Effect of different extracts of *Centella asiatica* on cognition and markers of oxidative stress in rats

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Abstract

*Centella asiatica*, a plant mentioned in Indian literature has been described to possess CNS effects such as stimulatory-nervine tonic, rejuvenant, sedative, tranquilizer and intelligence promoting property. In the present study aqueous, methanolic and chloroform extracts of *C. asiatica* were investigated for their effect on cognitive functions in rats. Male Wistar rats of 200–250 g were used to study the effect on learning and memory by using shuttle box, step through, step down and elevated plus maze paradigms. Only the aqueous extract of whole plant (200 mg/kg for 14 days) showed an improvement in learning and memory in both shuttle box and step through paradigms. Therefore, further experiments were conducted with aqueous extract using 100, 200 and 300 mg/kg doses in different paradigms of learning and memory. All doses of aqueous extract increased the number of avoidances in shuttle box and prolonged the step through latency in step through apparatus in a dose dependent manner, while only two doses 200 and 300 mg/kg of aqueous extract showed significant increase in the step down latency in step down apparatus and transfer latency (TL) in elevated plus maze. Among doses of aqueous extract tested on oxidative stress parameters, only 200 and 300 mg/kg showed a significant decrease in the brain levels of malondialdehyde (MDA) with simultaneous significant increase in levels of glutathione. There was a significant increase in the levels of catalase at the 300 mg/kg but no significant change in superoxide dismutase (SOD) levels were observed. The present findings indicate that the aqueous extract of *C. asiatica* has cognitive enhancing effect and an antioxidant mechanism is involved. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Centella asiatica*; Learning and memory; Active avoidance; Passive avoidance; Elevated plus maze; Antioxidant

1. Introduction

Oxidative stress due to increase in free radical generation or impaired endogenous antioxidant mechanism is an important factor that has been implicated in Alzheimer’s disease (Maxwell, 1995) and cognitive deficits seen is elderly (Cantuti et al., 2000). Thus the efforts have been directed to find therapeutic agents both synthetic compounds and the natural products that could reduce the oxidative stress and improve the memory.

In the Indian system of medicine Ayurveda, *Centella asiatica* (Umbelliferae) syn Hydrocotyl asiatica has been used in various parts of India for different ailments like headache, body ache, insanity, asthma, leprosy, ulcers, eczemas and wound healing (Chopra et al., 1956; Viala et al., 1977; Chatterjee et al., 1992; Shukla et al., 1999; Suguna et al., 1996). In course of pharmacological studies, the plant showed CNS depressant activity antitumor (Qian et al., 1982; Babu et al., 1995), and an inhibitory effect on the biosynthetic activity of fibroblast cells (Veechai et al., 1984).

The whole plant of *C. asiatica* has been shown to be beneficial in improving memory (Mukerji, 1953; Vaidyaratnam, 1994) and it is reported to improve general mental ability of mentally retarded children (Apparao et al., 1973; Kakkar, 1990). Nalini et al. (1992) have shown that fresh leaf juice improves passive avoidance task in rats. However, studies on the different extracts of the whole plant on paradigms of learning and memory are lacking. Therefore, in the present study the aqueous, methanolic and chloroform extracts of whole plant of *C. asiatica* was evaluated in different learning and memory paradigms in normal rats. Since cognitive deficits have been associated with increased oxidative stress, the effect of *C. asiatica* extracts was also studied.

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on markers of brain oxidative stress namely malondialdehyde (MDA), glutathione, superoxide dismutase (SOD) and catalase.

2. Methods

2.1. Animals

Studies were carried out using male Wistar rats weighing 200–250 g. They were obtained from the central animal house facility of All India Institute of Medical Sciences, New Delhi and stock bred in the departmental animal house. The rats were group housed in polyacrylic cages (38 × 23 × 10 cm) with not more than four animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C) with dark and light cycle (14/10 h). They were allowed free access to standard dry pellet diet (Golden Feeds, India) and tap water ad libitum. All behavioral tests were performed between 9:00 and 13:00 h. All procedures described were reviewed and approved by the Institutional committee for Ethical use of Animals.

2.2. Plant material and preparation of the extracts

Plants were procured from the commercial market at Khari Baoli, New Delhi. The samples were then authenticated for their correct botanical identity by Chief Botanist, Department of Drug Standardization, Dabur Research Foundation, Ghaziabad, India. The whole plant was dried and coarsely ground. For the preparation of aqueous extract, coarse powder of the plant was extracted with eight parts of water under boiling for 5 h and filtered to collect the extract. The extract was concentrated under vacuum and concentration was continued to completely remove the solvent, yielding a viscous liquid. The percentage w/w yields of aqueous, methanolic and chloroform extracts were 41, 12.48 and 1, respectively.

2.3. Treatment schedules

Initially to test the three extracts (aqueous, methanolic and chloroform) and the vehicle on the two paradigms shuttle box and step through apparatus the animals were divided in two sets of four groups each. In both the sets the four groups of animals were administered orally with vehicle (Tween 80–water (1:5)), aqueous extract 200, methanolic extract 200 and chloroform extract 200 mg/kg for 14 days. One set was tested on shuttle box and the number of avoidances to unconditioned stimulus (US) were noted and the other set was tested on step through apparatus and the prolongation of step through latency was determined. Of the three extracts tested aqueous extract showed a significant improvement in learning and memory in both the shuttle box and step through paradigms. Therefore, further experiments were conducted using three doses (100, 200 and 300 mg/kg) of only aqueous extract in shuttle box, step through apparatus, step down apparatus and the elevated plus maze behavioral paradigms. The rats were divided into three sets of the four groups each. The four groups of all the three sets were administered with vehicle (Tween 80–Water (1:5)), aqueous extract 100, 200 and 300 mg/kg, respectively for 14 days. First set of animals was tested on shuttle box and numbers of avoidances to US were noted. Second set was tested on step through paradigm and prolongation of step through latency was noted. In the 3rd set of experiments, two memory paradigms were tested. Firstly step down apparatus noting the step down latency followed by elevated plus maze for determining the effect on transfer latency (TL). Following the behavioral test on 14th day, the animals were sacrificed for estimation of markers of oxidative stress.

2.4. Behavioral test

2.4.1. Two-way active avoidance with negative (punishment) reinforcement: shuttle box

The apparatus was a box (29 × 23 × 21 cm) divided into two equal compartments with an opening of 8 × 8 cm in middle of the wall separating the two compartments. Light source (25 W bulb) and a buzzer (70 db) were fitted in both compartments and switched on alternatively in the two compartments. This was used as conditioned stimulus (CS). The US was an electric shock (50 V a.c.) applied to the grid floor for 2 s. Each trial consists of CS for 10 s followed by US of 2 s electric shock. The inter trial interval was 8 s. Thus the total time for one trial was 20 s. An avoidance response was recorded when the animal avoided the US (i.e. animals crossed to the other compartment) within 10 s after the onset of CS.

The rats were treated orally through an intragastric feeding tube with the drugs for 14 days. On the 6th day each rat was adapted to the shuttle box. For this purpose, it was placed in a compartment of the apparatus and 20 conditioned stimuli (12 s light and buzzer and 8 s interval) were applied without electrical reinforcement. On the 7th day, 1 h after the administration of the drugs each rat was trained with 50 trials with electrical shocks and termed as 'initial trials'. The avoided response (A) and unavoided response (UA) were recorded. Retention test (50 trials with electric shocks) was given 24 h, 48 h and 7 days after the initial trial and termed as 1st, 2nd and 3rd retention trial (RT), respectively (Vesselin et al., 1993).
2.4.2. Passive avoidance with negative (punishment) reinforcement: step-through

The apparatus consists of equal size light and dark compartments (30 × 20 × 30 cm). A 40 W bulb was fixed 30 cm above its floor in the center of the roof of light compartment. Floor consisted of metal grid connected to a shock scrambler. The two compartments were separated by a trap door that could be raised to 10 cm. Rats were treated orally through a intragastric feeding tube for 14 days. On 7th day of drug administration, rats were placed in the light compartment and the time taken to enter the dark compartment with all the four paws inside it, was recorded and termed as initial latency (IL). Immediately after the rat entered the dark chamber with all the four paws inside the dark chamber, the trap door was closed and an electric foot shock (50 V a.c.) was delivered for 2 s. Rats that had an IL of more than 60 s were excluded from experiments. Three hours, 24 h and 7 days later the latency (time lapse before each animal entered the dark compartment) was recorded in the same way as in acquisition trial and were termed as 1st, 2nd and 3rd retention latency (RL), respectively but the foot shock was not delivered, and the latency time was recorded to a maximum of 600 s (Vesselin et al., 1993).

2.4.3. Passive avoidance with negative (punishment) reinforcement: step-down

The apparatus consisted of a chamber (29 × 30 × 29 cm) with metal grid floor. A wooden platform (10 × 8 cm) was fixed in the center of the grid floor. The rats were treated orally through a probe for 14 days. On the 7th day of drug treatment the rats were placed on the platform and trained to remain on it for a longer time to escape the electric footshock. Each descent of the animal to the grid floor was punished by electric footshock for 2 s (50 V a.c.). Each training session consisted of five trials. Time taken to descent in the last trial (5th) was considered as ‘Initial latency’ (IL). Retention latencies were tested 3 h, 24 h and 7 days later by placing the animal onto the platform again and measuring the time of descent and were termed as 1st, 2nd and 3rd retention latency (RL), respectively (Vesselin et al., 1993).

2.4.4. Elevated plus maze

The plus maze consists of two opposite open arms (50 × 10 cm), crossed with two closed arms of the same dimensions with 40 cm high walls. The arms were connected with a central square (10 × 10 cm). On day 7 of drug treatment, rats were placed individually at one end of an open arm, facing away from the central square. Time taken for the rat to move from the open arm and enter into one of the closed arms was recorded and termed as ‘transfer latency’ (TL). Animal was allowed to explore the maze for 30 s after recording TL. After 24 h and 7 days, the rat is placed similarly on the open arm and retention latency (RTL) is noted again (Bhattacharya, 1994).

While doing the experiments with shuttle box, step through and step down apparatus the grid as well as the rat paw was moistened with water before delivering foot shock as it is known to reduce the wide inter animal variability in paw skin resistance of the rats.

2.5. Biochemical estimation of markers of oxidative stress

On 14th day following the behavioral testing animals were decapitated under ether anesthesia and the brains quickly removed, cleaned with ice-cold saline and stored at −80 °C.

2.5.1. Tissue preparation

Brain tissue samples were thawed and homogenized with 10 times (w/v) by homogenizer in ice-cold 0.1 M phosphate buffer (pH 7.4). Aliquots of homogenates from rat brain were separated and used to determine protein, lipid peroxidation and glutathione. Whereas the remaining homogenates were centrifuged at 15,000 rpm for 60 min and supernatant was then used for enzyme assays. Catalase activity was determined immediately after sample preparation and SOD was determined within 24 h. Protein concentration was determined according to Lowry et al. (1951) using purified bovine serum albumin as standard.

2.5.2. Estimation of MDA

Malondialdehyde (MDA) a measure of lipid peroxidation was measured as described by Jainkang et al. (1990). Reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulphate (8.1%) were added to 0.1 ml of processed tissue samples, then heated at 100 °C for 60 min. Mixture was cooled under tap water and 5 ml of n-butanol–pyridine (15:1), 1 ml of distilled water was added and vortexed vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was separated and absorbance was measured at 532 nm using a spectrophotometer and concentration of MDA was expressed as nmol/g tissue.

2.5.3. Estimation of glutathione

Glutathione was measured according to the method of Ellman (1959). The equal quantity of homogenate was mixed with 10% trichloracetic acid and centrifuged to separate the proteins. To 0.01 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 was added. Mixture was vortexed and the absorbance read at 412 nm within 15 min. The concentration of glutathione was expressed as μg/g tissue.
2.5.4. Estimation of catalase

Catalase activity was measured by the method of Aebi (1974). 0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 ml of freshly prepared 30 mM H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein.

2.5.5. Estimation of SOD

SOD activity of the brain tissue was analyzed by the method described by Kakkar et al. (1984). Assay mixture contained 0.1 ml of sample, 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml phenazine methosulphate (186 μM), 0.3 ml of 300 μM nitroblue tetrazolium, 0.2 ml NADH (750 μM). Reaction was started by addition of NADH. After incubation at 30 °C for 90 s, the reaction was stopped by the addition of 0.1 ml glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 ml of n-butanol. Mixture was allowed to stand for 10 min, centrifuged and butanol layer was separated. Color intensity of the chromogen in the butanol was measured at 560 nm spectrophotometrically and concentration of SOD was expressed as units/mg protein.

2.6. Statistical analysis

The data were processed by analysis of variance (ANOVA) followed by post-test and individual comparisons using Student’s t-test (two tailed).

3. Results

3.1. Shuttle box experiments

The number of avoidance response in 1st RT was higher as compared to the initial trial in vehicle treated group indicating an acquisition of the task. A gradual increase in number of avoidances response was observed till the 3rd RT. The difference was significant between initial trial and 3rd RT indicating acquisition and retrieval of the task. Upon administration of aqueous, methanolic and chloroform extract (200 mg/kg each extract), only the aqueous extract showed a significant increase in number of avoidance response in both initial trial as well as retention trails as compared to vehicle treated groups, which indicated that aqueous extract enhanced acquisition and retrieval of the task (Fig. 1). Therefore, subsequently only aqueous extract was studied in three doses 100, 200 and 300 mg/kg. All doses of aqueous extract showed a significant increase in number of avoidance response to US in both initial as well as retention trails. With 200 mg/kg dose the increase in number of avoidances were higher than 100 mg/kg but not significantly different than 300 mg/kg (Fig. 2).

3.2. Step through experiments

In vehicle treated group, the 1st RL was significantly higher as compared to IL signifying the acquisition and 2nd RL was significantly higher than the 1st RL which signified the retrieval of the task. On the contrary, the
Fig. 3. Step through. Effect of aqueous, methanolic and chloroform extracts of *C. asiatica* 200 mg/kg on learning and memory. Step through latency in seconds mean ± SE (n = 10). IL, initial latency on 7th day of drug administration; 1st RL, 1st retention latency 3 h after initial latency; 2nd RL, 2nd retention latency 24 h after initial latency; 3rd RL, 3rd retention latency after 7 days of initial latency. *; **; *** Significance of difference vs vehicle treated at *P* < 0.05; 0.01; 0.001, respectively. + + + Significance of difference between initial and retention trials in vehicle treated group at *P* < 0.001. (ANOVA followed by post-test and Student’s *t*-test.)

3rd RL had significantly decreased as compared to 2nd RL but was significantly higher as compared to IL, this decrease in step through latency might be due to the long duration between the IL and 3rd RL. Mean IL did not differ significantly between the vehicle treated group and the drug treated groups. The extracts at the dose of 200 mg/kg prolonged the step through latency in 1st retention testing as compared to vehicle treated group. However, only the aqueous extract showed statistically significant increase in the step through latency on both 2nd and 3rd retention testing (Fig. 3). Aqueous extract when tested at three different doses (100, 200 and 300 mg/kg) showed significant prolongation of retention latency. Increase in step through latency with 200 mg/kg was more as compared to 100 mg/kg but was not significantly different than 300 mg/kg (Fig. 4).

3.3. Step down experiments

The 1st and 2nd RL was significantly higher as compared to IL in the vehicle treated group indicating the acquisition and retrieval of the task. On the contrary, the 3rd RL decreased significantly as compared to 2nd RL. This decrease in step down latency might be due to the long duration between the IL and 3rd RL. Upon administration of aqueous extract (100, 200 and 300 mg/kg), only the 200 and 300 mg/kg treated rats showed significant increase in IL and the retention testings at 3 h, 24 h and 7 days as compared to vehicle treated group. There was no significant difference between the two doses (200 and 300 mg/kg) (Fig. 5).

Fig. 4. Step through. Effect of aqueous extract of *C. asiatica* 100, 200 and 300 mg/kg on learning and memory. Step through latency in seconds mean ± SE (n = 10). IL, initial latency on 7th day of drug administration; 1st RL, 1st retention latency 3 h after IL; 2nd RL, 2nd retention latency 24 h after IL; 3rd RL, 3rd retention latency after 7 days of IL. *; **; *** Significance of difference vs vehicle treated at *P* < 0.05; 0.01; 0.001, respectively. + + + Significance of difference between initial and retention trials in vehicle treated group at *P* < 0.001. (ANOVA followed by post-test and Student’s *t*-test.)

3.4. Plus maze experiments

The 1st and 2nd retention TL was decreased significantly as compared to IL in vehicle treated group signifying the acquisition and retrieval of the task. The mean IL did not differ significantly between the vehicle and drug treated groups. Aqueous extract when tested at three different doses, only 200 and 300 mg/kg showed a significant increase in the retention TL as compared to vehicle treated group in first retention latency, but only 300 mg/kg showed significant decrease in 2nd retention TL (Fig. 6).
4. Discussion

*C. asiatica* mentioned as ‘Medhya Rasayana’ in Ayurvedic texts of the Indian system of medicine has been described to counteract the effect of mental stress by tranquilizing the users and improving their memory span and intelligence (Chopra et al., 1956). Therefore, in the present study different extracts of the *C. asiatica* were evaluated for their effect on learning and memory in normal rats using four different paradigms. Normal rats were used because such a study may possess some predictive validity as compounds that have been found to improve cognitive function in normal subjects might also do so in demented patients (Riekkinen et al., 1998).

The conditioned reflex methods employed by us in the present study are widely used for behavioral characterization of the changes in learning and memory in laboratory animals. The increased number of avoidance response in the shuttle box upon training showed that the animals had acquired the task. On the contrary in step through and step down situation the behavior was regarded as learned when the rat remained in the illuminated compartment and platform as long as possible, respectively due to the negative reinforcement. In plus maze decrease in the TL upon training was considered to be the parameters of the learning and memory process.

In the preliminary screening study among the aqueous, methanolic and chloroform extracts tested, only the aqueous extract at a dose of 200 mg/kg showed increased number of avoidances in shuttle box and step through latency in step through paradigm indicating that the rats acquired the learning and memory task. This indicates that only the aqueous extract of *C. asiatica* is active extract in improving learning and memory. Therefore, the aqueous extract was evaluated in detail using three doses (100, 200 and 300 mg/kg) in different learning and memory paradigms, i.e. shuttle box, step through, step down and elevated plus maze.

3.5. Estimation of markers of oxidative stress in rat brain

In rats treated with *C. asiatica* (100, 200 and 300 mg/kg) MDA, glutathione, SOD and catalase were estimated on 14th day. In the group treated with 100 mg/kg, there was no significant change in the parameters except that a significant increase in glutathione level was observed as compared to vehicle treated group. In the 200 and 300 mg/kg treated group there was a significant decrease in MDA and increase in glutathione levels as compared to vehicle treated group. However, there was an insignificant difference between both the doses. The catalase level increased significantly in both 200 and 300 mg/kg treated groups. Increase in 300 mg/kg treated group was significantly higher than in 200 mg/kg treated group. No change in SOD levels was observed in both the drug and vehicle treated groups (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/g tissue)</th>
<th>Glutathione (µg/g tissue)</th>
<th>SOD (U/mg protein)</th>
<th>Catalase (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>236.5 ± 20.2</td>
<td>88.6 ± 7.7</td>
<td>3.59 ± 0.3</td>
<td>16.7 ± 3.2</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>255.3 ± 19.6</td>
<td>130.7 ± 8.2***</td>
<td>3.64 ± 0.2</td>
<td>21.6 ± 3.3</td>
</tr>
<tr>
<td>200 mg/kg</td>
<td>128.8 ± 9.5***</td>
<td>137.6 ± 7.5***</td>
<td>3.76 ± 0.3</td>
<td>37.0 ± 6.5*</td>
</tr>
<tr>
<td>300 mg/kg</td>
<td>134.7 ± 7.7***</td>
<td>142.8 ± 13.1***</td>
<td>4.20 ± 0.2</td>
<td>84.6 ± 14.0***</td>
</tr>
</tbody>
</table>

Each value represents mean ± SE (n = 10).

* P < 0.05,
** P < 0.01,
*** P < 0.001 significance of difference vs vehicle treated (ANOVA followed by post-test and Student’s t-test).
All doses increased the number of avoidances in shuttle box and prolonged the step through latency in step through apparatus thus indicating the facilitated learning and improved memory. With 200 mg/kg dose the increase in number of avoidances and prolongation of step through latency was higher than 100 mg/kg but not significantly different than 300 mg. In step down paradigm and elevated plus maze paradigms only animals treated with 200 and 300 mg/kg of aqueous extract showed significant increase in the step down latency (rat remained on the platform for longer time) and decreased TL as compared to vehicle treated group, indicating the acquisition of the step down and elevated plus maze task. Thus the aqueous extract of *C. asiatica* at doses 100, 200 and 300 mg/kg was found to improve learning and memory in normal rats and the effect was more pronounced at 200 and 300 mg/kg. This study is in accordance with the previous reports in which whole dried plant improves the general mental ability of mentally retarded children (Apparao et al., 1973).

Varying degrees of behavioral impairments are associated with aging and age associated neurodegenerative diseases (Reiter, 1995). Among the prime candidates responsible for producing the neuronal changes mediating these behavioral deficits appear to be free radicals and the oxidative stress they generate (Cantuti et al., 2000). Oxidative stress refers to the cytotoxic consequences of oxygen radicals like superoxide anion, hydroxyl radical, and hydrogen peroxide, which are generated as byproducts of normal aberrant metabolic processes during ageing and other neurodegenerative diseases and act on polyunsaturated fatty acids (PUFA) in brain, thereby propagating the lipid peroxidation (Coyle and Puttfarcen, 1993). Major antioxidant and oxidative free radical scavenging enzymes are glutathione, SOD and catalase.

Potential antioxidant therapy should therefore, include either natural antioxidant enzymes or agents, which are capable of augmenting the functions of these enzymes (Bast et al., 1991). Earlier reports have shown that the natural drugs like *Ginkgo biloba* (Christen, 2000; Sastre et al., 2000) and *Withania somnifera* (Bhattacharya et al., 1996), which improves cognition also shown to have antioxidant properties. Therefore, the different doses of aqueous extract (100, 200 and 300 mg/kg) of CA, which showed improvement in learning and memory paradigms were tested on the oxidative stress parameters i.e. levels of MDA, glutathione, SOD and catalase in brain of adult rats.

Among doses of aqueous extract tested, the dose of 200 and 300 mg/kg showed a significant decrease in the brain levels of MDA, which is the end product of lipid peroxidation and a measure of free radical generation. Also there was a simultaneous significant increase in levels of glutathione, a tripeptide found in all cells and reacts with the free radicals to protect cells from superoxide radical, hydroxyl radical and singlet oxygen (Schulz et al., 2000). In our study there was an insignificant increase in levels of SOD at 300 mg/kg, which is the only enzyme uses the superoxide anions as a substrate and produces the hydrogen peroxide as a metabolite, which is more toxic than O$_2^-$ radical and has to be removed by catalase (Harman, 1991; Carrillo et al., 1992). In the present study there was a significant increase in levels of catalase at 200 and 300 mg/kg as compared to vehicle treated group, indicating the aqueous extract scavenges the hydrogen peroxide, which is generated by SOD.

The present study therefore demonstrate that the aqueous extract of whole plant of *C. asiatica* have two pronounced effects, i.e. improving the learning and memory and, the antioxidant property by decreasing the lipid peroxidation and augmenting the endogenous antioxidant enzymes in brain.

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### References


