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Original Article

Pharmacological Evidences for the Antiamnesic Effects of *Desmodium gangeticum* in mice

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Abstract

Dementia is one of the age related mental problems and a characteristic symptom of various neurodegenerative disorders including Alzheimer's disease. *Desmodium gangeticum* DC. (Leguminosae) is widely used in Ayurveda for treating various neurological disorders. In the present study, the effectiveness of aqueous extract of *D. gangeticum* in attenuating scopolamine-induced amnesia in mice was investigated. Passive avoidance paradigm was used to assess long-term memory. In order to delineate the possible mechanism through which *D. gangeticum* elicits the anti-amnesic effects, we studied its influence on central cholinergic activity by estimating the acetylcholine content of the whole brain and acetylcholinesterase activity at different regions of the mouse brain, viz., cerebral cortex, midbrain, medulla oblongata and cerebellum. Pretreatment with *D. gangeticum* (100 mg/kg and 200 mg/kg, p.o.) for seven successive days, reversed scopolamine induced amnesia in mice. *D. gangeticum* increased mice brain acetylcholine content and decreased acetyl cholinesterase activity in a similar manner to the standard cerebro-protective drug piracetam. Hence, aqueous extract of *D. gangeticum* can be used to delay the onset and reduce the severity of the symptoms of dementia and Alzheimer's disease.

Keywords: Anticholinesterase activity; Acetylcholine; Memory; *Desmodium gangeticum.*

Introduction

Alzheimer's disease (AD) is a chronic, progressive, and disabling organic brain disorder characterized by disturbance of multiple cortical functions, including memory, judgment, orientation, comprehension, learning capacity and language (1). An estimated 4.5 million Americans have AD. 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 (2). The number of Americans with AD has more than doubled since 1980; by 2050 the number of individuals with Alzheimer's could range from 11.3 million to 16 million (3). 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 (4), has made their use limited and it is worthwhile to explore the utility of traditional medicines in the treatment of various cognitive disorders. Modulation of brain aging with complex extracts containing active phytochemicals has been used in the aging of wild-type rodents with encouraging results (5). Ayurvedic medicinal plants have been shown

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to successfully attenuate memory dysfunctions induced by scopolamine, ethanol and diazepam (6, 7).

Desmodium gangeticum DC. (Leguminosae) is commonly known as Shalparni in Sanskrit. It is abundantly found throughout India. It is one of the important plants used in indigenous system of medicine as a bitter tonic, febrifuge, digestive, and antiemitic in inflammatory conditions which are due to *vata* disorder (8). It is used in 'Ayurvedic' preparations like 'Dashmoolarishta' and 'Dashmoolakwaath'and for treatment of nervous disorders (9). Alkaloids isolated from aerial part comprise indol-3-alkyl-amines and β-carbolines, and have anticholinesterase, smooth muscle and CNS stimulant activities (10). Gangetin, a pterocarpan, shows anti-fertility (11), Anti ulcer (12), anti oxidant (13), cardiotonic (14), antidiabetic (15), anti-inflammatory and anti-nociceptive (16) activities.

In the present study, we employed a passive avoidance response paradigm for evaluating long time memory in mouse. Cholinergic activity determinants such as, acetylcholine (Ach) content and acetylcholinesterase (AchE) content in their brains were determined.

Experimental

Plant materials and extraction

The leaves and roots of *D. gangeticum* were collected from Gopeshwar, Tehri Garhwal district, Uttaranchal, India, during October 2003. The plant parts were identified and authenticated by taxonomists at Department of Systemic Botany, Forest Research Institute, Dehradun, Uttaranchal, India. Voucher specimens (HKJ/DG-39) of the collected samples were deposited in the Department of Pharm. Sciences, Guru Jambheshwar University, Hisar, Haryana, India. The shade-dried root and leaves were powdered and passed through 10-mesh sieve. The coarsely powdered materials (1000 g) were soaked in distilled water in a ratio of 1:16 (w/v). The extract was filtered, pooled and first concentrated on rotavapour and then freeze dried with high vacuum (yield 14.1% (w/w)). The chemical constituents of the decoction were identified by qualitative analysis and confirmed by thin layer chromatography (17), which indicated the presence of alkaloids and flavonoids. A suspension was prepared using distilled water containing 1% (w/v) carboxy methyl cellulose (CMC).

Chemicals

Scopolamine hydrobromide, acetylcholine chloride, gallamine triethiodide, eserine sulphate, 5, 5-dithio-bis2–nitrobenzoic acid, (Ellman's reagent), acetylthiocholine iodide (Sigma Aldrich, USA), piracetam (Nootropil®, UCB Pvt. Ltd., Vapi, Gujarat, India.), trichloroacetic acid (S. D. Fine Chemicals, Mumbai, India), and ingredients of frog ringer solution (Nice Chemical Co. Cochin, India) were used.

Animals

Adult albino mice of both sexes, weighing between 20–30 g were used for the study. Frogs (*Rana tigrina*) whose rectus abdominis muscle was used for bioassay of acetylcholine were procured from disease free animal house of CCS Haryana Agriculture University, Hisar Haryana, India. They were acclimatized to the laboratory conditions for 5 days before behavioral studies. The animals had free access to food and water and were exposed to alternate light and dark cycles of 12 h each. All experiments were carried out during daytime from 0900 to 1400 h. Institutional Animal Ethics Committee (IAEC) approved the experimental protocol and care of the animals was taken as per guidelines of CPCSEA.

Toxicity studies

D. gangeticum extract (DG) 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. 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.

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 (27×27×27 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 (20 V 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 (18, 19). 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 s) 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 s, electric shocks were delivered for 15 s. During the second test, animals were removed from shock free zone if they did not step down for a period of 60 s. 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.

Drug protocol

The animals were divided into 11 groups each consisted of a minimum of twelve mice. Separate animals were used for each experiment.

Group I: (Control group for mice): The vehicle was administered orally for seven successive days. Shock was delivered for 15 s after 90 min of vehicle administration on day seven, and SDL was recorded after 24 h (i.e. on eighth day).

Group II: Scopolamine (0.4 mg/kg) was injected intraperitoneally into mice and shock was delivered for 15 s after 45 min of injection and SDL was noted after 24 h (i.e. on eighth day).

Groups III: (Positive control for mice). Piracetam (200 mg/kg i.p.) was injected to mice for seven successive days. Shock was delivered for 15 s after 60 min of i.p. injection on day seven and SDL was examined after 24 h (i.e. on eighth day).

Groups IV and V: DG (100 and 200 mg/kg, respectively) were administered orally for seven successive days to mice. Shock was delivered for 15 s after 90 min of the extract administration on day seven and SDL was noted after 24 h (i.e. on eighth day).

Group VI & VII: DG (100 mg/kg and 200 mg/kg, p.o.) was administered for 7 days. After 45 min of administration of the last dose on 7th day, scopolamine hydrobromide (0.4 mg/kg, i.p.) was administered. SDL was recorded after 90 min of administration on 7th day and again after 24 h.

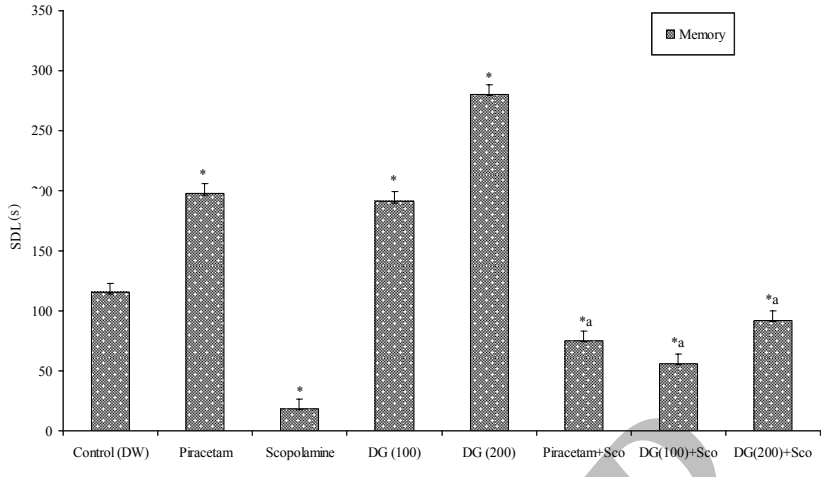
Group VIII: Piracetam (200 mg/kg, i.p.) was administered for 7 days. After 45 min of administration of the last dose on 7th day, scopolamine hydrobromide (0.4 mg/kg, i.p.) was administered. SDL was recorded after 60 min of administration on 7th day and again after 24 h.

Group IX, X and XI: Served as control, treated with DG (100 mg/kg and 200 mg/kg, p.o.) respectively. The locomotor function was assessed using photoactometer.

The mice of all groups were dosed once daily with the respective drugs for seven days. The extracts were dissolved in water and administered through the oral route. Out of 12 mice in a group, 6 were used for the acetylcholine estimation and 6 for determination of acetylcholinesterase activity.

Estimation of brain acetylcholine

Animals were killed by careful cervical dislocation to avoid any injuries to the tissue. The brain was removed and placed on ice. Acetylcholine was extracted into 10% ice-cold trichloroacetic acid (20). The concentration of the acetylcholine in the extract was determined by bioassay on frog Rectus abdominis muscle. Eserinised (10^{-6} M) Frog ringer was used as the physiological solution. The contraction of the rectus (skeletal) muscle was confirmed to be due to acetylcholine from the extract, by demonstrating its competitive reversible antagonism with gallamine. The concentration of the acetylcholine in the extract was calculated from the bioassay tracing by the method of equivalence, equating the height of contraction produced by a known concentration of standard



Values are each Mean ± SEM, ANOVA followed Dunnet's test,
* indicates p<0.001 compared to control
[a] indicates p<0.01 compared to scopolamine treated group

Figure 1. Effect of *D. gangeticum* on Step-down-latency using Passive avoidance paradigm.

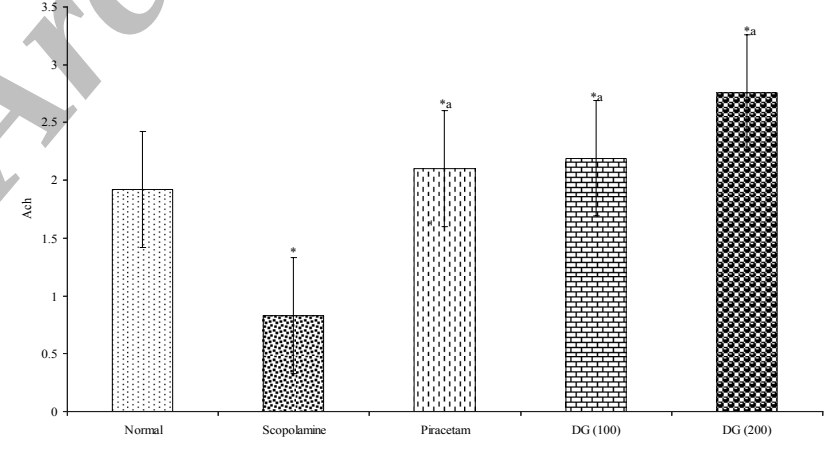
acetylcholine with the height produced by a known volume of the extract (21).

Determination of acetylcholinesterase activity in different parts of brain

The cerebral cortex, midbrain, medulla oblongata and cerebellum were dissected on ice as described earlier (22), suspended in phosphate buffer and weighed. The different parts of brain were homogenized in a tissue homogenizer using 20 mg/ml of phosphate buffer at a pH of 8.0. Reaction mixtures contained 0.4 ml of homogenate, 2.6 ml of phosphate buffer, 100 µl of Ellman's reagent, and substrate acetylthiocholine iodide 20 µl was added. The changes in optical absorbances were measured every minute at 412 nm in a Jasco 530 UV VIS spectrophotometer to provide a measure of enzyme activity (23, 24).

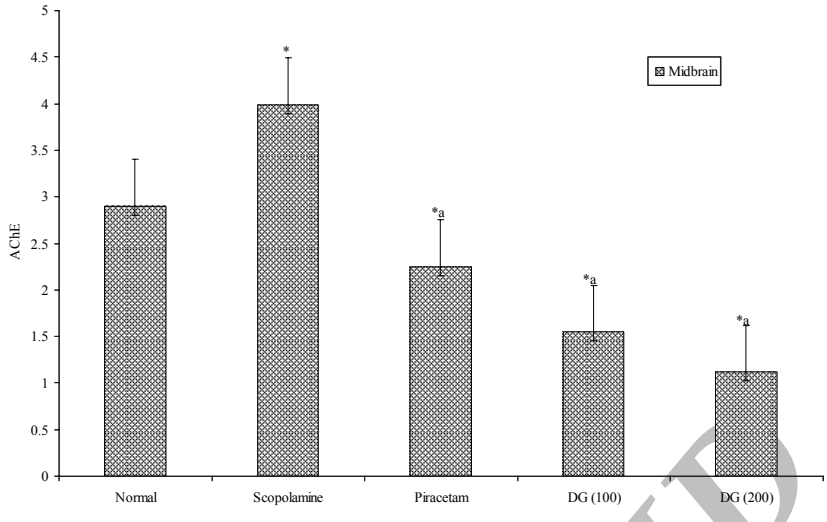
Locomotor function

Locomotor activities of control and drug treated animals were measured using a photoactometer (INCO, Ambala, India).



Values represent mean±SD; Dunnet's test
* indicates p<0.001 compared to control
[a] indicates p<0.01 compared to scopolamine treated group

Figure 2. Effect of *D. gangeticum* on Ach content (µg/g of brain).



Values represent mean±SD; Dunnet's test
* indicates p<0.001 compared to control
[a] indicates p<0.01 compared to scopolamine treated group

Figure 3. Effect of *D. gangeticum* on AChE activity (nmol/min/g of tissue) in midbrain.

Statistical analysis

All the results were expressed as mean± Standard error. The data were analyzed using one-way ANOVA followed by Dunnet's test for individual comparison of groups with control. P <0.01 was considered as significant.

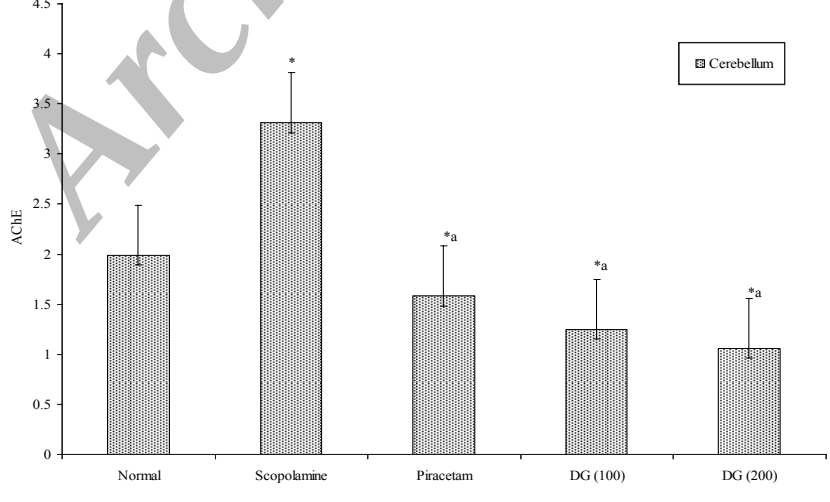
Results

Acute toxicity study

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

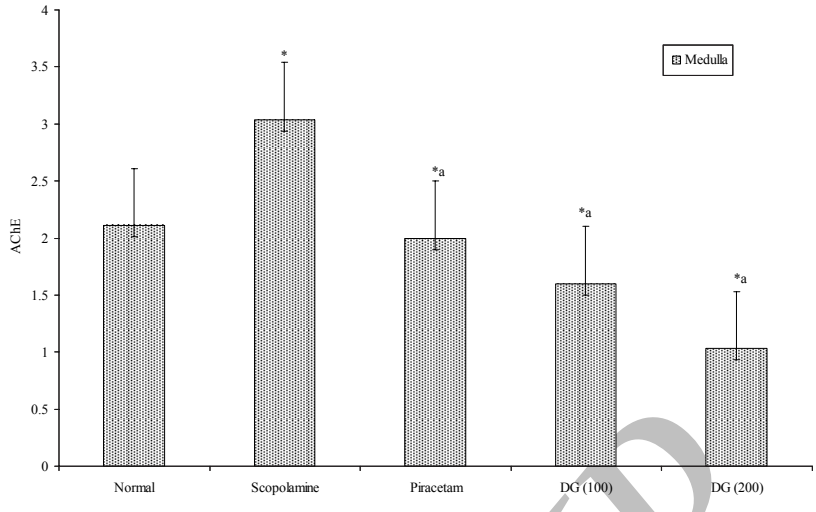
Effect on locomotor activity

In the present study, DG (100 and 200 mg/kg) did not show any significant change in the locomotor function of animals (score: 222.6±8 and 211±15) when tested on photoactometer as



Values represent mean±SD; Dunnet's test
* indicates p<0.001 compared to control
[a] indicates p<0.01 compared to scopolamine treated group

Figure 4. Effect of *D. gangeticum* on AChE activity (nmol/min/g of tissue) in cerebellum.



Values represent mean±SD; Dunnet's test
* indicates p<0.001 compared to control
[a] indicates p<0.01 compared to scopolamine treated group

Figure 5. Effect of *D. gangeticum* on AChE activity (nmol/min/g of tissue) in medulla.

compared to control group (score: 216.4±12).

Effect on SDL using Passive avoidance paradigm

DG (100 and 200 mg/kg, p.o.) treatment significantly (p<0.001) increased step down latency (SDL) as compared to control group on the second day indicating improvement in memory in mice. Scopolamine hydrobromide (0.4 mg/kg, i.p.) decreased SDL on second day after training, indicating impairment of memory. DG (100 and 200 mg/kg, p.o.) administered orally for seven days significantly (p<0.01) reversed amnesia induced by scopolamine (1).

Effect on Ach content

Pretreatment with DG (100 and 200 mg/kg, p.o.) for seven successive days, significantly (p<0.001) elevated ACh content as compared to control and scopolamine treated groups (p<0.001). The higher dose of DG (200 mg/kg, p.o.) profoundly increased ACh content in the mouse brain whereas scopolamine (0.4 mg/kg, i.p.) treated group significantly (p<0.01) reduced the ACh levels (Figure 2).

Effect on AChE activity in midbrain

Scopolamine (0.4 mg/kg, i.p.) significantly (p<0.001) increased mid brain AChE activity compared to control. DG (200 mg/kg and 100 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) exhibited significant decline in AChE activity in midbrain fraction of mice brain homogenate compared to control (p<0.001) and scopolamine treated (p<0.01) groups (Figure 3).

Effect on AChE activity in cerebellum

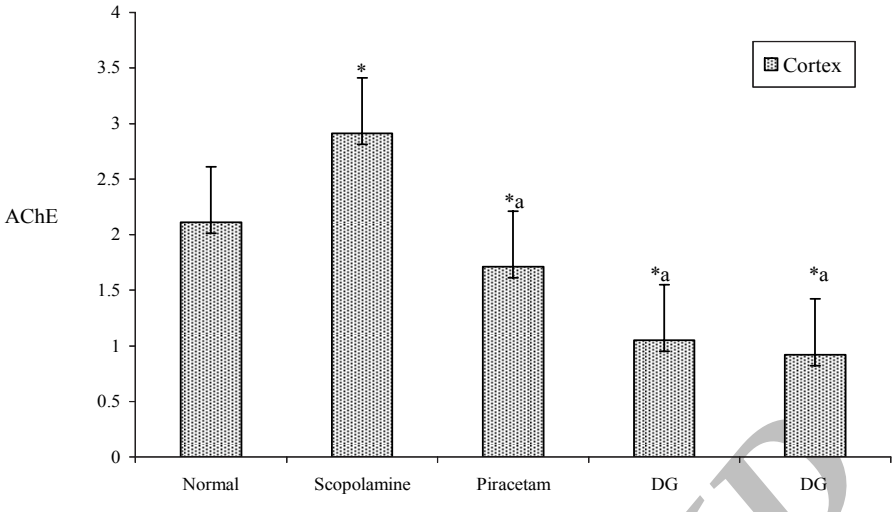
DG (200 mg/kg) significantly (p<0.001) decreased AChE activity in the cerebellum followed by DG (100 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) as compared to control and scopolamine treated (p<0.01)group (Figure 4).

Effect on AChE activity in medulla

Scopolamine (0.4 mg/kg, i.p.) significantly increased AChE activity in medulla as compared to control. DG (200 mg/kg and 100 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) exhibited significant (p<0.001) decline in AChE activity in medulla of mice brain (Figure 5).

Effect on AChE activity in cortex

DG (200 mg/kg) significantly decreased AChE activity in the cerebellum followed by DG (100 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) as compared to control (p<0.001) and scopolamine treated (p<0.01) groups (Figure 6).



Values represent mean±SD; Dunnet's test
* indicates p<0.001 compared to control
[a] indicates p<0.01 compared to scopolamine treated group

Figure 6. Effect of *D. gangeticum* on AChE activity (nmol/min/g of tissue) in cortex.

Discussion

The symptoms of all types of dementia are presumed to be related to impaired neuro transmission and degeneration of neuronal circuits in the brain areas affected (25). Cognitive deterioration occurring in patients with probable Alzheimer's disease (AD) is associated with a progressive loss of cholinergic neurons and a consequent decline in the levels of acetylcholine (ACh) in the brain particularly in the temporal and parietal neocortex and hippocampus (26). Acetylcholine is believed to affect the memory, sleep, and concentration abilities, and also to be involved in some severe diseases such as Alzheimer, Parkinson and epilepsy (27, 28).

Despite the severity and high prevalence of this disease, Allopathic system of medicine is yet to provide a radical cure for AD. Therefore, we were motivated to explore the potential of medicinal plants from Himalayan flora to manage this deadly disease (AD). In the present study, *D. gangeticum* extract administered orally for 7 days improved the memory of mice as reflected by enhanced SDL values as compared to control animals. Additionally, DG reduced central cholinesterase activity. Furthermore, pretreatment with DG for 7 days protected the animals from memory deficits produced by scopolamine. These findings suggest a possible neuroprotective role for *D. gangeticum*.

Nootropics represent a new class of psychotropic agents with selective facilitatory effect on integrative functions of the central nervous system, particularly on intellectual performance, learning capability and memory (29). Piracetam, the first representative of a class of nootropic agents, has been shown to improve memory deficits in geriatrics. Repeated injections of piracetam have been shown to improve learning abilities and memory capacities of laboratory animals. Passive avoidance behavior is based on negative reinforcement and is used to examine long-term memory. Both piracetam and *D. gangeticum* meet major criteria for nootropic activity, namely improvement of memory in absence of cognitive deficit (30).

It has been observed that elderly patients suffering from Alzheimer's disease show reduction in symptoms of Alzheimer's disease upon chronic use of anti-inflammatory drugs (30, 31). Epidemiological studies have almost confirmed that non steroidal anti-inflammatory drugs reduce the incidence of AD (32). *D. gangeticum* has been shown to produce anti-inflammatory activity (16). This anti-inflammatory effect of *D. gangeticum* would certainly help Alzheimer patients by taking care of the inflammatory component of the Alzheimer's disease. Oxygen free-radicals are

implicated in the process of age-related decline in cognitive performance and may be responsible for the development of Alzheimer's disease in elderly persons (33, 34). *D. gangeticum* has been reported to possess antioxidant property as well (35). The neuroprotective effect of DG may be attributed to its antioxidant property through of which susceptible brain cells get exposed to less oxidative stress resulting in reduced brain damage and improved neuronal function.

Acetylcholine is considered as the most important neurotransmitter involved in the regulation of cognitive functions. There are extensive evidences linking the central cholinergic system to memory (36). The symptoms of dementia are presumed to be related to impaired neurotransmission and degeneration of neuronal circuits in the affected brain areas of affected (25). Cognitive deterioration occurring in patients with probable AD is associated with progressive loss of cholinergic neurons and consequent decline in levels of acetylcholine (ACh) in brain (26). Selective loss of cholinergic neurons and decrease in cholinacetyltransferase activity was reported to be a characteristic feature of senile dementia of the Alzheimer's type (37). DG (200 mg/kg and 100 mg/kg, p.o.) significantly increased acetylcholine contents and inhibited the AChE activity in various parts of the mouse brain viz. midbrain, cerebellum, medulla oblongata and cortex, indicating its potential in attenuation of severity of Alzheimer's disease.

In the present study, we observed that *D. gangeticum* extract (i) inhibited acetylcholinesterase enzyme activity in mid brain, cerebellum, medulla and cortex, (ii) elevated acetylcholine concentration in brain homogenate and (iii) ultimately improved memory of mice when tested on exteroceptive behavioral model, passive avoidance paradigm. Thus, a combination of anticholinesterase, procholinergic, anti-inflammatory, antioxidant and neuroprotective effects exhibited by *D. gangeticum* may be responsible for the memory improving effect observed in the present study. However, investigations using more experimental paradigms may be required for further confirmation of nootropic potential of *D. gangeticum* in the treatment of dementia and Alzheimer's disease.

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