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Research Article

Evaluation of nootropic activity of *Curcuma longa* leaves in diazepam and scopolamine-induced amnesic mice and rats

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ABSTRACT

Background: The present study was undertaken to assess the nootropic activity of hydroalcoholic extract of *Curcuma longa* leaves (HAECL) in diazepam-induced amnesia in mice using Morris water maze method and scopolamine-induced amnesia in rats by using elevated plus maze behavioral paradigm and its effect on acetylcholinesterase (AChE) and reduced glutathione (GSH) level were carried out.
Methods: Amnesia was induced by administration of diazepam (1 mg/kg i.p.) and scopolamine (0.4 mg/kg i.p.) and treatment groups received HAECL (200 and 400 mg/kg p.o) for 14 and 10 days in scopolamine and diazepam-induced amnesia model, respectively. The extent of improvement in memory was measured by behavioral paradigm. Finally, animals were sacrificed, and the whole brain was isolated for estimation of concentration of AChE and reduced GSH levels.
Results: The oral treatment with HAECL with a dose 400 mg/kg has shown an enhancement in the memory function compared to 200 mg/kg.
Conclusion: This could be by inhibiting the levels of cholinesterase concentration of enzyme and thereby increasing the concentration of acetylcholine level in brain and improving cognition-memory performance.

Keywords: Hydroalcoholic extract of *Curcuma longa* leaves, Piracetam, Scopolamine, Diazepam, Morris water maze, Elevated plus maze, Acetylcholinesterase

INTRODUCTION

Memory is the ability of the brain to encode, store and retrieve information. Encoding refers to the initial perception and registration of information. Storage is the retention of encoded information over time. Retrieval refers to the processes involved in using stored information. Whenever people successfully recall a prior experience, they must have encoded, stored, and retrieved information about the experience,[1] conversely, memory failure is forgetting an important fact reflects a breakdown in one of these stages of memory. Memory and learning are closely related and the terms often describe roughly the same processes. The term learning is often used to refer the processes involved in the initial acquisition or encoding of information, whereas the term memory more often refers to later storage and retrieval of information.[2]

Cognition in a broad sense means processing of information. It denotes a relatively high level of processing of specific information including thinking, memory, perception, motivation, skilled movements, and language. The hippocampus contains the neural circuitry crucial for cognitive functions such as learning and memory, refers to the perceptual, and intellectual aspects of mental functioning.[3] Cognitive dysfunction, a major health problem in the 21st century one of the most functionally debilitating aspect. Age, stress, and emotion are conditions that may lead to memory loss, amnesia, anxiety, high blood pressure, dementia, and to more ominous threat neurodegenerative disorder such as schizophrenia, depression, Alzheimer's disease, dementia, cerebrovascular impairment, head injury, parkinsonism, Huntington's disease, Down's syndrome, Pick's disease, trauma, chronic insomnia, and attention deficit disorders.[4]

Recently, memory complaints and memory disorders are becoming more prevalent due to various factors such as natural (ageing, physical, and mental stress), environmental (excess levels of carbon monoxide, carbon dioxide, methyl

mercury in atmosphere, and aluminum in foods), and iatrogenic (electroconvulsive shock therapy and use of certain central nervous system depressants).[5]

The cholinergic neuronal system plays an important role in learning and memory in humans and animals. Acetylcholinesterase (AChE) is an enzyme which hydrolyses acetylcholine (Ach) to choline. Among the various approaches attempted to increase cholinergic activity, the inhibition of AChE is the most successful one. Phellandrene isolated from *Pinus* species has exhibited AChE inhibitory activity.[6] The constituents of *Curcuma longa* leaves are monoterpenes which include phellandrene and C8-aldehyde, with phellandrene.[7]

Hence, the study is planned to screen nootropic activity of *C. longa* leaves in diazepam and scopolamine-induced amnesic mice and rats using Morris water maze and elevated plus maze models with biochemical tests including estimation of reduced glutathione (GSH) and AChE activity.

METHODS

Plant

The leaves of *C. longa* were collected in Uttar Kannada. The plant herbarium specimen was identified and authenticated by Dr. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati - 517 502, Andhra Pradesh, India.

Preparation of extract

The hydroalcoholic extract of *C. longa* leaves (HAECL) was obtained as a gift sample from Olive life sciences, Bengaluru.

Experimental animals

Healthy female mice weighing (20-25 g) were used for acute toxicity studies. Healthy Albino Wistar rats of either sex of age 12-13 weeks, weighing between 150 and 200 g were taken for the evaluating nootropic activity. All the animals were procured from the drug testing laboratory, Bangalore. They were housed in polypropylene (32 cm × 24 cm × 16 cm) cages containing bedding material as husk and maintained under controlled conditions of temperature (25±2°C), humidity (55±5%) and 12 hrs light and 12 hrs dark cycles in animal house facility of Government College of Pharmacy. They were fed with commercial pelleted rat chow (Shri Venkateshwara Enterprises, Bengaluru). With water *ad libitum*. The experimental protocols were approved by the Institutional Animal Ethics Committee dated March 23, 2013, with ref. no. DCD/GCP/20/E.C/ADM/2012-13. All the procedures performed were in accordance with the CPCSEA guidelines.

Acute toxicity studies

Acute toxicity study (OECD 420 guidelines) was carried out using female albino mice (20-25 g) those maintained under standard husbandry conditions. The maximum upper limit dose 2000 mg/kg of *C. longa* extract was administered orally to 3 female mice. Animals were observed for 48 hrs to study the general behavior of animals, signs of discomfort, and nervous manifestation.

Evaluation of nootropic activity

Diazepam induced amnesia in mice using Morris water maze test[8-10]

Healthy Swiss albino mice of either sex were divided into five groups. Each group containing eight animals and treated as follows:

Group 1: Control group, received only vehicle, Group 2: Positive control (diazepam 1 mg/kg [i.p.]), Group 3: Standard (Piracetam 200 mg/kg p.o,) + diazepam (i.p.), Groups 4 and 5: Test groups, which receive HAECL 200 mg and 400 mg/kg, p.o, + diazepam (i.p) respectively. The animals were treated with HAECL and standard for 10 days. Diazepam was given by i.p. route from 6th to 10th day to all the group of animals except control group before 45 mins of acquisition trials for inducing amnesia and each mouse will be subjected to water maze task performance.

The water maze task is divided into two phases:

i. Acquisition trials: Each animal was subjected to four consecutive trials on each day (from 6th to 9th day) with an interval of 10 mins, during which mouse was allowed to escape on the hidden platform and was allowed to remain there for 20 sec. Escape latency time (ELT) to locate the hidden platform in water maze was noted as an index of acquisition and learning. In a preliminary study, the trial was conducted to familiarize the mouse with the task and was not counted. Mouse was subjected to acquisition trials for 4 consecutive days
ii. Retrieval trial: On the 10th day, hidden platform was removed and each mouse was allowed to explore the pool for 90 sec. Mean time spent by the mouse in each of four quadrants was noted. The mean time spent by the mouse in target quadrant for searching the hidden platform was noted as an index of retrieval.

After water maze task performance on 10th day animals were sacrificed and brains of mice were isolated for the estimation of reduced GSH and concentration of AChE.

Scopolamine induced amnesia in rats using elevated plus maze[11]

Healthy Albino Wistar rats of either sex were divided into five groups. Each group containing 8 animals and treated for 14 days as follows:

Group 1: Control group, received only vehicle, Group 2: Positive control (scopolamine 0.4 mg/kg [i.p]), Group 3: Standard (piracetam 200 mg/kg p.o,) + scopolamine (i.p.), Groups 4 and 5 test groups which receive HAECL 200 mg and 400 mg/kg, p.o, + scopolamine (i.p) respectively. The animals were treated with HAECL and standard for 14 days and at the end of treatment period, animals of respective groups are subjected to scopolamine (0.4 mg/kg i.p) treatment, 60 mins after administration of extract, except the first group which served as vehicle control. Transfer latency (TLT) was recorded after 45 mins of drug administration and after 24 hrs.

After elevated plus maze performance on 15th day animals are sacrificed and brains were isolated for the estimation of reduced GSH and concentration of AChE.

Biochemical estimation

Tissue preparation

Animals were sacrificed by decapitation under deep ether anesthesia, the whole brain dissected out, blotted dry and immediately weighed and rinsed with ice-cold isotonic saline. Brain samples were then homogenized with 10 times (w/v) ice cold 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 ×*g* at −15°C for 15 mins and aliquots of supernatant were separated and used for biochemical estimation.

Estimation of AChE concentration[12,13]

A 0.4 ml aliquot of the supernatant was added to a cuvette containing 2.6 ml phosphate buffer (pH 7.2, 0.05 M). To this, 100 µl of Ellman's reagent was added and then taken into the photocell. The absorbance was set at 412 nm when the fluctuations stopped. Of the substrate (acetyl thiocholine iodide) 20 µl was added. A change in the absorbance is recorded for a period of 10 mins at an interval of 2 mins.

The rate of moles of substrate hydrolyzed per minute per gram of tissue was later calculated as per the following equation:

$$R = \frac{\Delta A}{1.36(10^4)} \times \frac{1}{(400/3120)C_0} = 5.74(10^{-4})\frac{\Delta A}{C_0}$$

Where,

ΔA=Change in absorbance per minute
C_0=Original concentration of the tissue
R=Rate in moles substrate hydrolyzed per minute per gram of tissue

Estimation of reduced GSH[14]

The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5,5-dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled water were added. The mixture was vortexed and the absorbance was read at 412 nm within 15 mins. The concentration of reduced GSH was expressed as µg/g tissue.

Statistical significance

The data and other related values will be expressed statistically as mean±standard error of mean. The statistical difference in mean will be analyzed using one-way ANOVA followed by Tukey's multiple comparison tests and the $p<0.05$ value will be considered as statistically significant.

RESULTS

Diazepam induced amnesia using Morris water maze in mice

The animals treated with piracetam (200 mg/kg p.o.) has shown significantly decreased ELT on 6-9th day and increased time spent in the target quadrant (TSTQ) on 10th day. The treatment animal group with HAECL at 200 mg/kg p.o. and 400 mg/kg p.o. has shown significant decrease in ELT from 6th to 9th day and increase in TSTQ on 10th day, the extract also showed significant reduction of AChE enzyme activity and produced significant increase in GSH level compared to the positive control (diazepam) group. The groups of animals showing ELT and TSTQ are summarized in Figures 1 and 2.

Effect of oral administration of HAECL, piracetam, and i.p. administration of diazepam on AChE activity and GSH levels in mice brain

In the standard group, the animals treated with piracetam (200 mg/kg p.o.) produced a significant reduction of AChE enzyme activity. In the treatment group, the animals treated with HAECL at 200 mg/kg p.o and 400 mg/kg p.o. produced a significant reduction of AChE enzyme activity and as compared to positive control. Percentage inhibition of AChE activity of treatment group has shown a significant increase when compared to positive control. The extract also showed a

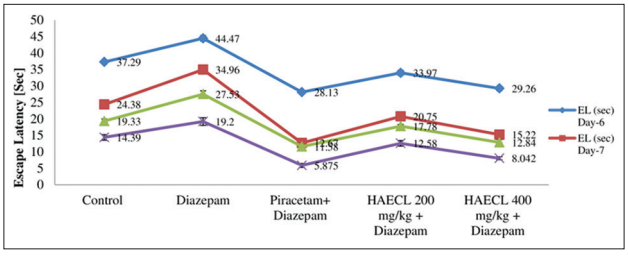

Figure 1: Effect of oral administration of hydroalcoholic extract of *Curcuma longa* leaves, piracetam and i.p. administration of diazepam on escape latency of mice using Morris water maze (n=8).

significant increase in the GSH levels as compared to positive control. The activity, percentage inhibition of AChE, and levels of GSH are shown in Table 1.

Scopolamine induced amnesia using elevated plus maze in rats

In the standard group, the animals treated with piracetam (200 mg/kg p.o.) produced a significant decrease in TLT. In the treatment group, the animals treated with HAECL at 200 mg/kg p.o. and 400 mg/kg p.o. produced significant decrease in TLT as compared to positive control. After 24 hrs, the TLT of HAECL was graphically represented in Figure 3.

Effect of oral administration of HAECL, piracetam and i.p. administration of scopolamine on AChE activity and GSH levels in rat brain

In the standard group, the animals treated with piracetam (200 mg/kg p.o.) produced a significant reduction of AChE enzyme activity. In the treatment group, the animals treated with HAECL at 200 mg/kg p.o. and 400 mg/kg p.o. produced a significant reduction of AChE enzyme activity and as compared to positive control. Percentage inhibition of AChE activity of treatment group has shown a significant increase when compared to positive control. The extract also showed a significant increase in the GSH levels as compared to positive control. The activity, percentage of inhibition, and GSH levels are shown in Table 2.

DISSCUSSION

In the present study, we have focused on exploring the potential of HAECL for its efficacy in reversing the memory deficits and improving acquisition and memory retention in diazepam-induced amnesia in mice by Morris water maze and scopolamine-induced amnesia in rats by elevated plus maze models.

ELT and TSTQ were the parameters assessed in diazepam-induced amnesia in mice using Morris water maze apparatus. The animals were trained to find the hidden platform for a period of 7 days. The animals were treated for a period of 10 days and ELT was observed and recorded on 6th, 7th, 8th, and 9th day and TSTQ was also observed and recorded on 10th day.

The dose of 400 mg/kg of HAECL has produced significant effect as seen in decreased ELT (8.042±0.44) on 9th day and increased TSTQ (23.74±0.87) on 10th day than 200 mg/kg as (EL - 12.58±0.84 and TSTQ - 17.43±0.66) as compared

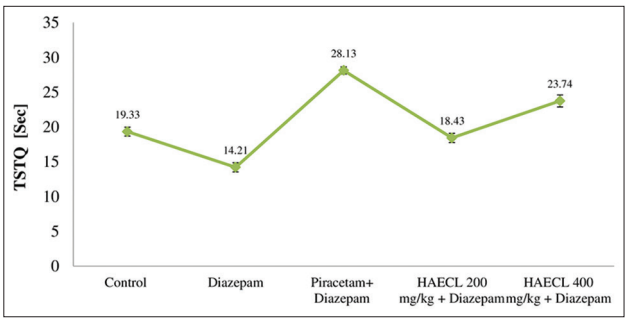

Figure 2: Effect of oral administration of hydroalcoholic extract of *Curcuma longa* leaves, piracetam and i.p. administration of diazepam on time spent in target quadrant of mice using Morris water maze (n=8).

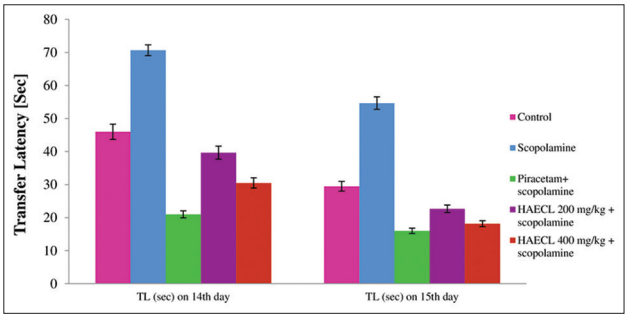

Figure 3: Effect of oral administration hydroalcoholic extract of *Curcuma longa* leaves, piracetam and i.p. administration of scopolamine on transfer latency of rats using elevated plus maze (n=8).

Table 1: Effect of oral administration of HAECL, piracetam and i.p. administration of diazepam on AChE activity and GSH levels in mice brain.

Treatments	Dose $(kg)^{-1}$	AChE concentrated (µMol/minute/g of tissue)	Inhibition of AChE activity (%)	GSH absorbance
Control	1 ml p.o.	6.742±0.18		1.009±0.036
Diazepam	1 mg (i.p.)	10.39±0.35	35.11	0.2043±0.015
Piracetam+diazepam	200 mg p.o.+1 mg (i.p.)	3.968±0.19	61.8	0.7155±0.031***
HAECL+diazepam	200 mg p.o.+1 mg (i.p.)	5.683±0.28	45.30	0.4142±0.028***
HAECL+diazepam	400 mg p.o.+1 mg (i.p.)	4.967±0.31	52.19	0.5760±0.034***

Values were expressed as mean±SEM. The statistical difference in mean will be analyzed using one-way ANOVA followed by Tukey's multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 compared with diazepam-induced amnesia. HAECL: Hydroalcoholic extract of *Curcuma longa* leaves, GSH: Glutathione, AChE: Acetylcholinesterase

Table 2: Effect of oral administration of HAECL, piracetam and i.p. administration of scopolamine on AChE activity and GSH levels in rat brain.

Treatments	Dose $(kg)^{-1}$	AChE concentration (µMol/minute/g of tissue)	Inhibition of AChE activity (%)	GSH absorbance
Control	1 ml p.o.	8.813±0.16		1.40±0.045
Scopolamine	1 mg (i.p.)	12.12±0.18	27.28	0.17±0.021
Piracetam+scopolamine	200 mg p.o.+1 mg (i.p.)	5.968±0.11***	49.38	1.12±0.036***
HAECL+scopolamine	200 mg p.o.+1 mg (i.p.)	7.717±0.27***	36.32	0.40±0.0096**
HAECL+scopolamine	400 mg p.o.+1 mg (i.p.)	6.967±0.31***	42.51	0.95±0.027***

Values were expressed as mean±SEM. The Statistical difference in mean will be analysed using one-way ANOVA followed by Tukey's multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 compared with scopolamine-induced amnesia, HAECL: Hydroalcoholic extract of *Curcuma longa* leaves, AChE: Acetylcholinesterase, GSH: Glutathione

to standard (EL - 5.875±0.60 and TSTQ - 28.13±0.52) and control (EL - 19.20±1.1 and TSTQ - 14.21±0.66) as shown in Figures 1 and 2.

The screening of nootropic effect of HACEL was carried out by using diazepam-induced amnesic model in mice by estimating AChE concentration and its inhibitory activity. The 400 mg/kg of HAECL has shown significant decrease in concentration of AChE (4.967±0.31) and increase in activity (52.19%) than 200 mg/kg of HAECL with concentrated - not required (5.683±0.28) and activity (45.30%) compared to diazepam positive control group concentrated (10.39±0.35) and activity (35.11%) as shown in Table 1.

Diazepam treated animals decreased the levels of GSH (0.2043±0.01) pre-treatment with different doses of HAECL (200 and 400 mg/kg p.o.) and piracetam (200 mg/kg p.o.) significantly elevated the levels of GSH (0.4142±0.02, 0.5760±0.03, and 0.7155±0.03) indicating that pre-treatment reduced the GSH depletion as shown in Table 2.

Diazepam produces memory loss in brain cell of mice by increasing the GABAergic inhibitory facilitation and or by causing oxidative stress by releasing free radicals in the brain cavity. GSH plays an essential role in protecting brain from free radical release by neutralizing these free radicals. The damage by free radicals to brain tissue is associated with neurodegenerative disorders. The potential effect of HAECL in enhancing the brain memory in terms of increasing the 43- concentration of ACh of Ach by decreasing the AChE enzyme, which helps in enhancing the cholinergic transmission and improving the nootropic effect. The effect may also responsible for its antioxidant property of plant because of plant extract comprising of essential phytoconstituents like flavonoids, tannins which inhibit lipid peroxidation.

TLT was the parameter assessed in scopolamine-induced amnesic rats by Elevated plus maze paradigm. TLT of 1st day reflected learning behavior of animals whereas, TLT of 2nd day reflected retention of information or memory.

The dose of 400 mg/kg of HAECL has produced significance in learning behavior and retention of memory (30.50±1.1 and 18.17±0.47) than 200 mg/kg (39.67±2.3 and 24.67±0.88) as seen in decreased TLT on 14th and 15th day as compared to standard (21.00±1.2 and 16.00±0.57) and control (70.67±1.6 and 54.67±1.8) as shown in Figure 3.

The screening of nootropic effect of HACEL was carried out by using scopolamine-induced amnesic model in rats by estimating AChE concentration and its inhibitory activity. The 400 mg/kg of HAECL has shown significant decrease in concentration of AChE (6.967±0.31) and increase in activity (42.51%) than 200 mg/kg of HAECL with concentrated - not required (7.717±0.27) and activity (36.32%) compared to scopolamine positive control group concentrated - not required (12.12±0.35) and activity (27.28%) as shown in Table 2.

Scopolamine treated animals decreased the levels of GSH (0.1755±0.02) pre-treatment with different doses of HAECL (200 and 400 mg/kg p.o.) and piracetam (200 mg/kg p.o.) significantly elevated the levels of GSH (0.4058±0.009, 0.9543±0.02, and 1.121±0.03) indicating that pre-treatment reduced the GSH depletion as shown in Table 2.

Ach is the most important neurotransmitter involved in the regulation of cognitive function. Scopolamine, a muscarinic antagonist readily crosses blood-brain barrier and blocks Ach receptors at synapse which impair memory and acquisition. GSH plays an essential role in protecting the brain from free radical release by neutralizing these free radicals. The damage by free radicals to brain tissue is associated with neurodegenerative disorders. The potential effect of HAECL in enhancing the brain memory in terms of increasing the concentration of Ach by decreasing the AChE enzyme, which helps in enhancing the cholinergic transmission and improving the nootropic effect. The effect may also responsible for its antioxidant property of plant because of plant extract comprising of essential phytoconstituents like flavonoids, tannins which inhibit lipid peroxidation.

CONCLUSION

The present study was taken up to evaluate HAECL memory enhancing activity.

The results of acute toxicity study suggested that the extract was safe up to 2000 mg/kg p.o. Pharmacological evaluations were conducted at 200 and 400 mg/kg p.o. Results of preliminary phytochemical tests indicated the presence of flavonoids, steroids, and tannins.

The HAECL at a dose of 400 mg/kg p.o. reversed the diazepam and scopolamine-induced amnesia in mice and rats and produced memory enhancing activity this was evident by decrease in TLT and increase in EL in elevated plus maze and Morris water maze behavioral paradigm, respectively.

The possible mechanism of nootropic activity may be due to the presence of essential phytoconstituents (phenolics, steroids, and flavonoids) present in HAECL and its antioxidant property.

The HAECL was subjected for preclinical evaluation using rats and mice; there is scope for clinical studies to validate further detailed study in human volunteers to find out the more detailed mechanism of action of this plant extract.

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