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Evaluation Of Antiamnesic Potentials Of [6]-Gingerol And Phyllanthin In Mice

Corresponding Author

Hanumanthachar Joshi
Department of Pharmacognosy,
Soniya Education Trust's College of Pharmacy,
S.R.Nagar, Dharwad-02, Karnataka, (INDIA)
Tel. : 91+9448632253,
Email : amanjoshi17@yahoo.com

Co-Author

Milind Parle
Division of Pharmacology,
Department of Pharm. Sciences,
Guru Jambheshwar University,
Hisar, Haryana, (INDIA).

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ABSTRACT

Nootropic activity of [6]-gingerol and phyllanthin was studied in mice using elevated plus maze and passive avoidance paradigm. [6]-gingerol (25 and 50 mg/kg, p.o.) and phyllanthin (7.5 and 15 mg/kg, p.o.) significantly attenuated amnestic deficits induced by diazepam, scopolamine (0.4 mg/kg, i.p.) and natural aging. [6]-gingerol and phyllanthin increased step down latencies significantly in the aged mice, diazepam and scopolamine induced amnesic mice as compared with piracetam (200 mg/kg, i.p.). To delineate the possible mechanism through which [6]-gingerol and phyllanthin elicit anti-amnesic effects, their influence on central cholinergic activity was studied by estimating the whole brain acetylcholinesterase activity. [6]-gingerol and phyllanthin significantly decreased whole brain acetyl cholinesterase activity. Hence, [6]-gingerol (25 and 50 mg/kg, p.o.) and phyllanthin (7.5 and 15 mg/kg, p.o.) might prove to be useful memory restorative agents in the treatment of dementia seen in elderly. The underlying mechanism of action can be attributed to their anti acetylcholinesterase properties.

KEYWORDS

Acetylcholine;
[6]-gingerol;
Phyllanthin;
Piracetam;
Scopolamine.

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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 the most common form of onset of adult dementia and attention deficit disorders[2-3]. Centrally acting antimuscarinic drugs (like Scopolamine) impaired learning and memory of rats[4] and human beings[5]. Nootropic agents such as Piracetam[6], Pramiracetam, aniracetam[7] and choline esterase inhibitors like Donepezil® are presently used for improving memory, mood and behavior. However, the resulting side effects associated with these agents have limited their use[8-9] and it is worthwhile to explore the utility of traditional medicines in the treatment of various cognitive disorders.

Zingiber officinale Roscoe. (family: Zingibe raceae) is commonly known as ginger and used in the traditional system of medicine since time immemorial. The dried rhizomes of ginger are implicated in the treatment of cardiac diseases, piles, colic, diseases of kapha, asthma and pitta[10]. Ginger is reported to possess antioxidant[11], hypoglycaemic[12], antimigraine[13] activity, enhanced learning and memory[14] and inhibited β-amyloid peptide-accumulation, thus may delay the onset and progression of neurodegenerative disorders[15]. Ginger is one of the most frequently used ingredients in Chinese folk medicine for treatment of various infectious diseases. gingerols, the active components of ginger, represent a potential new class inhibitor of platelet activators[16]. [6]-gingerol has been found to possess a variety of interesting pharmacological effects, viz., antipyretic, cardiotonic effects, and inhibition of spontaneous motor activity and prostaglandin biosynthesis[17-18]. [6]-gingerol has been used as a marker substance of ginger and possesses anti-obesity activity[19]. The [6]-gingerol contents in different preparations were evaluated for analgesic and anti-inflammatory effects[20].

Phyllanthus amarus Linn. (Euphorbiaceae) is commonly known as bhumi amla and is traditionally used to treat flu, dropsy, diabetes, and jaundice[21]. It is also used to treat hepatic and urolitic diseases and has diuretic activity. P.amarus is reported to possess antiviral[22], anticancer[23-24], hepatoprotective[25], antioxidant[26], anti-inflammatory[27] activity. P.amarus mainly contains phyllnathin and hypophyllanthin as active ingredients[28-29]. The aqueous extract of P. amarus has been applied for treatment of nervous debility, epilepsy, as medhya (intellect promoting) and in vata disorders.

In the present study, the nootropic effects of [6]-gingerol and phyllanthin were investigated by employing both exteroceptive and interoceptive behavioural models. The stimulus lies outside the body in exteroceptive behavioral models, whereas, it lies within the body in case of interoceptive behavioral models. Elevated plus maze is a neutral exteroceptive model used to assess short-term memory whereas, where as passive avoidance apparatus is a punishment based exteroceptive model used to test long-term memory[30]. Interoceptive behavioral models such as scopolamine and natural aging induced amnesia are widely cited as models simulating human dementia in general and Alzheimer's disease in particular[31]. To delineate the possible mechanism of action, effects on whole brain acetyl cholinesterase activity was also assessed.

MATERIALS AND METHODS

Animals

All the experiments were carried out using male, Swiss Albino mice procured from the disease-free small animal house of CCS Haryana Agricultural University, Hisar (Haryana), India. Young (3-4 months old) mice weighing around 20 g and older (12-15 months old) mice weighing around 35 g were used in the present study. The animals had free access to food and water, and they were housed in a natural (12 h each) light-dark cycle. Food given to mice consisted of wheat flour kneaded with water and mixed with a small amount of refined vegetable oil. The animals were acclimatized for at least 5 days to the laboratory conditions before behavioral experiments. Experiments were carried out between 9 am and 6 pm. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and the care of laboratory animals was

taken as per the guidelines of CPCSEA, Ministry of Forests and Environment, Government of India (registration number 0436).

Drugs and chemicals

[6]-Gingerol (Calbiochem®, Germany), phyllanthin (Natural Remedies, India). The companies guaranteed batch to-batch chemical consistency by high performance liquid chromatograph and biological reliability by lymphocyte proliferation assays. Its purity was more than 95% for gingerol and 98% for phyllanthin, which were further confirmed by spectroscopical studies. Diazepam (Calmpose®, Ranbaxy, India), Scopolamine hydrobromide (Sigma Aldrich, USA), piracetam (Nootropil®, UCB India Pvt. Ltd., Vapi, Gujarat) were diluted in normal saline and administered intra-periotoneally. Phenytoin (Dilantin® suspension, Parke Davis) were administered orally. [6]-gingerol and phyllanthin were suspended with 0.5% CMC and administered orally. Volume of administration was 1 ml/ 100 g. All the drugs were administered in the morning session i.e. 8am till 9am on each day. 5, 5'-dithiobis nitrobenzoic acid (DTNB, Ellman's reagent, Sigma, USA) and acetyl thiocholine (Sigma USA) were used for AChE activity.

Acute toxicity studies

[6]-Gingerol (5-500 mg/kg) and phyllanthin (1-500 mg/kg) were administered at different doses orally to normal mice. During the first four hours after the drug administration, the animals were observed for gross behavioral changes if any for 7 days. The parameters such as hyperactivity, grooming, convulsions, sedation, diarrhea, hypothermia and mortality were observed.

Exteroceptive behavioral models

Elevated plus maze

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 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 was elevated to a height of 25 cm from the floor. On the first day, each mouse 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 mouse 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 returned to its home cage. Memory retention was examined 24 h after the first day trial on the second day[32-33] .

Passive shock avoidance paradigm

Passive avoidance behavior based on negative reinforcement was recorded 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 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 300 sec[34-35].

Estimation of brain acetyl cholinesterase (AChE) activity

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The time frame of cholinesterase activity estimation was similar to behavioral tests i.e. 8am-11am on each day. On the 9th day the aged mice were euthanized by cervical dislocation carefully to avoid any injuries to the tissue. The tissues were homogenized, centrifuged (5000 rpm), centrifugate was collected and whole brain AChE activity was measured[36]. 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 colour 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:

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

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 coefficient = 13600 / M / cm.

Statistical Analysis: All the results were expressed as mean ± Standard error. The data was analyzed using ANOVA and Student'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 studies: [6]-gingerol and phyllanthin did not produce any mortality or behavioral changes even with highest dose (500 mg/kg, p.o.). However [6]-gingerol, at doses more than 250 mg/kg and phyllanthin at doses more than 150 mg/kg produced profuse watery stools. Two doses for [6]-gingerol (25 and 50 mg/kg) and Phyllanthin (7.5 and 15 mg/kg) were selected for further studies.

Effects of [6]-gingerol and phyllanthin on transfer latency by elevated plus maze

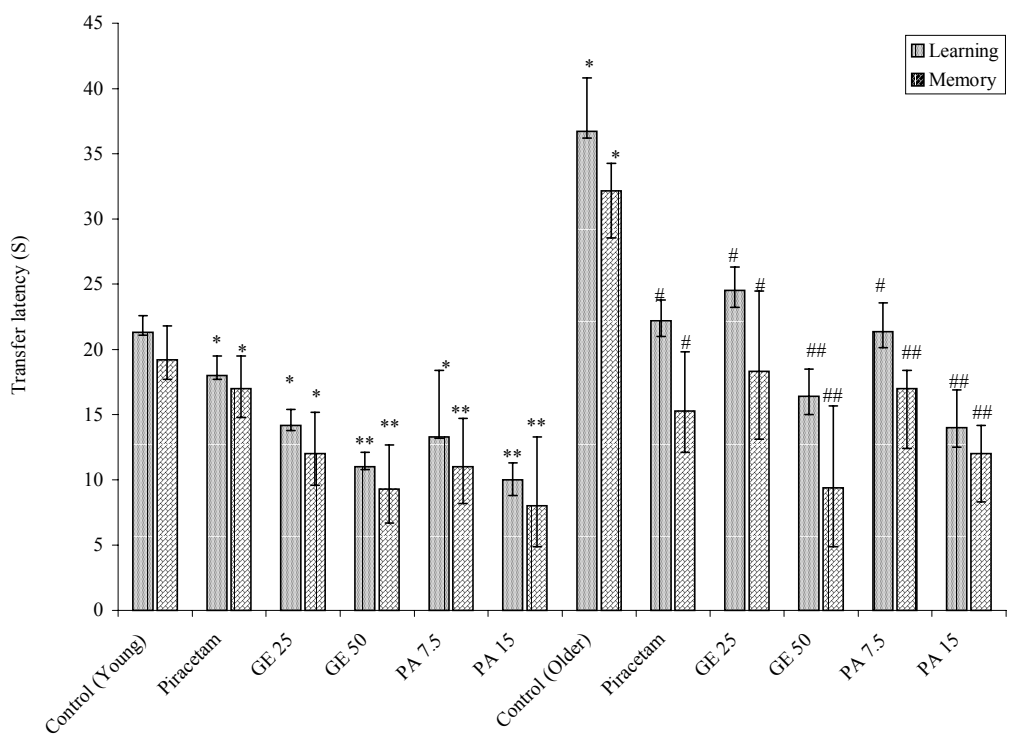

Figure 1: Effect of [6]-gingerol and phyllanthin on transfer latencies of young and aged mice on elevated plus maze

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 1st day and 2nd day as compared to distilled water treated group, indicating improvement in both learning and memory. Diazepam (1 mg/kg, i.p.) and 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 (Figure 1).

[6]-gingerol (25 mg/kg, p.o.) decreased the TL on 8th day and 9th day in both young and aged mice ($P < 0.01$) when compared to respective control groups. Higher dose of [6]-gingerol (50 mg/kg, p.o.) improved the learning and memory of aged animals rather than the young mice as reflected by marked decrease in TL on 1st day and 2nd day ($P < 0.001$), when subjected to elevated plus maze tests (Figure 1). [6]-gingerol (25 and 50 mg/kg, p.o.) pretreatment for 8 days protected the young mice against diazepam and scopolamine induced amnesia (Figure 2).

The young animals treated with phyllanthin (7.5 and 15 mg/kg, p.o.) showed dose- dependent reduction in TL of 9th day, indicating significant improvement in memory, when compared with control group. These concentrations of phyllanthin (7.5 and 15 mg/kg, p.o.) also produced significant improvement in memory ($P < 0.01$ and $P < 0.001$ respectively) of older mice (Figure 1). Scopolamine (0.4 mg/kg, i.p.) and diazepam (1 ml/kg, i.p.) injected before training significantly increased ($P < 0.01$) the TL of 9th day indicating impairment in memory (amnesia). The mice treated with phyllanthin (7.5 and 15 mg/kg, p.o.) for 9 successive days) reversed successfully the amnesia induced by both scopolamine and diazepam (Figure 2). Piracetam (used as the positive control) at the dose of 200 mg/kg, i.p. improved memory ($P < 0.01$) of both young and older mice and reversed the amnesia induced by scopolamine and diazepam as expected.

Effects of [6]-gingerol and phyllanthin on Step down latency

Step down latency (SDL) of second day (9th day

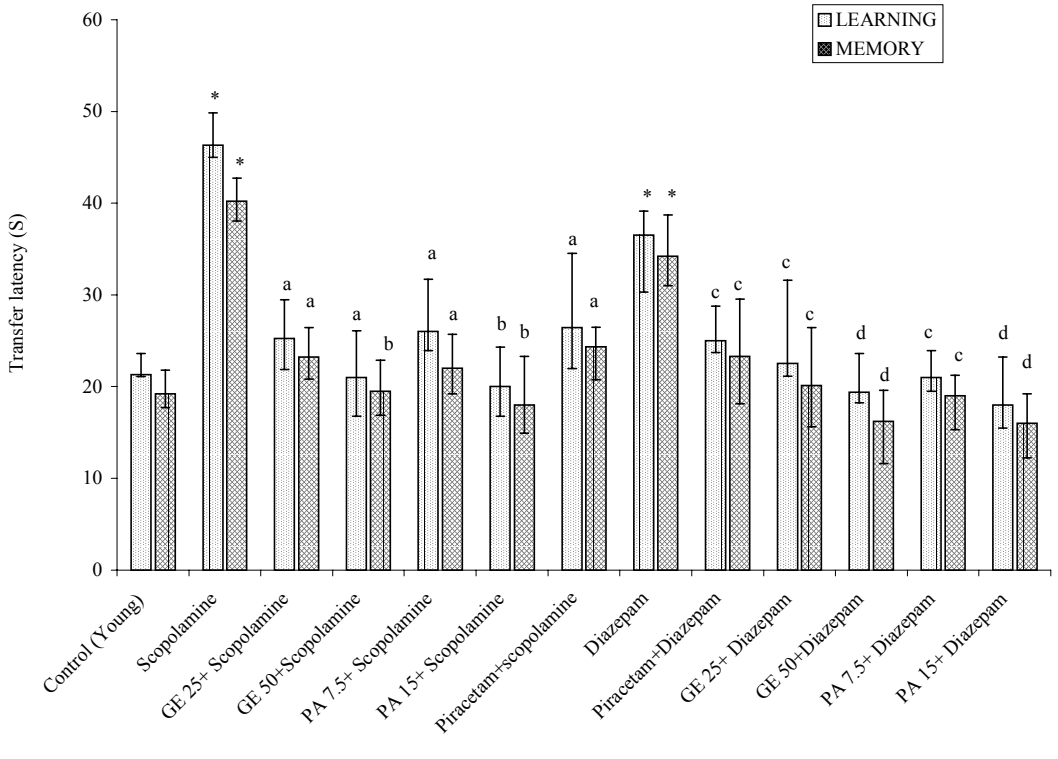

Figure 2: Effect of [6]-gingerol and phyllanthin on transfer latencies on scopolamine and diazepam induced amnesia

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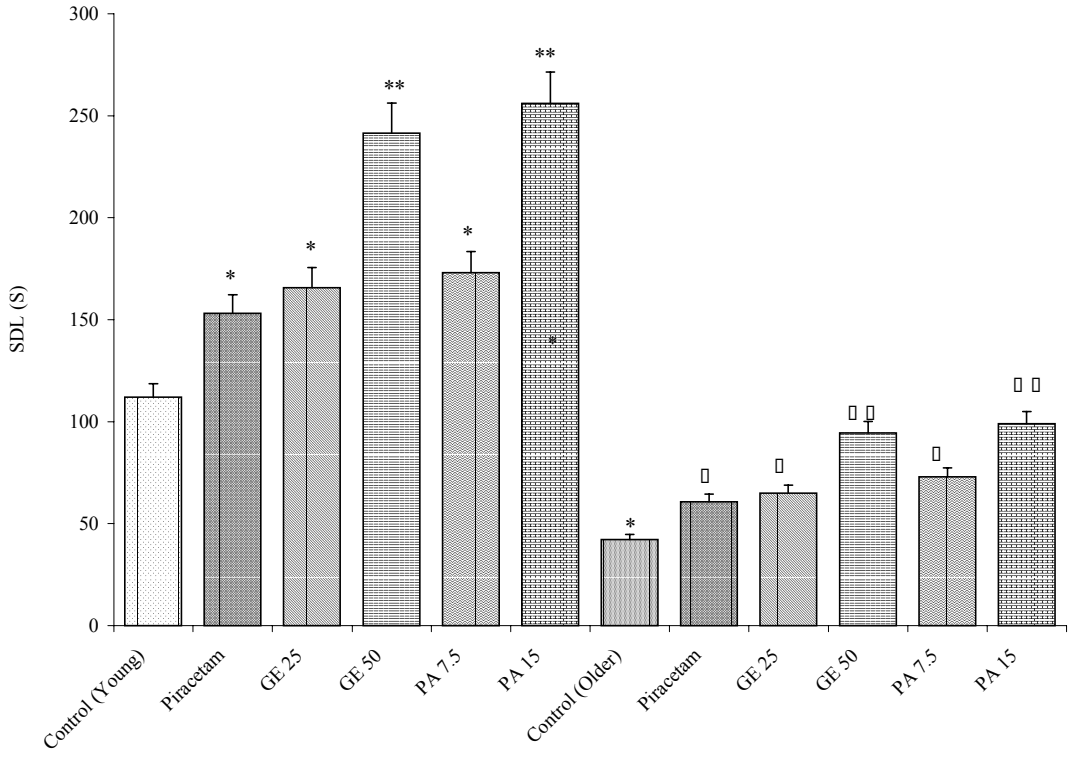

Figure 3: Effect of [6]-gingerol and phyllanthin on SDL of young and aged mice

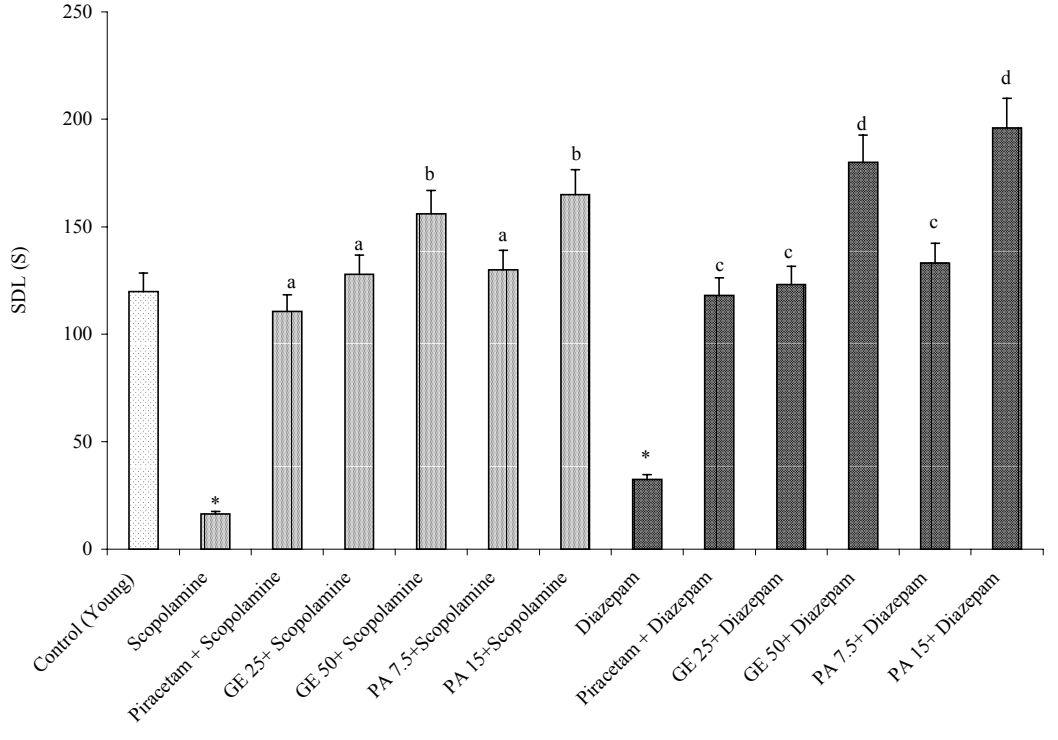

Figure 4: Effect of [6]-gingerol and phyllanthin on SDL of scopolamine and diazepam induced amnesia

of drug treatment) reflected the long-term memory of animals. [6]-gingerol (25 and 50 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. Diazepam (1 mg/kg, i.p.) and scopolamine hydrobromide (0.4 mg/kg, i.p.) decreased SDL on second day after training, indicating impairment of memory. [6]-gingerol (50 mg/kg, p.o.) administered orally for 8 days significantly (P<0.001) reversed amnesia induced by scopolamine and natural ageing (Figure 4).

Various doses of phyllanthin (7.5 and 15 mg/kg, p.o.) administered to young and older mice for 9 days, showed dose-dependent increase in SDL values as compared to respective control groups. Phyllanthin (7.5 and 15 mg/kg, p.o.) administered for 9 days reversed memory deficits due to ageing induced amnesia (Figure 3). The groups of mice, which were treated with piracetam (200 mg/kg, i.p.) for seven successive days showed improvement in memory of young as well as older mice. Higher dose of Phyllanthin (15 mg/kg, p.o.) protected the mice by significantly increasing SDL (P <0.001) followed by phyllanthin (7.5 mg/kg, p.o) in scopolamine and diazepam induced amnesic mice (Figure 4).

Effects of [6]-gingerol and phyllanthin on brain AChE activity

The acetylcholinesterase 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 [6]-gingerol (25 mg/kg, p.o.) significantly lowered AChE activity (P <0.01). [6]-gingerol (50 mg/kg, p.o.) profoundly decreased (P <0.001) whole brain AChE activity compared to control (Figure 5). Phyllanthin (7.5 and 15 mg/kg, p.o.) showed a remarkable reduction in brain cholinesterase activity in young and older mice, as compared to respective control groups (Figure7).

DISCUSSION

Alzheimer's disease is a neurodegenerative disorder associated with a decline in cognitive abilities. Patients often show non-cognitive symptoms, such as depression, apathy and psychosis that impair their day-to-day activities[37]. The symptoms of all types of dementia were presumed to be related to impaired

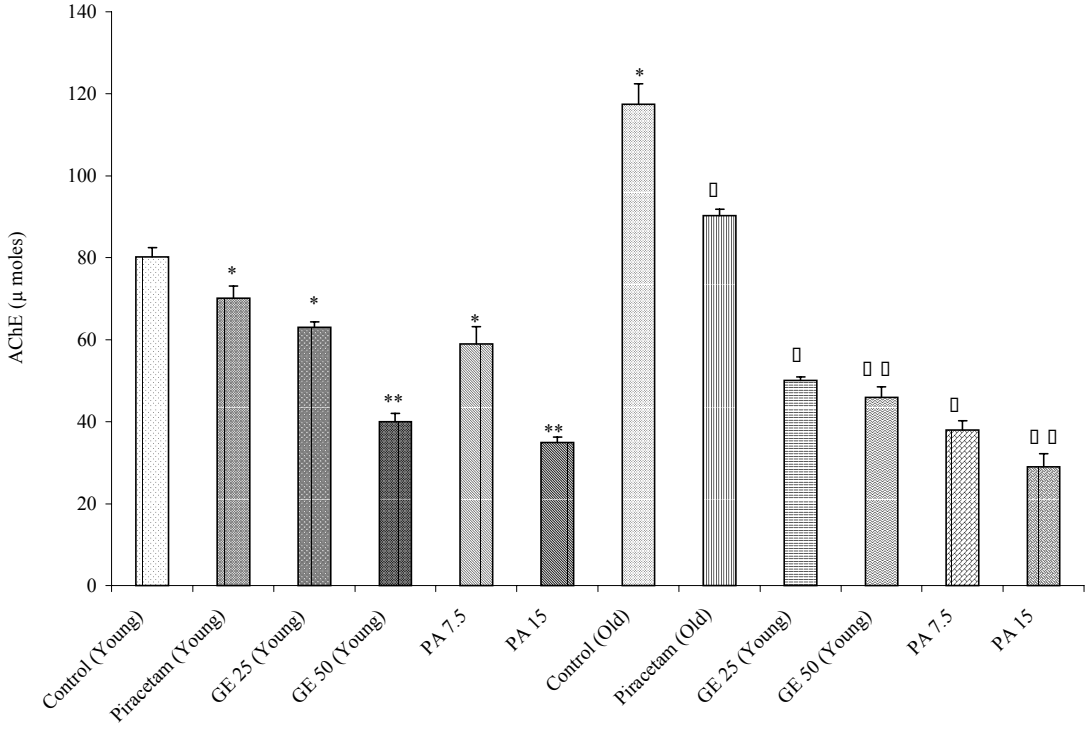

Figure 5: Effect of [6]-gingerol and phyllanthin on AChE activity of young and aged mice

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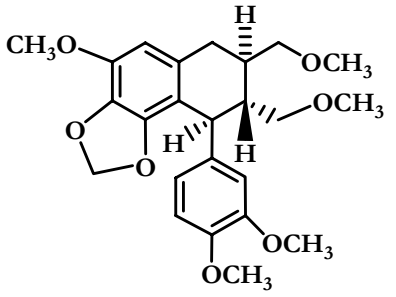

$C_{24}H_{30}O_7$ Mol.WT.430.49

Figure 6: Structure of phyllanthin

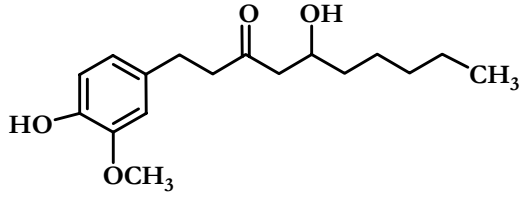

Figure 7: Structure of [6]-gingerol

neurotransmission and degeneration of neuronal circuits in the affected brain areas[38]. Cognitive deterioration occurring in patients with probable AD is associated with a progressive loss of cholinergic neurons and a consequent decline in levels of acetylcholine (ACh) in the brain, particularly in the temporal and parietal neocortex and hippocampus[39]. Acetylcholine is a neurotransmitter inhibited mainly by acetylcholinesterase (AChE) and is considered to play a crucial role in the pathology of AD[40]. Despite the unknown etiology of AD, elevation of acetylcholine amount through AChE enzyme inhibition has been accepted as the most effective treatment strategy against AD[41].

The present study suggests that [6]-gingerol and phyllanthin are potential anti-cholinesterase agents. They also possess nootropic activity in view of their facilitatory effect on retention of learned task. In our study, phenytoin per se (12 mg/kg, p.o.) significantly elevated brain AChE activity. Piracetam (200 mg/kg, p.o.), phyllanthin (7.5 and 15 mg/kg, p.o.) and [6]-gingerol (25 and 50 mg/kg, p.o.), on the other hand significantly ($P<0.01$) lowered this activity indicating the counteracting activity of these drugs on the cholinergic system. Phyllanthin and [6]-gingerol also reversed the diazepam and scopolamine-induced amnesia and aging-induced impairments of learning and memory, when assessed on elevated plus maze and passive avoidance paradigm.

CONCLUSION

The present study indicates that [6]-gingerol and phyllanthin can be of enormous use in the treatment and management of early dementia of Alzheimer's disease and other cognitive disorders. However, further investigations are necessary employing more models and other neurotransmitters for the confirmation of nootropic potentials of [6]-gingerol and phyllanthin.

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