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Antiamnesic evaluation of *C. phlomidis* Linn. bark extract in mice

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Clerodendron phlomidis *Linn. (Verbenaceae) is known as Agnimantha in sanskrit. Bark of the plant is used in treating various nervous disorders. In the present study* C. phlomidis *was investigated for its potential as a nootropic agent in mice. The aqueous extract of the* C. phlomidis *(100 and 200 mg/kg, p.o.) was administered for 6 successive days to both young and aged mice. Exteroceptive behavioral models such as elevated plus maze and passive avoidance paradigm were employed to evaluate short term and long term memory respectively. Scopolamine (0.4 mg/kg, i.p.), diazepam (1 mg/kg, i.p.) were employed to induce amnesia in mice. To delineate the mechanism by which* C. phlomidis *exerts nootropic action, its effect on brain acetyl cholinesterase levels were determined. Piracetam (200 mg/kg, i.p.) was used as a standard nootropic agent. Pretreatment with* C. phlomidis *(100 and 200 mg/kg, p.o.) for 6 successive days significantly improved learning and memory in mice. It reversed the amnesia induced by scopolamine, diazepam and natural ageing. It also decreased the acetyl cholinesterase levels in the whole brain. The bark of* C. phlomidis *can be of enormous use in the management of treatment of cognitive disorders such as amnesia and Alzheimer's disease.*

Unitermos
- Alzheimer's disease
- Nootropic agent
- Acetyl cholinesterase
- *Clerodendron phlomidis*

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INTRODUCTION

Neurodegenerative diseases are now generally considered as a group of disorders that seriously and progressively impair the functions of the nervous system through selective neuronal vulnerability of specific brain regions. Alzheimer's disease (AD) is the most common neurodegenerative disease (Scatena *et al*., 2007), which affects the brain and hence memory. It is a chronic, progressive organic brain disorder characterized by disturbance of multiple cortical functions, including memory, judgement, orientation, comprehension, learning capacity and language (Jay, 2005). Clinically, the disorder is characterized by a gradual but progressive decline in memory and other cognitive domains and the frequent occurrence of noncognitive behavioral symptoms. Neuropathologically, the cardinal features of AD include neuritic plaques, neuro-fibrillary tangles, and the loss of synapses and neurons (Caselli *et al*., 2006). AD has been identified as a protein misfolding disease due to the accumulation of abnormally folded amyloid beta protein in the brains of AD patients (*Hashimoto et al., 2003)*. Amyloid beta (A^2), is a short peptide that is an abnormal proteolytic byproduct of the transmembrane protein amyloid precursor protein (APP), whose function is unclear but thought to be involved in neuronal development (*Kerr, Small*, 2005). Neuropathogenesis is proposed to be a result of the accumulation of amyloid beta peptides in the brain together with oxidative stress mechanisms and neuroinflammation

(Newman *et al*., 2006). AD begins as a deficiency in the production of the neurotransmitter acetylcholine. Patients with AD show loss of cognitive, intellectual,functional and social abilities, and therefore become fully dependent on their caregiver. It is estimated that in 2010 over five million people will be diagnosed with probable AD in the United States alone. Increasing age is the greatest risk factor for AD; one-tenth of elderly over 65 years of age develop AD, whereas nearly half of those over age 85 are diagnosed with probable AD. Certain people in the population are at greater risk of developing AD due to various genetic risk factors associated with AD such as apolipoprotein (APO) polymorphism (Francesco *et al*., 2006). A person with AD is expected to live an average of 8 years and up to 20 years after the onset of symptoms. An association between cholesterol and the development of AD was suggested in the early 1990s and ever since, an increasing amount of research has confirmed that there is a link between cholesterol and the development of AD. A high cholesterol levels in mid-life is a risk for AD (Sjogren *et al*., 2006). The National Institute of Health predicts, if the current trend continues, there will be more than 8.5 million AD patients by the year 2030 in USA alone (Anonymous, 2000). Although there is no cure for dementia of AD type at present, alternative pharmacologic treatment modalities can reduce the symptoms of cognitive impairment and slow disease progression (Geldmacher, 2003). Nootropic agents like, piracetam and cholinesterase inhibitors like, Donepezil® are commonly used for improving memory, mood and behavior. However, the resulting adverse effects of these drugs such as diarrhea, insomnia, nausea, bronchitis, loose stools, muscular cramps and other known side effects (Doody *et al*., 2001), has made their use limited and it is worthwhile to explore the utility of traditional medicines in the treatment of various cognitive disorders. In the early stages of this neurodegenerative process it is more pronounced in cholinergic-type brain centres. One of the most notable of these is the amount of attention recently being paid to the enzyme AChE, which increases the bioavailability of the neurotransmitter in the cholinergic synapses by preventing the hydrolysis of acetylcholine (Gandia *et al*., 2006). Herbal medicines can be used in the treatment of AD (Izzo, Capasso, 2007). Higher intake of folate and vitamins B6 (pyridoxine hydrochloride) and B12 (cyanocobalamin) may decrease the risk of AD through the lowering of homocysteine levels (Luchsinger *et al.,* 2007).

Clerodendron phlomidis Linn. (verbenaceae) is known as Agnimantha in Sanskrit. Bark of the plant is used for treating various nervous disorders (Chopra, 1956). A decoction of *C. phlomidis* leaves is used along with other parts for inflammation and is effective in treating bronchitis, headache, weakness, drowsiness and digestive problems (Nadkarni, 1976). *C. phlomidis* has also shown antidiarrhoeal activity (Rani *et a*l., 1999) and antifungal activity (Rajasekaran *et al*., 2006). A new chalcone glycoside, together with pectolinarigenin, 7-hydroxyflavone and 7-hydroxyflavanone 7-O-glucoside have been isolated from the flowers and leaves of *C. phlomidis* (Roy, Pandey, 1994).

In the present study *C. phlomidis* was investigated for its potential as a nootropic agent. Elevated plus maze and passive avoidance paradigms were the exteroceptive behavioral models to assess short-term and long term memory respectively. To delineate the mechanism by which *C. phlomidis* exerts nootropic action, its effects on brain cholinesterase levels were also determined.

MATERIALS AND METHODS

The plant material

The bark of *Clerodendron phlomidis* (Family - Verbenaceae) was obtained from Dharwad, Karnataka, India. The plant was authenticated and identified by Dr. S. Hebbar, taxonomist, Department of Botany, Karnatak University, Dharwad. The specimen has been kept at Dept. of Pharmacognosy, SET'S college of Pharmacy, Dharwad, Karnataka, India.

Preparation of extract

The bark was dried in shade; cleaned, powdered and aqueous extract was prepared by simple maceration process using 1000 g of powder. The extract was concentrated using rotary flash evaporator followed by freeze drying. The yield of the dry extract from crude powder of *C. phlomidis* was 2%. A suspension was prepared using tween 80 and administered orally.

Drugs and chemicals

Scopolamine hydro bromide (Sigma Aldrich, USA), diazepam (Valium®, Ranbaxy laboratories Ltd., Mumbai, India), piracetam (Nootropil® UCB India pvt. Ltd., Vapi, India) and phenytoin (Zydus Neurosciences, Ahmedabad, India) were diluted in normal saline and injected intraperitoneally (i.p.). Volume of injection was 1 ml/100 g body weight of the mouse.

Animals

Swiss mice of either sex weighing around 18 g (younger, 8 weeks old) and 25 g (older, 28 weeks old) were used in the present study. Animals were procured from

disease free animal house, BLDEA Medical College, Bijapur. They were acclimatized to the laboratory conditions for 5 days before behavioral studies. The animals had free access to food and water and maintained under 12**:**12 h light and dark cycles. All experiments were carried out during day time from 900 to1900 hours. The Institutional Animals Ethics Committee (IAEC) approved the experimental protocol and care of animals was taken as per guidelines of CPCSEA, Dept. of Animal Welfare, Govt. of India (Nº. 412).

MEMORY MODELS

Exteroceptive behavioral model

Elevated plus maze

The elevated plus maze served as the exteroceptive behavioral model (where in stimulus existed outside the body) to evaluate learning and memory in mice (Joshi, Parle, 2006). The apparatus consists of two open arms (16 cm x 5 cm) and two covered arms (16 cm x 5 cm x 12 cm). The arms extended from a central platform (5 cm x 5 cm), and maze is elevated to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of open arm, facing away from the central platform. Transfer latency (TL) was taken as the time taken by mouse to move into one of the covered arm 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 is gently pushed into one of the two covered arms and the TL was assigned as 90 seconds. The mouse was allowed to explore the maze for 10 seconds and then returned to its home cage. Memory retention was examined on the second day, 24 hours after the first day's trial (Dhingra *et al*., 2004; Itoh *et al*., 1990; Parle, Dhingra, 2003; Joshi *et al.*, 2006).

Passive shock avoidance paradigm

Passive avoidance behavior based on negative reinforcement was used to examine the long term memory. The apparatus consisted of a box (27 x 27 x 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 x 7 x 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 mouse was gently placed on the wooden platform set in the center of the grid floor. When the mouse stepped down and 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 wee 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 300 s (Joshi, Parle, 2005; Parle *et al*., 2004; Joshi, Parle, 2006a).

Interoceptive behavioral models:

Scopolamine induced amnesia - Amnesia was induced by administration of scopolamine hydrobromide on 6th day and the TL recorded. Retention was recorded after 24 hr. CP (100 and 200 mg/kg) and piracetam (200 mg/kg) were administered for 6 days successively. On 7th day, after 45 min of administration of doses, scopolamine (0.4 mg/kg, p.o.) was administered and TL and SDL noted after 45 min.

Diazepam induced amnesia – Diazepam (1mg/kg, i.p.) was administered to young mice and TL was noted after 45 min of injection on 6th day and after 24 hr. CP (100 and 200 mg/kg, p.o.) and piracetam (200 mg/kg, ip) were administered for 6 successive days. After 60 min of administration of the last dose on 6th day, diazepam was administered. TL and SDL were noted after 45 min of administration of diazepam and after 24 hr (Joshi, Parle, 2006 b).

Estimation of brain Acetyl cholinesterase (AChE) activity

On the 7th day the animals were euthanized by cervical dislocation carefully to avoid any injuries to the tissue. The whole brain AChE activity was measured using the Ellman method (Ellman *et al*., 1961). 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 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 minute for a period of three minutes was measured. Protein estimation was done using Folin's method. AChE activity was calculated using the following formula:

$$R = \frac{\text{d O.D. x volume of assay (3ml)}}{\text{E x mg of protein}}$$

Where R= Rate of enzyme activity in 'n' mole of acetylcholine iodide hydrolyzed / minute / mg protein. d O.D. = Change in absorbance / min and E = Extinction coefficient = 13600 / M / cm.

Statistical Analysis

All the results were expressed as mean ± Standard error. The data was analyzed using ANOVA followed by Tukey- kramer test. $P < 0.01$ was considered as statistically significant.

RESULTS

Acute toxicity studies

C. phlomidis aqueous extract at different doses (50-1000 mg/kg) was administered orally to the mice with the help of a specially designed oral needle connected to a polythene tube. Mice, which received extracts in doses above 1000 mg/kg, exhibited ptosis (dropping of upper eyelids) and were found lethargic. The parameters such as hyperactivity, grooming, convulsions, sedation, hypothermia and mortality were observed. The doses selected were 100 mg/kg and 200 mg/kg/day.

Effect on transfer latency (TL) using elevated plus maze

Aged mice showed higher transfer latency (TL) values on first day and on second day (after 24 hr) as compared to young mice, indicating impairment in learning and memory. Piracetam (200 mg/kg, i.p.) pretreatment for 6 days decreased TL on 6th day and after 24 hrs i.e. on 7th day as compared to control, indicating improvement in both learning and memory (Figure 1). Scopolamine (0.4 mg/kg) and Diazepam (1 mg /kg) increased TL significantly ($P < 0.05$) in young mice on first day and second day as compared to control, indicating impairment of memory (Figure 2).

C. phlomidis (100 and 200 mg/kg, p.o.) decreased the TL on 6th day and 7th day in young and aged mice ($P<0.05$) when compared to control groups. Higher doses of CP (200 mg/kg, p.o.) more significantly enhanced the learning and memory of aged animals rather than the young mice as reflected by marked decrease in TL on 6th and 7th day when subjected to elevated plus maze tests. The higher doses of CP pretreatment for 6 days successively to young mice protected them against scopolamine, diazepam and ageing induced amnesia.

Effect on SDL using Passive avoidance apparatus

C. phlomidis extract (200 mg/kg, p.o.) profoundly increased SDL as compared to control group on second day

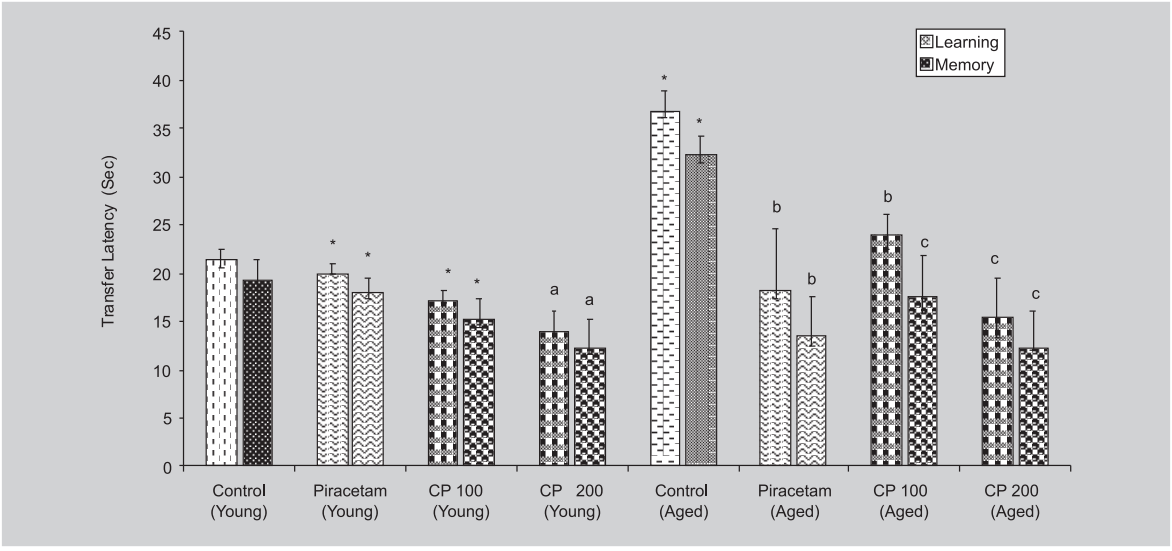

FIGURE 1 - Effect of *C. phlomidis* (CP) on transfer latencies of young and aged mice.

All values are mean ± SEM : ANOVA followed by Tukey- Kramer test

* denotes $P<0.01$ as compared to control (Young)

a denotes $P<0.001$ as compared to control (Young)

b denotes $P< 0.01$ as compared to control (Aged)

c denotes $P<0.001$ as compared to control (Aged)

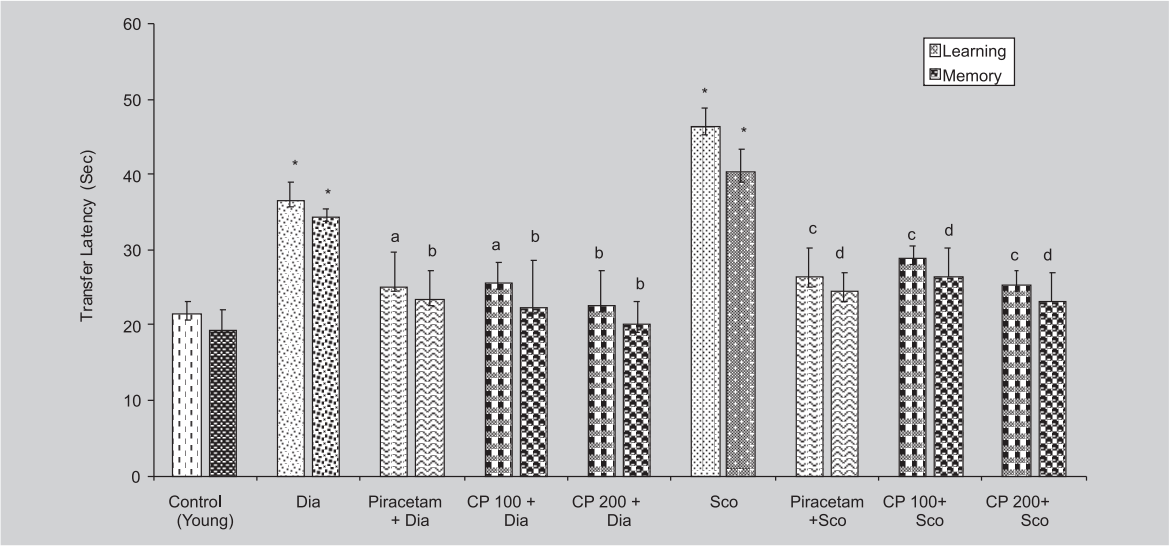

FIGURE 2 - Effect of *C. phlomidis* (CP) on transfer latencies of diazepam and scopolamine induced mice.

All values are mean ± SEM : ANOVA followed by Tukey- Kramer test
* denotes P<0.01 as compared to control (Young)
a denotes P<0.01 as compared to diazepam treated mice
b denotes P< 0.001 as compared to diazepam treated mice
c denotes P<0.01 as compared to scopolamine treated mice
d denotes P<0.001 as compared to scopolamine treated mice

indicating improvement in the memory of young mice. Furthermore, this dose of *C. phlomidis* reversed diazepam and scopolamine significantly decreased SDL on second day indicating impairment of memory (Figure 3).

Effect on whole brain acetylcholinesterase (AChE) activity

The whole brain AChE activity with phenytoin (12 mg/kg, p.o.) exhibited significant elevation to AChE activity as compared to control and piracetam (200 mg/kg, p.o.). *C. phlomidis* (200 mg/kg, p.o.) significantly reduced AChE activity (Figure 4).

DISCUSSION

Alzheimer's disease (AD) is a progressive disease of the brain. It is a common type of dementia in the elderly, which can have devastating outcomes on the diagnosed patient, on the caregiver and family, and on society at large. AD is a major cause of disability and mortality, and its impact on health care costs, including direct and indirect medical and social service costs, is estimated to be greater than $100 billion per year. AD typically presents with an insidious decline in memory that progresses to affect language, visuospatial perception, calculations, and executive functioning. Behavioral and psychiatric symptoms are also frequent in AD (Yaari, Corey-Bloom, 2007). The treatment of AD is a clinical challenge (Gustavo *et al*., 2006). Many other conditions can lead to similar memory loss, confusion, agitation and metabolic disturbances (Francesco *et al*., 2006). The symptoms of dementia are oxidative damage, impaired neurotransmission and degeneration of neuronal circuits in the affected brain areas (Joshi, Parle, 2006a). Oxidative damage accompanies AD, and cholinesterase inhibitors are recommended for use in mild to moderate AD (Joshi, Parle, 2006b). In exteroceptive behavioral models, the stimulus lies outside the body where as, it lies within the body in case of interoceptive behavioral models. Passive avoidance behavior is a classic paradigm to assess memory with strong aversive component (Poirier, 2002), based on negative reinforcement and is used in the present study to examine long-term memory where as Elevated plus maze was used to examine short term memory (Doody *et al*., 2001). Interoceptive behavioral models such as scopolamine and natural aging induced amnesia are widely cited as models simulating human dementia in general and AD in particular (Cahil *et al*., 1998). Modulation of brain aging with complex extracts containing active phytochemicals has been useful in the aging of wild type rodents with encouraging results (Giacobini, 1998).

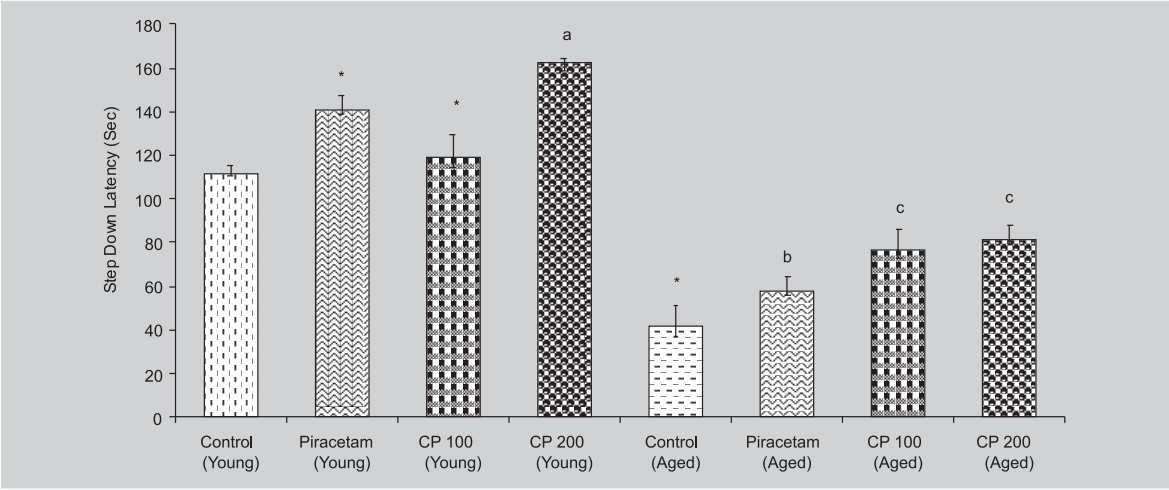

FIGURE 3 - Effect of *C. phlomidis* (CP) on step down latency of young and aged mice.

All values are mean ± SEM : ANOVA followed by Tukey- Kramer test
* denotes P<0.01 as compared to control (Young)
a denotes P<0.001 as compared to control (Young)
b denotes P< 0.01 as compared to control (Aged)
c denotes P<0.001 as compared to control (Aged)

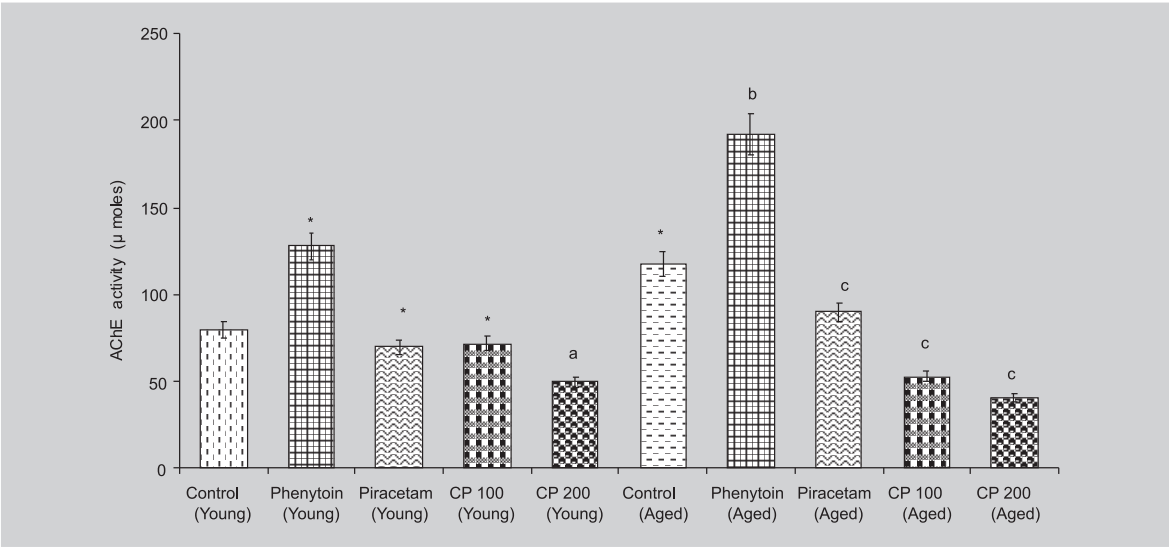

FIGURE 4 - Effect *of C. phlomidis* (CP) on acetylcholinesterase activity of young and aged mice.

All values are mean ± SEM : ANOVA followed by Tukey- Kramer test
* denotes P<0.01 as compared to control (Young)
a denotes P<0.001 as compared to control (Young)
b denotes P< 0.01 as compared to control (Aged)
c denotes P<0.001 as compared to control (Aged)

Neurodegenerative disorders, such as AD, are often characterised by the degeneration of the cholinergic system. Thus, the aim of many treatment regimens is to support this system either by means of muscarinic agonists or by inhibitors of acetylcholinesterase (AChE), the latter being able to increase the concentration of acetylcholine (Holzgrabe *et al*., 2007).

The present study indicates that *C. phlomidis* is a potential anti-cholinesterase agent. It also possesses nootropic activity in view of its facilitatory effect on retention of acquired learning. Cognitive deterioration occurring in patients with probably AD is associated with progressive loss

of cholinergic neurons and consequent decline in levels of acetylcholine (Ach) in brain. Cholinergic deficits occur in the brain of patients with AD and vascular dementia (Mckeith *et al*., 2003; Tohgi *et al*., 1990;). Altered hippocampal neurogenesis may also play a pathophysiological role in neurodegenerative disorders such as AD (Elder *et al*., 2006). Phenytoin is known to reduce hippocampal ACh concentration and causes cognitive impairment (Sudha *et al*., 2001). The aqueous extract of *C. phlomidis* significantly inhibited the AChE activity in the whole brain homogenate of mice, indicating its potential in the attenuation of learning and memory deficits especially in aged mice. Considering the lack and need of drugs with proven effectiveness in improving learning and memory (Bhattacharya *et al*., 2000), the specific memory improving effects of *C. phlomidis* reported in the present study is of enormous interest and deserves further investigations using more experimental paradigms for further confirmation of memory improving potential of *C. phlomidis* in the treatment of various cognitive disorders.

CONCLUSION

Considering the lack and the need of the drugs with proven effectiveness in improving learning and memory, the specific memory improving, anticholinesterase and anticholesterol effects of *C. phlomidis* can be of enormous use in the management of preliminary symptoms of dementia and Alzheimer's disease.

RESUMO

Avaliação da atividade antiamnésica da casca de *C. phlomidis* Linn. em camundongos

Clerodendron phlomidis *Linn. (Verbenaceae) é conhecida como Agnimantha em sânscrito. A casca da planta é utilizada no tratamento de várias disfunções neurológicas. No presente estudo,* C. phlomidis *foi investigada pelo seu potencial como agente nootrópico em camundongos. O extrato aquoso de* C. phlomidis (100 e 200 mg/kg, p.o.) *foi administrado por seis dias consecutivos tanto para camundongos jovens quanto para idosos. Modelos comportamentais exteroceptivos, tais como labirinto em cruz elevada e paradigma de esquiva passiva foram empregados para avaliar memória recente e tardia, respectivamente. Escopolamina (0,4 mg/kg i.p.), diazepam (1 mg/kg i.p.) foram empregados para induzir amnésia em camundongos. A fim de delinear o mecanismo pelo qual* C. phlomidis *exerce ação nootrópica, determinaram-se seus efeitos nos níveis cerebrais de acetilcolinesterase. Utilizau-se piracetam (200 mg/kg i.p.) como nootrópico padrão. O pré-tratamento com* C. phlomidis *(100 e 200 mg/kg, p.o.) por seis dias sucessivos melhorou, significativamente, o aprendizado e a memória em camundongos. Ela reverteu a amnésia induzida por escopolamina, diazepam e pelo envelhecimento normal. Também, diminuíram-se os níveis de acetilcolinesteraseem todo o cérebro. A casca de* C. phlomidis *pode ser de grande uso no tratamento de disfunções cognitivas, como amnésia e doença de Alzheimer.*

UNITERMOS: Doença de Alzheimer. Agente nootrópico. Acetilcolinesterase. Clerodendron phlomidis.

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