Treatment with *Centalla asiatica* (Linn) fresh leaf extract enhances learning ability and memory retention power in rats

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ABSTRACT

Objectives: To investigate the role of *Centella asiatica* (CeA) fresh leaf extract treatment on the behavior, especially learning and memory, of adult rats.

Methods: Adult rats (2.5 months old) were fed with 2, 4, and 6 ml/kg body of fresh leaf extract of CeA for 2, 4, and 6 weeks. After the treatment period the rats were subjected to spatial learning (T-Maze) and passive avoidance tests along with age matched normal and saline control rats. The data were compared with those of age matched control rats. The study was conducted at the Melaka Manipal Medical College, Manipal Academy of Higher Education, Manipal, Karnataka, India between December 2001 and February 2005.

Results: The rats treated with a higher dose (6 ml) of CeA showed improvement in spatial learning performance, namely, increased (p<0.001) number of alternations and decreased (p<0.001) percentage bias during spontaneous alternation test and increased (p<0.001) percentage bias during rewarded alternation test. They also showed enhanced memory retention power, namely, less (p<0.001) time spent in the small compartment during the retention period of passive avoidance test.

Conclusion: This indicates that treatment with higher doses of CeA fresh leaf extract enhances learning ability and memory retention power in adult rats.

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Various types of traditional medicines are being used throughout the world. Ayurveda is one of the ancient medicinal systems, predominantly practiced in India as an alternate system of medicine. In Ayurveda, several plants are being used for the treatment of a variety of diseases including diseases of the nervous system. Among the many groups of medicines, which act on the nervous system, "Medhya rasayana" is a well-known mixture of many plant extracts. It mainly contains the extracts from plants such as *Centella asiatica* (CeA), *Acorus calamus, Jatamansi,* and *Baccopa monnieri.* The medhya rasayana has been claimed to improve mental ability.[1] *Centella asiatica* grows as an herb, in wet places throughout India and other South Asian countries. In Ayurvedic preparations, CeA will be used either as whole plant, or leaves in fresh or extract form.[1] The learning and memory enhancing properties of CeA have been well documented.[2-4] Additionally, treatment with CeA in mentally retarded children has been shown to improve general mental ability.[3,5-7] It has also been used in people suffering from cognitive disorders.[3,5-7] Though the extract of CeA has been claimed to improve learning and memory in different clinical studies,[2,3,5,6] there is no evidence to show the effect of this plant extract on improvement of behavior, especially learning and memory in adult rats. We hypothesize that treatment with fresh leaf extract of CeA will bring about behavioral changes, especially in learning and memory in adult rats. Thus, this study was designed to investigate the effect of different doses of CeA fresh leaves extract treatment for different durations on learning and memory in adult rats. In this study, we aimed to conduct the experiment in the same way as explained in the classic texts of Ayurveda,[1] namely, without extraction, but using fresh leaf extract.

Methods. The present study was conducted in the Department of Anatomy, Melaka Manipal Medical College, Manipal University, Manipal, Karnataka, India. The experiment was started in December 2001 and was completed in February 2005. Prior approval was taken from the institutional animal ethical committee before the commencement of the experiment. The experimental animals used in this study were 2.5-month-old adult Wistar rats of both genders. These rats were bred and maintained in the

central research animal house of the institution. They were fed with food and water ad libitum and maintained in 12:12 hour dark and light cycle with access to food and water in excess. The temperature of the animal house was kept constant at 25°C. The minimum number of rats was used and handled in a humane way to generate significant data. The experimental rats were divided into 2, 4, and 6-week treatment groups. Rats belonging to each of these groups were divided into 2ml/kg (CeA 2ml), 4ml/kg (CeA 4ml), and 6ml/kg (CeA 6ml) body weight dose group (n=8 for each dose). The rats were fed, through gastric intubation with a given amount of fresh leaf extract of CeA daily for 2, 4, or 6 weeks. Experimental groups were accompanied by normal control group (NC) and saline (vehicle) control group (SC) (n=8 in both groups). After identification of the plant, a voucher specimen number "525PP" was assigned and entered in the registry in the Department of Pharmocognosy, Manipal College of Pharmaceutical Sciences, Manipal, India. In the present experiment, we used CeA plant extract from the leaves collected from the plants cultivated in uniform soil conditions, in order to maintain the same plant source throughout the experiment. The fresh leaf juice was extracted as explained in a previous publication.[8] Briefly, fresh, 15-20 days mature leaves of CeA were collected in the morning. Fresh leaf juice was extracted from these leaves after washing, air drying, and homogenizing in a glass vessel, and finally filtered through a sterile gauge cloth. The fresh leaf extract was fed to the rats through a gastric tube, a capillary tube attached to a 1ml hypodermic syringe. The control rats remained undisturbed in their home cage, and the saline control rats were fed with a volume of saline equivalent to the volume of extract that their age matched experimental rats received on each day. As explained in the earlier paper,[8] the standard extraction procedure, which involves boiling in water, ethyl alcohol, or other organic solvents, may alter the structure of bioactive components; hence, we opted to avoid standard extraction protocols. We accept that there may be minor variation in daily preparations; which will be minimal as leaves of equal maturation are collected from the same place on all days. However, this minor daily variation will be compensated by the long period (2, 4, and 6 weeks) of treatment. Soumyanath et al,[9] showed that a *Centella asiatica* plant extract obtained from ethanol extraction, is different from water extraction in its biological activity.

Behavioral tests. Rats in all the groups (CeA, NC, and SC) were subjected to spatial learning (T- Maze) test and passive avoidance test after the treatment period.

Spatial learning (T-maze) tests. The spatial learning ability of the rats was assessed in this test. This test included spontaneous alternation and rewarded alternation tests. Detailed methodology of the spatial learning test is given in our earlier report.[8]

Spontaneous alternation test.[8,10] The rats were starved for 48 hours in order to motivate them for the food reward. Subsequently, the rat's body weight was maintained at 85% of pre-test weight. The rats were oriented to the maze to familiarize them with the T-maze. During this, the rats were placed in the start box for 60 seconds. The sliding door was then opened to allow the rat to explore the T maze for 30 minutes, and to eat all (15) pellets (10 mg each) in each goal area. Rats will be returned to the start box and procedure was repeated once again. The orientation sessions are followed by 6 daily trails for the next 4 days. In each trial, the rat was first placed in the start box and by opening the sliding door it was allowed to enter the stem and allowed to choose any of the arms. When a rat enters a particular arm with all 4 limbs it will be deemed to have entered that arm. As soon as the rat ate the pellet in the goal area of a given arm, it was replaced back in the start box for the next trial. The trail was repeated with an inter-trial interval of one minute. The arm chosen by the rat in each trail was noted. At the end of test days (namely, 24 trials in 4 days), the total number of alternations for each rat was noted. The percentage bias was calculated for each rat using the following formula. Percentage bias = total number of choices of more frequently chosen side x 100 / total number of trials. A higher number of alternations and less percentage bias was considered as an index for improved learning ability.

Rewarded alternation test.[8,10] After completion of the spontaneous alternation test, rats were subjected to rewarded alternation test. During this test, rats were subjected to 6 trials on each day for 4 days. Each trial had 2 runs, a forced run and a choice run. In the forced run, the rat was forced to one of the arms by blocking the other arm and was allowed to consume the pellet in that goal area. The rat was placed back into the start box, immediately after it had consumed the pellet in the goal area for a choice run. In this choice run, the goal area of the forced arm was kept empty and pellets were placed in the goal area of the opposite arm. Both the arms were free for the rat to choose. Each forced run and the choice run were separated by an interval of one minute. The inter trail interval was also one minute. The sequence of the forced arm was predetermined and was the same for all the rats for a given day. During the choice run, if the rat entered the arm opposite to the forced arm, then that response was considered as "correct response." If it entered the same arm to which it was forced during the forced run, it was considered as a "wrong response." Using the following formula, the percentage of correct responses was calculated for each

rat as follows: percentage of correct responses = total number of correct responses x 100 / total number of trials. An increase in the percentage of correct response was considered as an index of improved learning and memory.

Passive avoidance test (modified from Bures et al).[8,11] The passive avoidance test was carried out as detailed in our previous report.[8] Briefly, the behavioral experiment included 3 parts, a. exploration test, b. aversive stimulation, and learning (passive avoidance acquisition), and c. retention test. During the exploration test, the door between the 2 compartments was kept open. The rat was allowed to explore the apparatus (both larger and smaller compartments) for 3 minutes. Total time spent by the animal in the smaller compartment in each trail was noted. At the end, the rat was replaced in the home cage, where it remained during the inter-trial interval of 5 minutes. After the last exploration trial, the rat was placed in the smaller compartment and the door between the 2 compartments was closed. Foot shocks (3 shocks, 50Hz, 1.5mA, one second duration) were given at 5-second intervals. The door was then opened, and the rat was returned to its home cage. The retention test was carried out 24 hours after foot shock. The rat was placed in the center of the larger compartment facing away from the entrance to the smaller compartment, and the sliding door between the 2 compartments was open. The rat was allowed to explore the larger and smaller compartments for 3 minutes. After 3 minutes the rat was replaced back in the home cage. The trial was repeated 3 times with an inter trail interval of 5 minutes. In each trial, the time spent in the smaller compartment was noted. A decrease in the time spent in the smaller compartment during retention test was considered as good memory retention performance.

Data analysis. Data were analyzed using analysis of variance (ANOVA) followed by Bonferroni's post test using Graph Pad in Stat (GPIS) software, version 1.13.

Results. The rats treated with all the doses of CeA remained healthy throughout the treatment period. They gained better body weight than that of control and saline treated rats (data not shown).

Results of 6 weeks treatment group. Spatial learning (T-Maze tests). During spontaneous alternation test, only the rats treated with 6 ml of CeA showed a significant increase in the number of alternations (14.33 ± 2.94 in normal control versus 21.85 ± 1.67 in CeA 6 ml group, [52.5% increase] $p<0.001$) (**Figure 1**). They also showed significantly less percentage bias in comparison with the normal control group (64.85 ± 3.13 in normal control versus 43.37 ± 2.22 in CeA 6 ml group, [33.1% decrease] $p<0.001$) (**Figure 2**). During rewarded alternation test, rats treated with 2 and 4 ml of CeA extract showed no significant difference in the percentage of correct responses when compared to the normal control group. Rats treated with 6 ml of CeA extract showed a significant increase in percentage of correct response when compared to the normal control group (59.57 ± 7.79 in normal control versus 94.64 ± 7.1 in the CeA 6 ml group, [58.9% increase] $p<0.001$) (**Figure 3**).

Passive avoidance test. Total time spent in small compartment. During exploration, there was no significant difference between the animals treated with CeA extract (2, 4, and 6ml) and normal control animals. However, during the retention test, the animals treated with 6ml CeA extract spent significantly less time in the small compartment (111.66 ± 32.12 seconds in the normal control versus 9.57 ± 3.52 seconds in the CeA 6ml group, [91.4% decrease] $p<0.001$) (**Figure 4**).

Total number of crossings. During the time of exploration there was no significant difference between the rats treated with different doses of CeA (2, 4, and 6 ml) and the rats of normal control group in total number of crossings. However, the number of crossings was significantly decreased in the 6 ml CeA treated animals during retention test (3.33 ± 1.03 in the normal control group versus 0.85 ± 0.21 in the CeA 6 ml group, [74.8% decrease] $p<0.001$) (**Figure 5**).

Results of 2 and 4 weeks treatment group. There was no significant change in the results of the spontaneous alternation, rewarded alternation, and passive avoidance tests in rats treated with CeA for 2 and 4 weeks in any (2, 4, and 6 ml/kg) of the dose groups.

Discussion. During spontaneous alternation test, rats treated with a higher dose (6 ml/kg body weight) of CeA fresh leaf extract for a longer duration (6 weeks) showed a 52.5% increase in number of alternations, and a 33.1% decrease in the percentage bias. Similarly, during rewarded alternation tests they showed a 58.9% increase in percentage of correct response. These results clearly indicate the improved learning behavior in these rats. During passive avoidance retention test, these rats also spent 91.4% less time in the small compartment and showed a 74.5% decrease in the number of crossings indicating improved memory retention power. However, rats treated with lower doses (2 and 4 ml) for a longer duration (6 weeks) did not show significant change in their behavior. Similar performance was observed in all dose groups (2, 4, and 6 ml) treated with CeA fresh leaf extract for a shorter duration (2 and 4 weeks).

These results clearly indicate that oral administration of fresh leaf extract of CeA improved learning and memory in adult rats. This effect was marked in animals treated with higher doses of CeA. The memory enhancing effect of CeA fresh leaf extract in neonatal rats

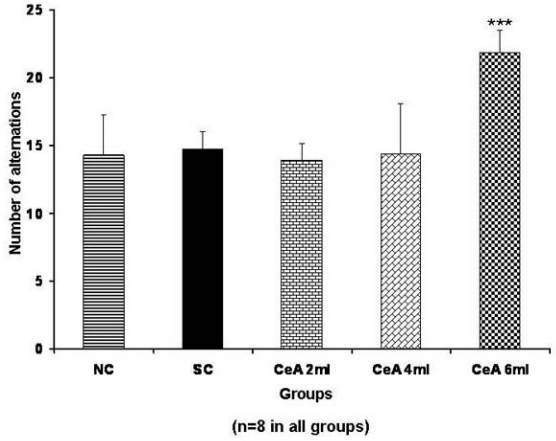

Figure 1 - Number of alternations during spontaneous alternation test in rats treated with 2, 4, and 6 ml/day/kg body weight of CeA for 6 weeks and age matched control and saline treated rats. Each bar represents mean ± standard deviation, F-value - 15.97.

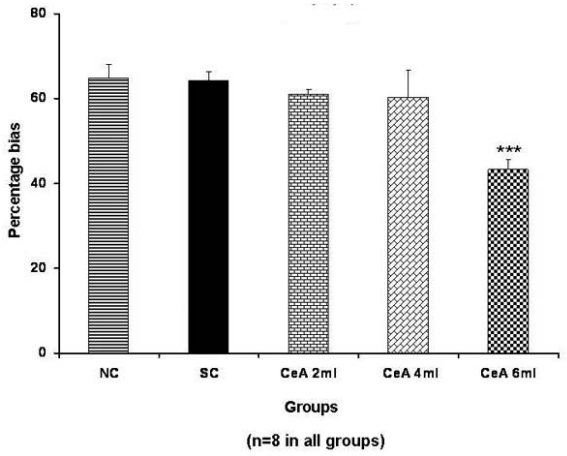

Figure 2 - Percentage bias shown by rats treated with 2, 4, and 6 ml/day/kg body weight of CeA for 6 weeks and age matched control and saline treated rats during spontaneous alternation test. Each bar represents mean ± standard deviation, F-value: 48.25.

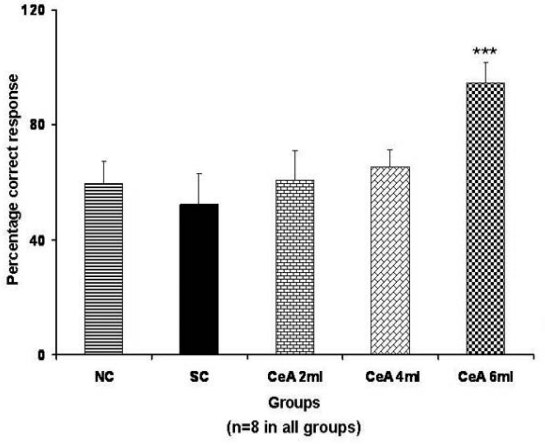

Figure 3 - Percentage of correct response shown by rats treated with 2, 4, and 6 ml/day/kg body weight of CeA for 6 weeks and age matched control and saline treated rats during rewarded alternation test. Each bar represents mean ± standard deviation, F-value: 29.79.

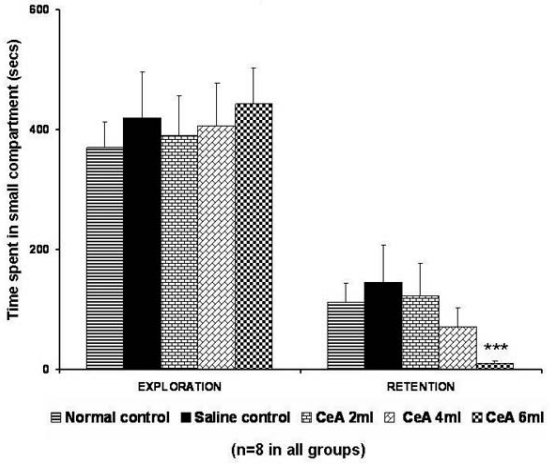

Figure 4 - Total time spent in small compartment by the rats treated with 2, 4, and 6 ml/day/kg body weight of CeA for 6 weeks and age matched control and saline treated rats during passive avoidance test. Each bar represents mean ± standard deviation, F-value: 1.49 for exploration test and 12.77 for retention test.

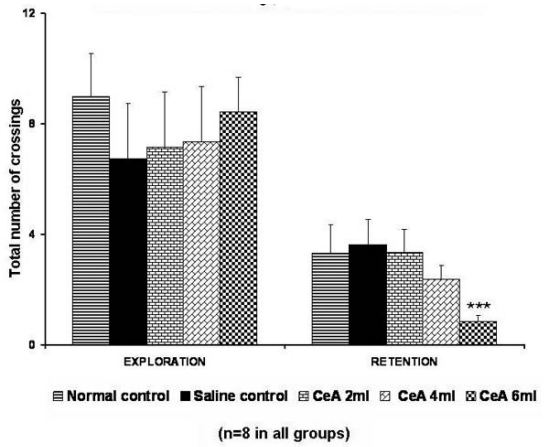

Figure 5 - Number of crossings by the rats treated with 2, 4, and 6 ml/day/kg body weight of CeA for 6 weeks and age matched control and saline treated rats during passive avoidance test. Each bar represents mean ± standard deviation, F-value: 1.52 for exploration test and 18.3 for retention test.

has been reported before.[8] The use of CeA in preventing radiation induced behavioral changes during clinical radiotherapy has been reported earlier.[12] Asiatic acid, a triterpene of CeA is used in the treatment of dementia and as an enhancer of cognition. Three derivatives obtained from CeA are found to be efficacious in protecting neurons from oxidative damage caused by exposure to excess glutamate.[13] An aqueous extract of CeA has an enhancing effect on cognitive functions.[14] Centella asiatica is also reported to improve general mental ability and behavioral pattern in mentally retarded children.[6-8] Nalini et al[15] reported the memory enhancing effect of aqueous extract of CeA in adult rats. However, the results of the present study are the first experimental evidence regarding the memory enhancing property of CeA fresh leaf extract in adult rats.

Treatment with *Clitoria ternatea* root extract has been shown to enhance memory in neonatal rats.[16] The exposure to the new learning experiences,[17] intracranial self stimulation,[18] and living in an enriched environment,[19-21] has been shown to alter the cyto-architecture of the hippocampus, which is a part of the brain concerned with learning and memory. Similarly, fresh leaf extract of CeA has been shown to improve dendritic arborization of hippocampal CA3 neurorns.[22] Improved learning behavior and enhanced memory retention in the present study is probably because of the structural changes in these brain regions.[17,23,24]

It has been observed that CeA treatment increases the level of neurotransmitter GABA that is known to act on the hippocampus.[25,26] Similarly, CeA may also affect the biosynthesis of other neurotransmitters involved in learning and memory such as Ach, noradrenaline, 5HT, and dopamine.[27-29] However, these morphological, neurophysiological, and neurochemical changes need to be investigated further.

We conclude by saying that oral administration of higher doses of CeA fresh leaf extract in adult rats improves learning ability and enhances their memory retention power, which is probably due to the structural, neurochemical, and neurophysiological changes induced by CeA in the brains of these rats.

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