

CLITORIA TERNATEA (Linn) ROOT EXTRACT TREATMENT DURING GROWTH SPURT PERIOD ENHANCES LEARNING AND MEMORY IN RATS

KIRANMAI S. RAI*, K. DILIP MURTHY**, K. S. KARANTH*** AND MUDDANNA S. RAO****

Departments of Physiology*, Anatomy****,
K. M. C. Manipal - 576 119,

Department of Physiology**,
I. C. H. S Manipal - 576 119

and

Department of Pharmacology***,
K. S. Hegde Medical Academy,
Deralkatte, Mangalore - 575 001

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Abstract : Neonatal rat pups (7 days old) were intubated with either 50 mg/kg body weight or 100 mg/kg body weight of aqueous root extract of *Clitoria ternatea* (CTR) for 30 days. These rats were then subjected to open field, two compartment passive avoidance and spatial learning (T-Maze) tests (i) immediately after the treatment and (ii) 30 days after the treatment, along with age matched normal and saline control rats. Results showed no change in open field behaviour, but showed improved retention and spatial learning performance at both time points of behavioural tests, indicating the memory enhancing property of CTR which implicates a permanent change in the brain of CTR treated rats.

Key words : *Clitoria ternatea*
spatial learning-T maze

passive avoidance open field
learning and memory

INTRODUCTION

In the Ayurvedic system of medicine "Medhya drugs" -a group of herbal medicines are known for their actions on the nervous system. These "Medhya drugs" mentioned in Ayurvedic texts are said to improve mental abilities

(1). Some of these herbal drugs reported to act on the nervous system include-*Clitoria ternatea* (2, 3), *Acorus calamus*, *Centella asiatica* (3, 4, 5), *Withania somnifera*, *Celastrus panniculatus*, *Guduchi* and *Areca*. "Medhya rasayana" a rejuvenating recipe containing *Clitoria ternatea* is considered wholesome for intellect (6).

*Corresponding Author

Clitoria ternatea (commonly called Shanka pushpi) is a twining herb with white flowers resembling a conch-shell (2, 3). Extracts of *Clitoria ternatea* has been used since time immemorial to treat mental disorders since it has property of being a good nervine tonic (3). Our preliminary study on *Clitoria ternatea* root extract showed a memory enhancing property in rats (7). Though *Clitoria ternatea* root extract is claimed to improve learning and memory (3) there are no experimental or clinical studies to show that this herb affects learning and memory. Thus, this study was designed to find the effect of *Clitoria ternatea* root extract treatment on the general behaviour and learning and memory in rats. Further, our aim was also to study, whether behavioural changes occurring in these experimental rats were transient or permanent. The present experiment was designed on neonatal rat pups, since in rats, active brain growth occurs during pre weaning and post weaning period (growth spurt period, 8).

METHODS

Aqueous root extraction

Fresh roots of *Clitoria ternatea* were collected, cleaned, cut into small pieces and sunshade dried. It was then hand powdered. Dry powder was weighed and mixed with distilled water at 1:10 ratio and boiled over a low flame for half an hour. It was then cooled and decanted. Residue was mixed with distilled water (1:10) and boiled for 30 minutes, cooled and decanted. The above procedure was repeated twice. The clear supernatant obtained each time was decanted and then centrifuged (3000 rpm

for 5 minutes) and the supernatant was evaporated on low flame, to get a thick paste like extract, which was later dried in an incubator at 37° C and the dry powder so obtained was stored in a dessicator.

Animals and experimental groups

Wistar rats provided with food and water *ad libitum*, maintained under 12 hours dark and 12 hours light cycle were used for the experiments. Seven days old neonatal rat pups were divided into three groups. 1) Normal control (NC) - These rats remained undisturbed in their home cage for 30 days. 2) Saline control (SC) - These rats received orally, a known volume of saline each day for 30 days (period of intubation). 3) *Clitoria ternatea* root extract (CTR) treated group of rats, were divided into two subgroups, receiving orally, either CTR 50 mg/kg or CTR-100 mg/kg, respectively, every day, for 30 days.

Behavioural tests

All four groups of rats were subjected to the following tests- a) open field behavioural test, b) two compartment passive avoidance test and c) spatial learning test (T-Maze)-commencing from postnatal day 38 (i.e. a day after the last dose, early test). These rats were later once again subjected to the same tests on postnatal day 68 (i.e. 30 days after the last dose, delayed test) in order to test whether the behavioural changes, if any, is transient or permanent.

a) Open field behaviour test

This test was carried out as explained by Buresova O and Bures J (9). Open field

behavioural test apparatus consisted of a large wooden box 100x100 cm with 40 cm high walls. The floor consisted of a black painted wire mesh grid dividing the field into 25 equal squares 5 x 5 cm with 16 peripheral squares (PS) close to the wall and a central squares (CS). A 100 watts bulb was placed centrally, 5 feet above, for bright illumination.

The rat was placed in a corner of the apparatus and was allowed to explore the apparatus for 5 minutes. During this period the number of peripheral and central squares entered by the rat and the number of boli of excreta excreted were counted. Incidences of expression of rearing grooming/preening behaviours were also noted. Increase in the number of peripheral squares entered and more time spent in the peripheral area was considered as an increase in motor activity. Increased number of central squares entered and more time spent in the centre of the field indicates decreased fear and anxiety and emotional disturbance. Increased number of grooming (10), number of boli of excreta (9) and decreased rearing (10) are the expressions of emotionality, which are measures of autonomic function in the animals.

b) Passive avoidance test

Modified procedure of Buresova O and Bures J (9) was adopted.

The passive avoidance apparatus consisted of a box/compartment with 50 x 50 cm grid floor and wooden walls of 35 cm height. A 100 watts bulb placed 5 feet above, illuminated this box. A smaller dark compartment 15x15x15 cm, with electrified

grid floor, connected to a constant current stimulator, opened into this box on one side. A 6x6cm opening in the wall between the two boxes/compartments could be closed using a transparent plexiglass sliding door.

On the first day of the test, each rat was allowed to explore the two compartment for 5 minutes. On the second day latency to enter the dark compartment for the first time was noted for each rat. The learning session was followed immediately. The plexiglass door between the two compartments was closed and the rat was confined to the dark compartment. Three inescapable electric foot shocks (50 Hz, 1.5 mA, 1 sec) were delivered to the rat. The rat was then returned to its home cage. Retention performance of each rat was tested by noting the latency to enter the dark compartment after a period of 48 hours and 30 days respectively. Increase in the latency to enter the dark compartment during retention test (i.e. 48 hrs/30 days) after inescapable foot shock, was interpreted as good retention performance.

c) Spatial learning test (T-Maze test, 11)

To assess the spatial learning ability, rats were subjected to spontaneous alternation and rewarded alternation tests.

The T-Maze consisted of a start box 15x12 cm, a stem 35x12 cm, a choice area 15x12 cm and two arms 35x12 cm each, at the end of which were the goal areas 15x12 cm each, containing the food pellets. The side walls were 40 cm in height. A sliding door separated the stem, from the start box. The T-maze was kept in a sound attenuated room.

i) Spontaneous alternation test

Rats were starved for two days prior to the test in order to motivate them for food reward. Subsequently food was restricted so that the body weight was maintained at 85% of pretest weight.

Rats were placed in the T-maze for 30 minutes daily, for 2 days, to orient them to the T-maze environment. During these sessions 15 pellets of food (10 mg each) were kept in each goal area.

On the following 4 days, six trials were given daily. In each trial, the rat was placed in the start box and the door opened, thus allowing it to enter into the stem and arms of the T-maze. After the rat ate the pellet in the goal area, it was replaced back in the start box. In each trial, the arm chosen by the rat and number of alternations made, were noted. The inter-trial interval was one minute. The rat was deemed to have entered into a particular arm when it entered that arm with all its four limbs. Percentage bias was calculated for each rat using the following formula:

$$\% \text{ bias} = \frac{\text{Total number of choices of more frequently chosen side} \times 100}{\text{Total number of trials}}$$

More number of alternations and less % bias was considered as an index of improved learning ability.

ii) Rewarded alternation test

This test was done after completion of spontaneous alternation test. Test consisted

of 6 trials/day, for four consecutive days. Each trial had two runs viz. forced run and choice run. In the forced run, the rat was forced to one of the arms by blocking the other arm and allowing it to consume the pellet there. In the choice run, the forced arm was kept empty and pellet was placed in the opposite arm. Both the arms were free for the rat to run. Now, the rat had to enter into the arm, opposite, to the forced arm, if it had to be considered as "correct response". The forced arm was predetermined and it was same for all rats on any given day. It was changed on subsequent days. Experiment was repeated on 4 successive days. "Percentage of correct responses" was calculated for each rat by using the following formula:

$$\% \text{ correct response} = \frac{\text{Total number of choices of correct response} \times 100}{\text{Total number of trials}}$$

Increase in mean % correct response was considered as improved learning and memory.

Statistics

Data obtained from the above tests were analysed by applying One Way ANOVA followed by post test (Dunnet test).

RESULTS

a) Open field test

Results of open field behaviour is shown in Table I and II. There was no statistical difference in any of the parameters studied either immediately after the treatment or 30 days after the treatment.

TABLE I: Open field test performance.

Groups	Peripheral Area				Central Area			
	No. of squares entered		Time spent (sec)		No. of squares entered		Time spent (sec)	
	Early	Delayed	Early	Delayed	Early	Delayed	Early	Delayed
NC n=17	100.71±4.66	49.38±6.75	290.48±1.4	292.79±3.3	11.41±1.11	5.63±1.75	9.41±1.43	5.96±3.33
SC n=24	95.79±3.26	78.25±9.18	274.13±4.89	288.9±2.72	13.42±1.14	14.13±1.99	25.87±4.86	11.1±2.72
CTR50 n=20	90.45±5.5	74.67±7.43	289.99±1.12	291.37±1.76	11.85±1.12	11.44±2.41	10.01±1.12	8.63±1.76
CTR100 n=22	92.36±2.91	67.8±7.74	291.39±0.75	282.94±2.78	11.45±0.83	13.3±2.67	8.61±0.75	17.06±2.78

Open field test performance (number of squares entered and time spent) in peripheral and central area of the open field apparatus, during early (at postnatal day 38) and delayed (at postnatal day 68) tests. Each value represents Mean ± SEM. There was no statistical significance in any comparisons (ANOVA). NC= Normal Control; CTR 50/100= *Clitoria ternatea* root extract treated -50 or 100 mg/kg body weight per day

b) Passive avoidance test

Results of two compartment passive avoidance retention performance is shown in Fig. 1. The rats which received CTR100 mg/kg body weight per day showed very good memory retention after 48 hrs (77.94 ± 17.37 sec. in NC vs. 150.05 ± 7.99 sec. in CTR100, P<0.01). These rats also showed improved memory retention, even 30 days after treatment (27.78± 6.54 sec. in NC vs. 103.69±16.1 sec. in CTR100 P<0.05).

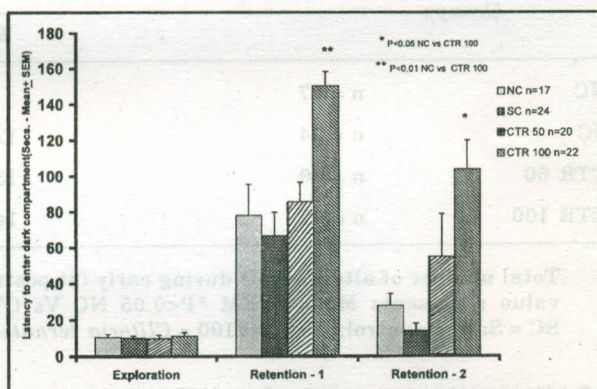


Fig. 1: Two compartment passive avoidance test performance (latency to enter dark compartment) during early and delayed post treatment periods, in controls and *Clitoria ternatea* root extract treated rats.

c) Spatial learning test (T-Maze test)

i) Spontaneous alternation test

Table III shows the number of alternations made by different groups of rats during spontaneous alternation test. There was no significant difference in total number

TABLE II: Open field test performance.

Groups	No. of rearing		No. of grooming		No. boli of Excreta	
	Early	Delayed	Early	Delayed	Early	Delayed
NC n=17	25.53±1.14	12.5±1.86	4±0.39	5±1.13	0.41±0.21	1.5±0.8
SC n=24	19.71±1.41	15.5±2.7	4.79±0.42	5.86±0.74	0.04±0.04	1.88±0.64
CTR 50 n=20	15.75±1.05	10±2.22	3.45±0.29	9.44±2.59	0.5±0.21	0±0
CTR 100 n=22	22.86±1.68	16.2±2.5	3±0.32	3.3±0.7	1.09±0.33	0.3±0.3

Open field test performance (number of rearing, grooming and number of boli excreted) in open field apparatus, during early (at postnatal day 38) and delayed (at postnatal day 68) tests. Each value represents Mean ± SEM. There was no statistical significance in any comparisons (ANOVA); NC=Normal Control; SC= Saline control; CTR 50/100=*Clitoria ternatea* root extract treated-50 or 100 mg/kg body weight per day.

TABLE III: Spontaneous alternation test-Total number of alternations.

Groups		Total no. of alternations	
		Early test	Delayed test
NC	n = 17	12±0.72	11.38±1.45
SC	n = 24	12.46±0.61	11±1.11
CTR 50	n = 20	13.55±0.67	16±0.55*
CTR 100	n = 22	14.32±0.5	12.3±0.54

Total number of alternations during early (at postnatal day 38) and delayed (at postnatal day 68) tests. Each value represents Mean ± SEM *P<0.05 NC Vs CTR 50 ANOVA, F = 5.609; (df = 82) NC = Normal Control; SC = Saline control; CTR 50/100 = *Clitoria ternatea* root extract -50 or 100 mg/kg body weight per day.

of alternations among the different groups when tested immediately after the root extract treatment. However, rats which received 50 mg CTR/kg body weight per day showed slightly improved alternation when tested 30 days after the treatment (11.38 ± 1.45 in NC vs 16.00 ± 0.55 in CTR 50, P<0.05).

Mean % bias data is shown in Fig. 2. The rats that received 100 mg CTR/kg body weight per day showed significantly less % bias as compared to normal control rats when tested immediately after the treatment (62.25 ± 1.87 in NC vs

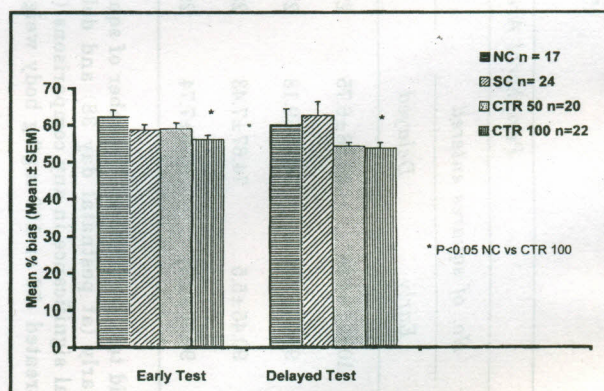


Fig. 2: Spontaneous alternation test performance (Mean % bias) during early and delayed post treatment periods, in controls and *Clitoria ternatea* root extract treated rats.

56.06 \pm 1.12 in CTR100, $P < 0.05$) and even, 30 days after treatment (61.80 \pm 5.32 in NC vs 53.64 \pm 1.46 in CTR 100, $P < 0.05$).

ii) Rewarded alternation test

Fig. 3 shows mean % correct response of various groups of rats. CTR50 as well as CTR100 groups of rats showed increased mean % correct response when tested on day 38 (67.89 \pm 3.64 in NC vs 80.7 \pm 2.45 in CTR50, $P < 0.05$, and 67.89 \pm 3.64 in NC vs 86.11 \pm 2.14 in CTR100, $P < 0.001$) The improved behaviour remained as such, even on day 68 i.e after 30 days (59.37 \pm 2.7 in NC vs 82.87 \pm 2.73 in CTR50, $P < 0.001$, and 59.37 \pm 2.7 in NC vs 83.75 \pm 3.43 in CTR100 $P < 0.001$).

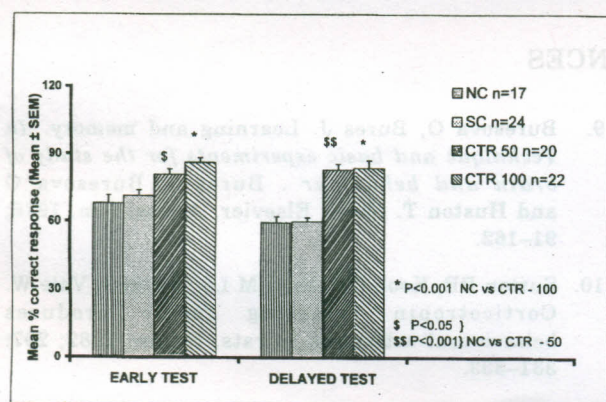


Fig. 3: Rewarded alternation test performance (Mean % correct response) during early and delayed post treatment periods, in controls and *Clitoria ternatea* root extract treated rats.

DISCUSSION

The results of the present study revealed that CTR root extract had memory enhancing properties which had no or little effect on the general motor activity and other behaviours in rats. This memory

enhancing property was marked in neonatal rats (which were in their brain growth spurt period) treated with CTR 100 mg/kg body weight for 30 days. Thus it appears that treatment with CTR extract must have certain permanent changes in the brain, which were responsible for the improved learning and memory.

This is the first report regarding, memory enhancing property of *Clitoria ternatea* aqueous root extract treatment, studied experimentally. The effect of alcoholic extract of *Clitoria ternatea* plant (i.e. stem, leaves and flowers) on the CNS has been reported (12). Treatment with alcoholic extract of *Clitoria ternatea* plant reduced spontaneous motor activity, increased sedation, diminished alertness and responsiveness to acoustic, tactile and nociceptive stimuli, and significantly prolonged time taken to traverse the water maze.

The exposure to new learning experiences (13), intracranial self stimulation (14) and environment within the pyramid model (15) has been shown to alter the cyto-architecture of brain regions concerned with learning and memory (i. e hippocampus). Fresh leaf extract of *Centella asiatica* has been shown to improve learning and memory correlated with an increase in dendritic arborization of amygdala and hippocampus (16,17). The synapse numbers in the regions of hippocampus and cingulate cortex, was shown to be affected, when the rats were chronically undernourished (18)

Emotional behavior of the rat, is altered by many factors such as toxins (19) and exposure to ultra sound (20). In the present

study there was no significant difference in number of rearing, grooming and number of boli excreted and also in the general motor activity of rats, which were treated with CTR, either immediately after the treatment or 30 days after the treatment. This suggests that CTR had no or very little effect on motor and autonomic nervous system of the rats. The treated rats appeared to be emotionally undisturbed.

Results from passive avoidance and spatial learning tests showed improved retention, increased percentage correct response and total number of alternations and decreased % bias in CTR treated rats. This suggests that CTR affects the brain structures concerned with learning and memory

namely hippocampus and amygdala (13, 21, 22). These behavioral changes are relatively permanent indicating that CTR brings about permanent changes in the brain as reported in earlier studies (13,14). It may also affect the biosynthesis of neurotransmitters like acetylcholine, which has been implicated in the process of learning and memory (23). On the other hand, CTR may also induce long term potentiation. However, these morphological, neuro-physiological and neuro-chemical changes needs to be investigated.

We conclude that, *Clitoria ternatea* aqueous root extract treatment of neonatal rats, enhances their memory, which appears to be due to permanent changes, in the brain of these rats.

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