Antiamnesic potentials of ethyl acetate fraction of methanolic extract of Ayurvedic drug *Desmodium triquetrum* in mice

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Abstract

Alzheimer's disease is a neurodegenerative disorder associated with a decline in cognitive abilities. Dementia is one of the aged related mental problems and a characteristic symptom of Alzheimer's disease. Nootropic agents like piracetam and cholinesterase inhibitors like Donepezil® are used in situations where there is organic disorder in learning abilities, but the resulting side-effects associated with these agents have limited their utility. Desmodium triquetrum is widely used in Indian traditional systems of medicines and also as a folk remedy for nervous debility. The present work was undertaken to assess the potential of D. triquetrum as a nootropic and anti-cholinesterase agent in mice. Exteroceptive behavioral models such as Elevated plus maze and Morris water maze were employed to assess short term and long term memory in mice. To delineate the possible mechanism through which D. triquetrum elicits the anti-amnesic effects, its influence on central cholinergic activity was studied by estimating the whole brain acetylcholinesterase activity. Pretreatment of ethyl acetate fraction of Methanolic extract of roots of D. triquetrum (50 and 100 mg/kg, p.o.) for 8 successive days, ameliorated the amnesic effect of scopolamine (0.4 mg/kg) and aging induced memory deficits in mice. D. triquetrum (100 mg/kg, p.o) significantly decreased transfer latencies of young mice and aged mice, decreased escape latency, TSTQ and exhibited significant anti-acetyl cholinesterase effects, when compared to piracetam, scopolamine and control groups of mice. D. triquetrum might prove to be a useful memory restorative agent in the treatment of dementia seen in the elderly. The underlying mechanism of its action may be attributed to its antioxidant, anti-inflammatory and acetyl cholinesterase inhibition property.

Key words: Acetylcholine, *D. triquetrum* , memory, piracetam, scopolamine.

Introduction

Alzheimer's disease is a progressive neurodegenerative brain disorder that is slow in onset but leads to dementia, unusual behavior, personality changes and ultimately death (Jewart et al., 2005). The personality distortions interfere with the patient's professional life, social activities and relationships (Katzman et al., 1998). Oxygen free radicals, the harmful byproducts of oxidative metabolism are known to cause organic damage to the living system, which may be responsible for the development of Alzheimer's disease in elderly (Smith & Luo, 2003). Nootropic agents such as piracetam, aniracetam and choline esterase inhibitors like donepezil® are being used for improving memory, mood and behavior (Bhattacharya, 1993), but the resulting side effects (Rogers, 1998) associated with these agents have made their applicability limited. Indian system of medicine emphasizes use of herbs, nutraceuticals or life style changes for controlling age related neurodegenerative disorders.

Desmodium triquetrum belongs to family Leguminosae, Subfamily-Papilionaceae, is an erect or suberect undershrub, distributed throughout central, eastern Himalayas, South India and Sri Lanka. The leaves are used as a substitute for tea by hill tribes in upper Assam. The leaf extracts or pills are used for the treatment of piles (Anonymous ,1952). The chloroform and alcohol extracts of DT were reported for their antibacterial activity (Shirwaikar,2003). The ethanol extract of leaves of this plant has been reported for its wound healing activity. Preliminary phytochemical investigations on the DT revealed the presence of flavonoids, glycosides, steroids, saponins, phenolic compounds, amino acids and fixed oils. It was also reported that the whole plant is boiled and used against the treatment of liver parasite by Lao people (Mujumdar, 1990) .

In the present study, the nootropic effects of *Desmodium triquetrum* were investigated by employing both exteroceptive and interoceptive models. The stimulus lies outside the body in exteroceptive behavioral models, whereas, it lies within the body in case of interoceptive behavioral models. Interoceptive behavioural models such as scopolamine, diazepam and natural aging induced amnesia are widely cited as models simulating human dementia in general and Alzheimer's disease in particular (Joshi et al., 2017).

Materials and methods

Plant materials and extraction

Roots of *Desmodium triquetrum* were collected from Chamundi hills, Mysuru, Karnataka, India. The plant was authenticated at Dept. of Pharmacognosy, Sarada Vilas College of Pharmacy, Mysuru, Karnataka. Roots of *Desmodium triquetrum* were shade-dried root were powdered and passed through 10-mesh sieve. The coarsely powdered materials (1000 g) were soaked in distilled water in the 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).

Drugs and reagents

Scopolamine hydrobromide (Sigma Aldrich, USA), diazepam (Calmpose®, Ranbaxy, India) and piracetam (Nootropil®, UCB India Pvt. Ltd., India) were diluted in normal saline and administered intra-peritoneally. Volume of administration was 1 ml/ 100 g. 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.

Animals

Swiss mice of either sex weighing around 18- 20 g (younger ones, aged 3-4 months) and more than 30 g (aged ones, aged 12-15 months) were used in the present study. Animals were acclimatized to the laboratory conditions for 5 days before behavioral studies. The animals had free access to food and water and were maintained under 12:12 h light and dark cycles. All experiments were carried out during day time from 0900 to 1400 h. Institutional Animals Ethics Committee (IAEC) had approved the experimental protocol and care of animals was taken as per guidelines of CPCSEA, Dept. of Animal Welfare, Govt. of India.

Acute toxicity studies

Desmodium triquetrum (DT) methanolic extract at different doses (10-2000 mg/kg) was administered orally to mice with the help of a specially designed oral needle connected to a polythene tube. DT 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. The parameters such as hyperactivity, grooming, convulsions, sedation, hypothermia and mortality were observed. The doses selected were 50 and 100 mg/kg/day.

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 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 arm 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 (Itoh et al., 1990; Joshi et al., 2005; Parle et al., 2004b).

Morris water maze (MWM):

The MWM test was employed to assess learning and memory of the animals. MWM is a swimming model where the animals learn to escape on to a hidden platform. In the present study the target quadrant was Q4. Each animal was subjected to four consecutive trials on each day with a gap of 5 minutes for four consecutive days, during which they are allowed to escape onto the hidden platform and to remain there for 20 seconds. In case the animal was unable to locate the hidden platform within 120 seconds, it was gently guided to the platform and allowed to remain on the platform for 20 seconds. Escape latency time to locate the hidden platform in water maze was taken as an index of acquisition or learning (Morris, 1984). The starting position

on each day to conduct four acquisition trials was changed as described below and Q4 was maintained as the target quadrant in all the acquisition trials. The starting point for dropping the mice into water maze on day 1 for four consecutive acquisition trials was in the sequence Q1, Q2, Q3 Q4 and so on. The sequence change in starting point was as follows. Day 1: Q1, Q2, Q3, Q4 Day 2: Q2, Q3, Q4, Q1 Day 3: Q3, Q4, Q1, Q2 Day 4: Q4, Q1, Q2, Q3. Mean escape latency time (ELT) was calculated for each day of the trial. On the fifth day the platform was removed, and each mouse was allowed to explore the pool for 120 seconds. The animal was subjected to four such trials with 5-minute interval time and each trial had a different starting point covering all the four quadrants. The mean time spent by animal in all four quadrants was recorded. The time spent in the target quadrants Q4 compared to time spent in other quadrants in search of missing platform was taken as an index of retrieval. Care was taken that the relative location of water maze with respect to other objects in laboratory serving as visual clues was not disturbed during the total duration of the study. All the trials were completed between 09:00 and 17:00 hours (D' Hooge, 2001).

Locomotor function

Locomotor activity of control and drug-treated animals was measured with the help of Photoactometer (INCO, Ambala, India).

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 9^{th} 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).

Statistical analysis

The data were expressed as mean ± SEM. The normally distributed data were subjected to one-way ANOVA followed unpaired 't' test. Kruskal Wallis one-way ANOVA followed by multiple range tests was used for the analysis of non-normally distributed data. $P < 0.05$ considered significant.

RESULTS

Acute toxicity studies: All the doses (5, 50, 250, 500 and 2000 mg/kg, p.o.) of *D. triquetrum* (DT) did not produce any mortality even with the highest dose (2000 mg/kg, p.o.) employed. Two submaximal doses (50 and 100 mg/kg, p.o.) were selected for further psychopharmacological and biochemical studies.

Effect on locomotor activity

In the present study, DT (50 and 100 mg/kg, p.o.) did not show any significant change in the locomotor function of animals (score 232±1.8 and 210±08) as compared to control group (score 219.2±09) when tested using a photoactometer.

Effect on transfer latency (TL) using elevated plus maze: DT (50 and 100 mg/kg, p.o.) showed dose-dependent reduction in TL of 8^{th} day and 9^{th} day, indicating remarkable improvement in learning ability and memory of the young and aged mice as compared to respective control groups (Fig. 1). Diazepam (1 mg/kg, i.p.) and scopolamine (0.4 mg/kg, i.p.) significantly increased ($P < 0.01$) the TL of 9^{th} day indicating impairment in memory (amnesia).

DT (50 and 100 mg/kg, p.o.) successfully (P< 0.001) reversed the amnesia induced by both diazepam and scopolamine (Fig. 2).

Effect on Escape latency and TSTQ

In MWM extreroceptive model, there was a significant fall in ELT of standard drug and DT (50 and 100 mg/kg, p.o.) treated groups as compared to control group which infers the improvement in learning ability (acquisition) of mice. Further there was a significant rise in TSTQ of standard drug and DT (100 mg/kg, p.o.) on 25th day compareD to TSTQ of normal control group. Even though there was a profound decrease in ELT and increase in TSTQ of DT (50 mg/kg, p.o.) was not significant as compared to normal control group (Table 1).

Effect on brain cholinesterase activity

DT (50 and 100 mg/kg, p.o.) showed a remarkable reduction in the brain acetyl cholinesterase activity in young and aged mice, as compared to respective control groups. Whereas, phenytoin (12 mg/kg, p.o.) significantly (P< 0.01) increased the acetyl cholinesterase activity.

Discussion

Alzheimer's disease Alzheimer's disease (AD), a chronic, progressive disabling organic brain disorder characterized by disturbance of multiple cortical functions, including memory, judgment, orientation, comprehension, learning capacity and language (Jay, 2005; Joshi 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).The present study indicates that ethyl acetate fraction of *D. triquetrum* is a potential anti-cholinesterase agent. It also possesses nootropic activity in view of its facilitatory effect on retention of acquired learning. *D. triquetrum* decreased transfer latencies, increases SDL in mice when subjected to passive avoidance paradigm, indicating its potent anti amnesic activity.

Central cholinergic system plays an important role in learning and memory (Biegon, 1986; Perry, 1994). Phenytoin is known to reduce hippocampal ACh concentration (Agarwal et al., 1964; Sudha et al., 2001; Vohora et al., 2004) and causes cognitive impairment (Aldenkamp et al., 1994). In our study, phenytoin per se (12 mg/kg, p.o.) significantly elevated brain AChE activity. Piracetam (250 mg/kg, p.o.) and FV (50, 100 and 200 mg/kg, p.o.), on the other hand significantly (P<0.05) lowered this activity indicating the counteracting action of the two drugs on the cholinergic system. The precise mechanism by which piracetam exerts its nootropic effects is not known, but multiple mechanisms have been suggested such as enhancement of oxidative glycolysis (Verbnyi Yal et al., 1996), an effect on the Ca^{2+} channels (McGeer et al., 1999) and an effect on the cholinergic system (Verbnyi Yal et al., 1996). Both piracetam and *D. triquetrum* meets major criteria for nootropic activity, namely improvement of memory in absence of cognitive deficit and anticholinesterase effects (Bhadania et al., 2012, Joshi et al., 2009, 2017, 2008). Furthermore, *D. triquetrum* also reversed the scopolamine-induced impairment in learning and memory, when assessed on passive avoidance paradigm. Therefore, it seems that *D. triquetrum* improved learning and memory probably due to its effect on cholinergic transmission.

Numerous epidemiologic studies have indicated that individuals who consume diets containing large amounts of fruits, vegetables may be at a reduced risk of developing age related diseases like Ad (Joseph et al., 2005). A cognitive impairment has been associated with lower

vitamin intakes, fruits and vegetables have been demonstrated to have protective effects against stroke and vascular dementia (Gillman et al., 1995). Many free radical scavengers are present in food, particularly in fruits, vegetables and grains and the regular consumption of these nutritive substances may therefore have a beneficial impact. Therefore, the memory improving activity of *D. triquetrum* may be attributed to its anti-inflammatory, neuroprotective, pro-cholinergic and anti-acetylcholinesterase properties of *D. triquetrum* and hence may be of enormous use in delaying the onset and reducing the severity of Alzheimer's disease. However, further investigations are warranted to explore the possible involvement of other neurotransmitters like glutamate, GABA, catecholamines etc. (Parle et al., 2004c) responsible for memory improving property of *D. triquetrum*.

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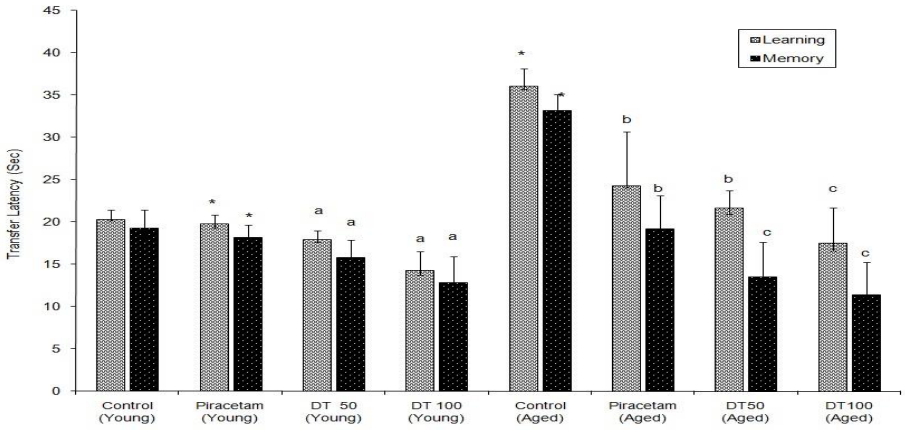

Fig. 1. Effect of *D. triquetrum* (DT 50 and 100 mg/kg) on transfer latency of young and aged mice using elevated plus maze.

Values are mean ±S.E.M. (n=6); * indicates P< 0.01 as compared to control group of young mice; a indicates P< 0.001 as compared to control group of young mice; b indicates P< 0.01 as compared to control group of aged mice; c indicates P< 0.001 as compared to control group of aged mice; (One way ANOVA followed by Tukey-kramer multiple comparison tests)

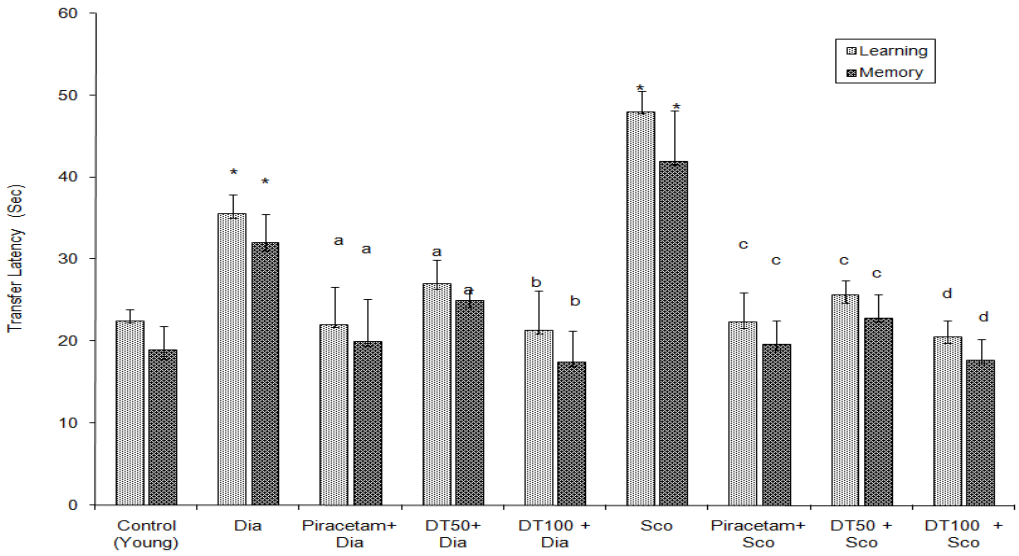

Fig. 2. Effect of *D. triquetrum* (DT 50 and 100 mg/kg) on transfer latency of diazepam and scopolamine induced amnesic mice.

Values are mean ±S.E.M. (n=6); * indicates P< 0.01 as compared to control group of young mice; a indicates P< 0.01 as compared to diazepam (Dia) group alone; b indicates P< 0.001 as compared to diazepam (Dia) group alone.c indicates P< 0.01 as compared to scopolamine (Sco) group alone; d indicates P< 0.001 as compared to scopolamine (Sco) group alone; (One way ANOVA followed by Tukey-kramer multiple comparison tests)

Table 1. Effect of *D. triquetrum* (DT 50 and 100 mg/kg) on Escape Latency time (ELT) & Time Spent in Target Quadrant (TSTQ) in young mice.

Group	Treatment	Dose	ELT (21st day)	ELT (24th day)	TSTQ (25th day)
I	Control	10 ml/kg p.o	91.06±0.57	49.13±0.91	59.05±0.4
II	Piracetam	400mg/kg i.p.	73.10±1.9*	34.05±1.8*	70.69±1.1*
III	DT	2mg/kg p.o	79.13±02 a	43.05±0.2 a	51.12±0.2a
IV	DT	5 mg/kg p.o	73.11±0.7a	41.16±0.8a	39.19±0.8a

Each values represents mean±S.E.M. **P<0.001compared to Normal control. One-way ANOVA followed by Tukey's post test

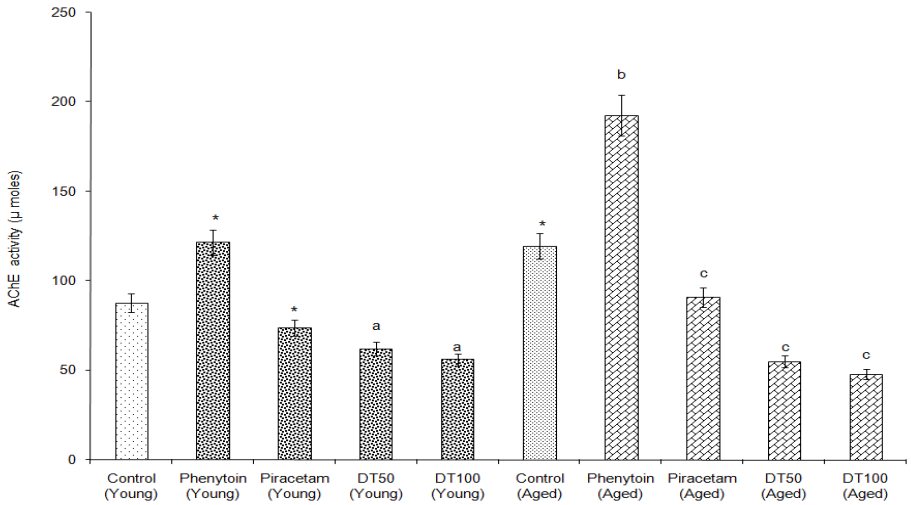

Fig. 3. Effect of *D. triquetrum* (DT 50 and 100 mg/kg) on ACh activity .
Values are mean ±S.E.M. (n=6); * indicates P< 0.01 as compared to control group of young mice; a indicates P< 0.001 as compared to control group of young mice; b indicates P< 0.01 as compared to control group of aged mice; c indicates P< 0.001 as compared to control group of aged mice; (One way ANOVA followed by Tukey-kramer multiple comparison tests)