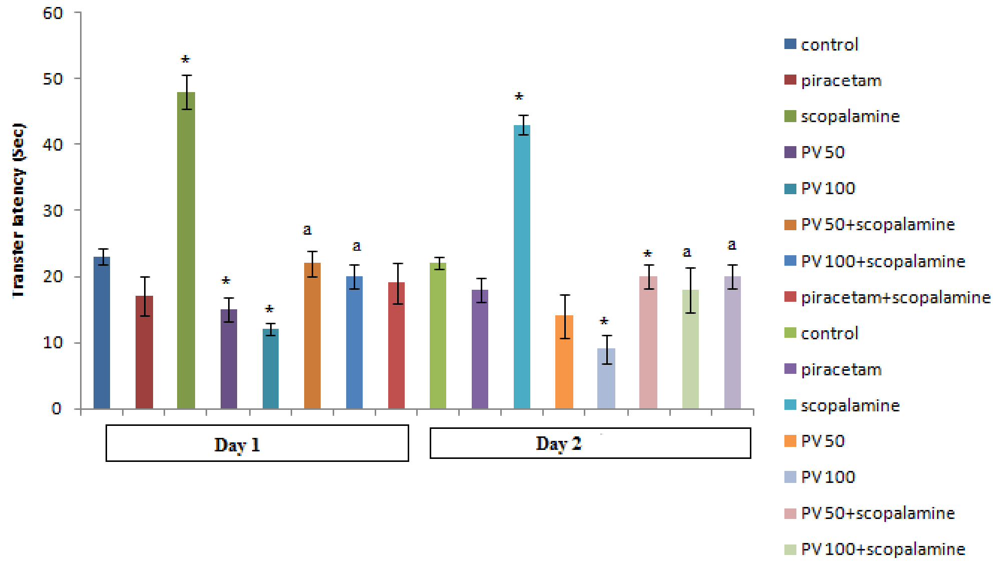

Fig 1. Effect of *P. Vulgaris* on transfer latencies of young mice. Each group com-prised of a minimum of 6 animals. Values are expressed as Mean ±SEM, ANOVA followed unpaired 't' test, * indicates p<0.05 com-pared to respective controls, a indicates p<0.05 compared to scopolamine treated group.

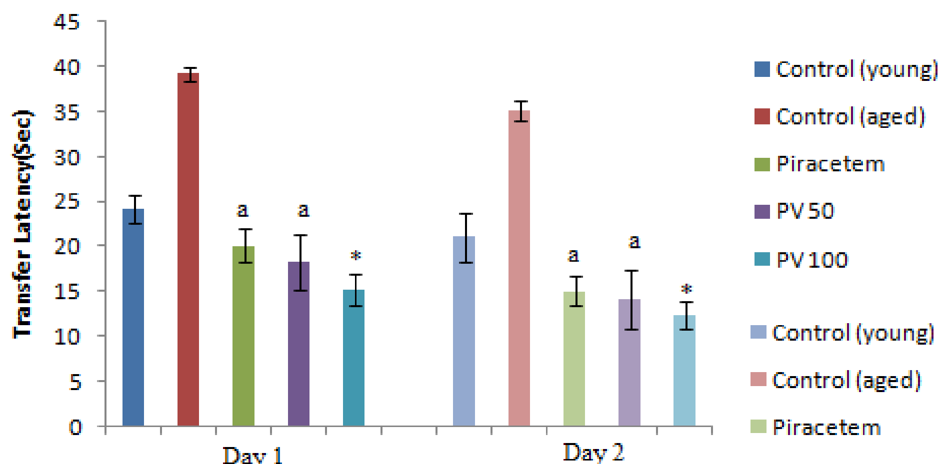

Fig 2. Effect of *P. vulgaris* on transfer latency of aged mice. Each group comprised of a minimum of 5 animals. Values are expressed as Mean ± SEM, ANOVA followed unpaired 't' test, * indicates p<0.05compared to young mice, a indicates p<0.05 compared to aged mice.

Effect on SDL Using Passive Avoidance Paradigm

PV (50 and 100 mg/kg, p.o.) treatment profoundly increased step down latency (SDL) as compared to control group on the second day indicating improvement in memory of young mice. Scopolamine hydrobromide (0.4 mg/kg, i.p.) decreased SDL on second day after training, indicating impairment of memory. PV (100 mg/kg, p.o.) administered orally for 8 days significantly ($p < 0.05$) reversed amnesia induced by scopolamine and natural ageing (Fig 3).

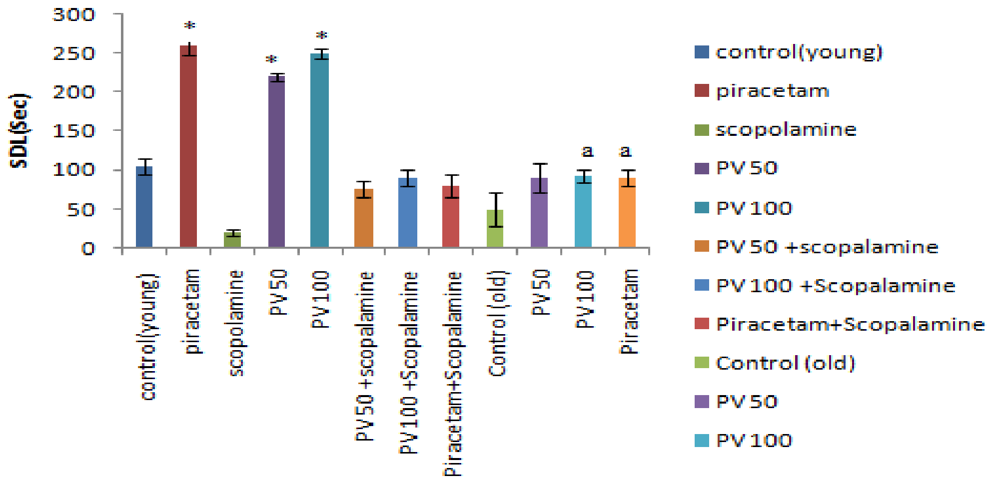

Fig 3. Effect of *P.Vulgaris* on SDL using passive aviodance para-digm.Each group comprised of a minimum of 6 animals. Values are expressed as Mean ± SEM, ANOVA followed unpaired 't' test, * indicates $p<0.05$ compared to control (young mice),[a] indicates $p<0.05$ compared to scopolamine treated group, [b] indicates $p<0.05$ compared control (aged mice)

Effect on Acetyl cholinesterase Activity

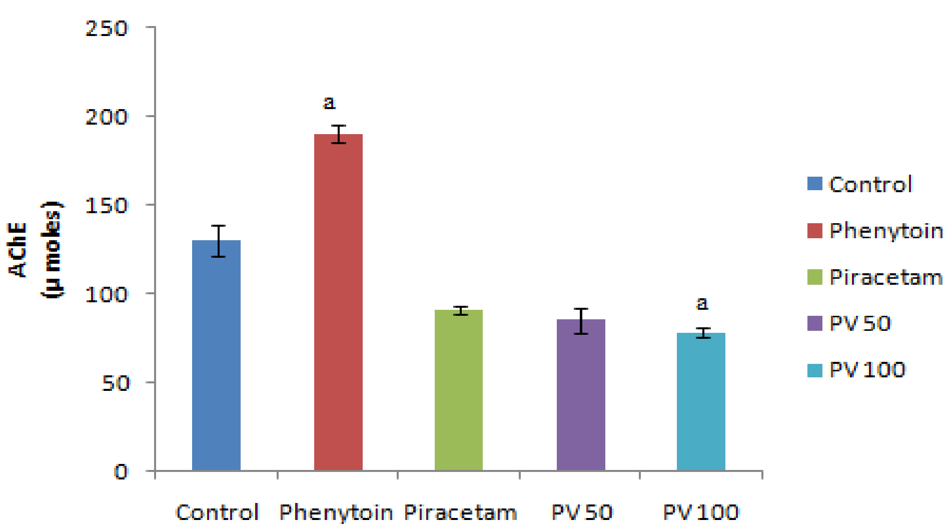

Fig 4. Effect of P.Vulgaris on AChE activity. Values are mean ±SEM of 6 animals, AChE- whole brain AChE activity, [a] indicates $p<0.05$ Vs control (multiple range test)

The acetyl choliesterase activity of whole brain was markedly elevated ($p < 0.05$) after Phenytoin (12 mg/kg, p.o.) treatment. Piracetam (200 mg/kg, p.o.) and PV (50 and 100 mg/kg, p.o.) significantly lowered AChE activity (Fig 4). Values are mean ± SEM, AChE- whole brain AChE activity, [a] indicates $p<0.05$ Vs control (multiple range test), $p < 0.05$.

DISCUSSION AND CONCLUSION

Dementia is one of the major causes of disability in late-life. Many diseases can result in dementia, the most common one being Alzheimer's disease [20]. Alzheimer's disease is a neurodegenerative disorder associated with a decline in cognitive abilities; patients also frequently have non-cognitive symptoms, such as depression, apathy and psychosis that impair daily living, Alzheimer's disease can occur at any age, even as young as 40 years, but its occurrence is much more common as the years go by [21]. It is estimated that there are currently about 18 million people worldwide with Alzheimer's disease. This figure is projected to nearly double by 2025 to 34 million. Much of this increase will be in the developing countries, and will be due to the ageing population. Currently, more than 50% of people with Alzheimer's disease live in developing countries and by 2025, this will be over 70% [22]. The present study suggests that *P.Vulgaris* is a potential anti-cholinesterase agent. It also possesses nootropic activity in view of its facilitatory effect on retention of learned task. Central cholinergic system plays an important role in learning and memory [23]. Phenytoin is known to reduce hippocampal ACh concentration and causes cognitive impairment [24]. In our study, Phenytoin (12 mg/kg, p.o.) significantly elevated brain AChE activity. Piracetam (250 mg/kg, p.o.) and PV (50 and 100 mg/kg, p.o.), on the other hand significantly ($p<0.05$) lowered this activity indicating the counteracting actions of two drugs on the cholinergic system. PV also reversed the scopolamine-induced impairment in learning and memory, when assessed on passive avoidance paradigm. Nootropic agents have selective facilitatory effect on integrative functions of the central nervous system particularly on intellectual performance, learning capacity and memory [25]. Piracetam, the first representation of a class of nootropic agents, has been shown to improve memory deficits in geriatric individuals. Repeated injections of piracetam had improved learning abilities and memory capacities of laboratory animals [26]. Passive avoidance behavior is based on negative reinforcement and is used to examine long-term memory [27, 28]. Apart from central neurotransmitters, impairment of cerebral metabolism and cerebral blood flow are known to induce cognitive deficits, and, it is proposed that the beneficial effect of nootropics may be the result of improvement in cerebral circulation and brain metabolism [29]. Both piracetam and *P.Vulgaris* meet major criteria for nootropic activity, namely improvement of memory in absence of cognitive deficit [30, 31]. In the present study, *P.Vulgaris* significantly inhibited the AChE activity in the mice whole brain homogenate, indicating its potential in the attenuation of symptoms of cognitive deficits. Further investigations using more experimental paradigms are required for further confirmation of nootropic potential of leaves of *P.Vulgaris in* the treatment of various cognitive disorders.

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