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Original Article

The effect of beet leaf (*Beta vulgaris*) extract on learning and memory after cerebral ischemic reperfusion in rats

Kobra Mirahmadi[1], Najmeh Asgharzadeh[2], Zahra Lorigooini[3], Marzieh Mardani[3], Zahra Rabiei[3], Diana Shahrani[4], Mohamad Shahrani[5], Mehrdad Shahrani Korrani[3*]

[1]Department of Education, Chaharmahal and Bakhtiari Province, Shahrekord, Iran
[2]Department of Biology, Falavarjan Branch, Islamic Azad University, Medicinal Plants Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran
[3]Medicinal Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran
[4]Student Research Committee, Shahrekord University of Medical Sciences, Shahrekord, Iran
[5]Student Research Committee, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background and aims: Cerebral ischemia causes tissue oxidative damage and leads to cell death. Antioxidants can reduce the adverse effects of ischemia in the brain. This study was conducted to determine the effect of *Beta vulgaris* hydroalcoholic extract on learning and memory caused by transient cerebral ischemia.

Methods: After preparing the herbal extract, the rats were divided into six groups: control, sham, ischemia, and 3 ischemic groups that received the extract. Ischemia was induced by bilateral carotid artery occlusion for 30 minutes. The control, sham, and ischemia groups received normal saline for two weeks. Three treatment groups received 50, 100, and 200 mg/kg *B. vulgaris* extract. After conducting behavioral tests, the antioxidant capacity and malondialdehyde (MDA) levels of the rat's serum and brain were measured.

Results: The results showed that the hydroalcoholic extract of *B. vulgaris* leaves increases the antioxidant capacity of serum and brain. It also reduces the amount of MDA in serum and brain. *B. vulgaris* extract enhances learning and improves motor balance.

Conclusion: This study showed that the hydroalcoholic extract of beet leaves firmly protects brain damage caused by cerebral ischemia. The antioxidant property of this extract may explain this effect.

Keywords: *Beta vulgaris*, Beet leaves, MDA, Antioxidant, Rat

***Corresponding Author:**
Mehrdad Shahrani Korrani,
Emails: shahrani.m@skums.ac.ir, mehrdadeshahrani2000@gmail.com

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Introduction

Cerebral ischemia means not getting enough oxygen in the brain. During ischemia, the levels of antioxidants and ATP decrease. The distribution of ions inside the cell changes, cell swelling, cytoskeleton disorganization, and cell acidosis occur. During reperfusion, blood flow returns to the brain, which causes cellular edema, excessive intracellular Ca^{2+} release, and cell membrane damage. Cell damage after reperfusion of ischemic tissues leads to the release of free radicals (1).

Endogenous antioxidants such as glutathione have preventive effects on reperfusion injury after ischemia (2). Recently, plant extracts containing polyphenols, oleuropein, etc., have been included in the food groups that protect against ischemia-reperfusion injury. Their mechanism of action is mainly related to their antioxidant power and ability to balance oxidative stress. Increased ROS causes oxidative damage and leads to cell death. ROS-induced damage also induces an inflammatory response, production of prostaglandins, and interstitial edema (3). Brain damage after cardiac arrest is one of the leading causes of disability and death in patients with cardiac arrest syndrome (4).

Acute inflammatory reactions cause neuronal damage in cerebral ischemia-reperfusion. Cerebral ischemia causes inflammatory cells to infiltrate the ischemic area and cause inflammation within a few hours to a few days (5). *Beta vulgaris* is a plant in the family Betoideae of the Amaranthaceae. A variety of *B. vulgaris* components can control many cellular pathways. *B. vulgaris* contains various beneficial compounds such as flavonoid, polyphenol, betanin, vitamins such as thiamin, riboflavin, pyridoxine, and ascorbic acid (6).

Several reports have shown that beet leaves have antioxidant and antidiabetic properties and a protective effect against tissue damage caused by hyperglycemia. *B. vulgaris* extracts possess anti-inflammatory antioxidants, and they can improve memory disorders (7). Phytochemical studies of *B. vulgaris* have shown the presence of some saponins and flavonoids that are hypoglycemic agents (8, 9). This study was conducted to investigate the effect of *B. vulgaris* on brain antioxidant

capacity, learning, and helping to heal tissue damage caused by cerebral ischemia.

Materials and Methods

Preparation of Beta Vulgaris Extract

The leaves of *the B. vulgaris* plant were identified by the Herbarium Unit of the Medicinal Plants Research Center of Shahrekord University of Medical Sciences, Iran. After washing, the collected leaves of *B. vulgaris* were shade-dried and powdered. One thousand grams of *B. vulgaris* leaf powder was mixed in 1200 cc of 70% ethanol and extracted by maceration method. The filtered liquid extract was dried using a rotary device at 50 °C, and 53 g of dry extract was obtained. Then, by dissolving the extract in distilled water, different doses of the extract were prepared.

Determination of antioxidant activity of Beta vulgaris extract by DPPH method

The inhibitory radical DPPH is an unstable free radical due to the presence of electrons in the structure of DPPH; this radical has a good absorption at the wavelength of 517 nm. Its color changes when placed in an antioxidant compound with free radical scavenging activity. To evaluate the cleaning activity of the extract, six different concentrations (10, 20, 40, 60, 80, and 100 µg/mL) of plant extract were prepared. 2 ml of DPPH solution was added to each, and their absorbance (A) was read at a wavelength of 517 nm. After 15 minutes, the percentage of DPPH inhibition (I) by the combination of antioxidants can be calculated from the following equation. A butylated dihydroxytoluene antioxidant compound was used as a positive control.

$$I\ (\%) = 100 \times \frac{\text{A control} - \text{A sample}}{\text{A control}}$$

A butylated hydroxytoluene antioxidant compound was used as a positive control (10).

Animal experimental design and protocols

In this study, 48 rats were divided into 6 groups (N = 8): 1) Control group, which received 0.9% sodium chloride without surgery and medicine, 2) Sham group, which underwent surgery without medication and carotid artery occlusion and received 0.9% sodium chloride, 3) Ischemic group, which were subjected to ischemia without receiving drugs and only received 0.9% sodium chloride, 4, 5, and 6) Treatment groups: After induction of ischemia, three groups received *Beta vulgaris* leaf extract in doses of 50, 100, and 200 mg/kg (11). Injections for 14 days after induction of ischemia in the treatment group were performed simultaneously in all groups. All animals were kept in normal environmental conditions with 12 hours of light and 12 hours of darkness, temperature 18-25 °C and humidity 30%-70%. Behavioral tests were performed from the 15th day onwards in all groups. After performing the behavioral tests, the rats were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg), and then blood samples were taken. Each group's brain (brain cortex, sub-cortex, and hippocampus) was removed for biochemical evaluation (Figure 1) (12).

Transient global cerebral ischemia

After anesthesia, an incision was made in the anterior region of the neck. The carotid sheath was identified, and the common carotid arteries were carefully identified and separated from the vagus nerve. Then, by closing the common carotid artery with a micro-bulldog clip, ischemia was created for 30 minutes (12).

Morris water maze test

This test was done to evaluate spatial memory. The Morris

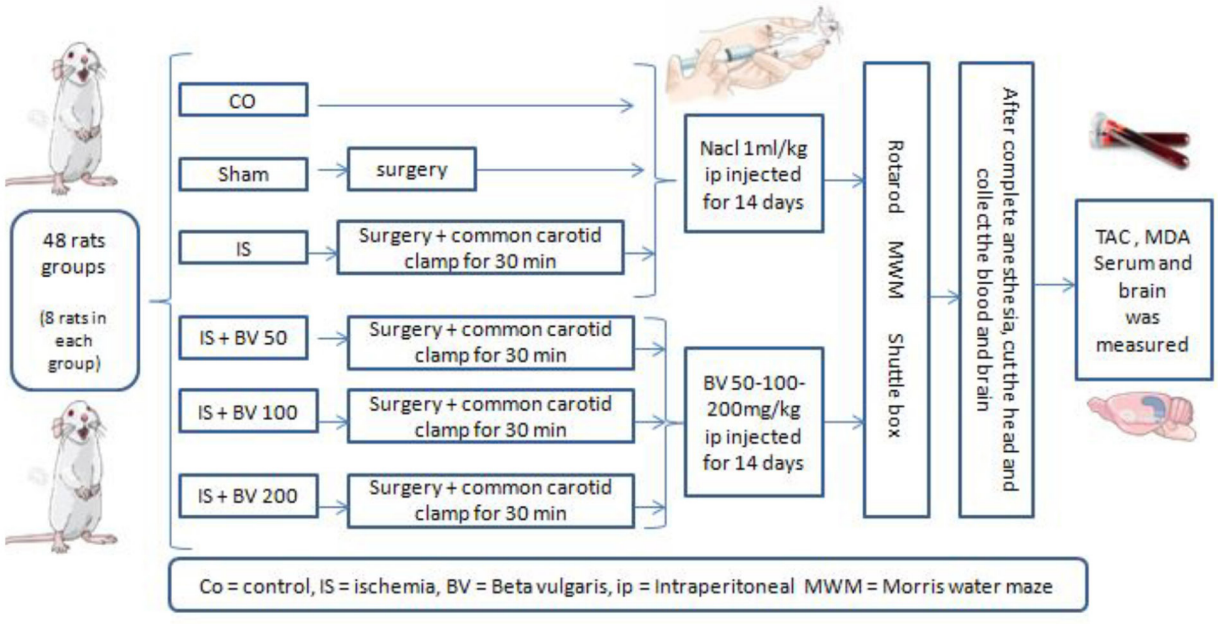

Figure 1. Schematic figure of the project execution method

water maze test placed rats in a water tank. The platform was not visible to the animals. When the animal was placed in the tank, it usually remembered the platform's location from the view it received from the outside. If the mouse found the platform faster, it was smarter (13).

Passive avoidance task

Passive avoidance memory was performed using a shuttle box device (Borjsanat Co. Iran). The device consisted of a light chamber made of clear plastic and a dark chamber with a dark plastic ceiling and walls. There was an opening between the chambers. The floors were made of stainless steel bars in both chambers, spaced 1 cm apart, and the darkroom floor could be electrified. After 10 minutes of adaptation, the rats were placed in the light chamber for training. The door was closed after the animals entered the dark chamber, then they received an electric shock (3 s, 0.5 mA) from the bottom of the steel bar. 24 hours later, after placing the animal in the light chamber, the latency to enter the dark chamber was measured (14).

Evaluation of motor coordination and balance

The rotarod (Borjsanat Co. Iran) consists of a rotating spindle with a diameter of 7.3 cm. To train the mouse to stay on the rod, each mouse was placed on the device for 10 minutes on two consecutive days. For the rotarod test, the rats were placed on the rod and tested for a maximum of 5 minutes at different rotation speeds. The movement speed starts from 5 rpm and reaches a maximum of 40 rpm. The duration of stay on the rotating rod was recorded (15).

Measurement of brain antioxidant capacity

The antioxidant capacity of the brain was measured using the Ferric reducing antioxidant power (FRAP) method. The FRAP reagent was prepared by mixing acetate buffer (25 mL), TPTZ solution (2.5 mL), FeCl3, and 6H2O solution (2.5 mL). The homogenized tissue sample (in cold 2.5% KCl solutions) was centrifuged, and the supernatant (50 µL) was mixed with 1.5 mL of fresh FRAP solution. After 10 minutes at 37 °C, the absorbance of the mixture was recorded at a wavelength of 590 nm (16).

Measurement of serum antioxidant capacity

Three different solutions were used to measure the antioxidant capacity of serum: Solution number 1: 1.5 mL of sodium acetate and 8 ml of concentrated acetic acid diluted to 5000 mL with distilled water. Solution number 2: 270 mg of iron chloride diluted to a volume of 50 ml with distilled water**.** Solution number 3: 47 mg of treeazin dissolved in 40 mL of HCl. The working solution was prepared by mixing 10 mL of solution number 1, 1 mL of solution number 2, and 1 mL of solution number 3. Twenty-five microliters of serum samples were added to 1.5 mL of working solution. The reaction mixture was kept at 37 °C for 10 minutes, and then the absorbance was read at 593 nm (17).

Measurement of serum MDA

Fifty microliters of serum was mixed with 50 µL of BHT (butylated hydroxytoluene) (0.05% in 95% ethanol), 400 µL of H3PO4 (0.44 M), and 100 µL of TBA and heated at 100 °C for 1 hour. After cooling for 5 minutes at 0 °C, butanol was added and centrifuged at 14000 rpm for 5 minutes. Then, the absorbance of the supernatant was read at a wavelength of 532 nm (18).

Measurement of brain MDA

One gram of brain cortex, sub-cortex, and hippocampus tissues were homogenized separately in 10 mL of 2.5% cold KCl solution. One milliliter of the homogenate was transferred into a 20 mL tube and incubated at 37 °C for 60 minutes. After that, the suspension was mixed with 1 mL TCA (5%) and 1 mL TBA (thiobarbituric acid) 67% and centrifuged for 15 minutes at 2000 rpm. The supernatant was transferred to a new tube and placed in a boiling water bath for 10 minutes. After cooling, the absorbance at 535 nm wavelength was read using a spectrophotometer for each sample (19,20).

Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). The statistical significance was determined using a one-way variance analysis followed by Tukey's post hoc test. Results were considered statistically significant between the two groups when $P < 0.05$.

Results

Antioxidant capacity of Beta vulgaris extract

The antioxidant capacity of *B. vulgaris* based on the DPPH method IC50 of *B. vulgaris* extract was 160.58 µg /mL (Figure 2a).

The effect of Beta vulgaris extract on passive avoidance memory

The duration of the delay in entering the dark room where they had previously received an electric shock was significantly reduced in the ischemia group compared to the control group ($P < 0.001$). The plant extract increased this time in the groups treated with *B. vulgaris* extract by improving memory (Figure 2b).

The Effect of Beta vulgaris extract on motor coordination and balance

The latency to fall from the rotating rod in the ischemic group was significantly lower than in the control group ($P < 0.001$). Administration of 100 and 200 mg/kg/d *of B. vulgaris* extract significantly increased motor coordination in the ischemic rats ($P < 0.01$ and $P < 0.001$) (Figure 2c).

The effect of Beta vulgaris extract on special memory

The spatial memory in ischemic rats was significantly reduced compared to the control group ($P < 0.001$). *B. vulgaris* extract improved the spatial memory in animals

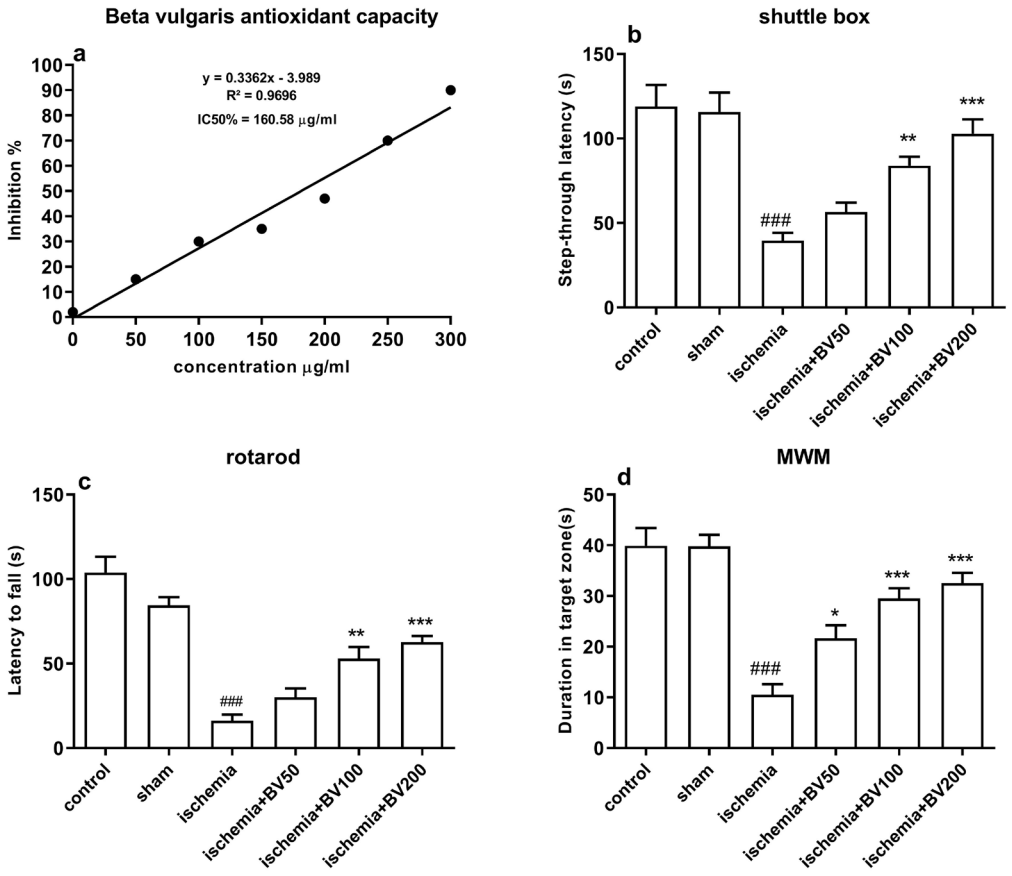

Figure 2. a. Standard curve of *Beta vulgaris* antioxidant capacity. **b.** The duration of delay in the shuttle box device. **c.** Latency to fall from the rotating rod. **d.** Duration of being in the target zone of MWM. ### $P<0.001$ compared to control and * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared to ischemia

receiving different doses (50, 100, and 200 mg/kg) (Figure 2d).

The effect of Beta vulgaris extract on serum antioxidant capacity

As can be seen, the antioxidant capacity of the ischemia group has significantly decreased compared to the control group. The plant extract with doses of 100 and 200 mg/kg could significantly increase the antioxidant capacity of serum compared to the ischemia group (Figure 3a).

The effect of Beta vulgaris extract on hippocampus antioxidant capacity

According to the graph below, the hippocampus's antioxidant capacity significantly decreased in the ischemia group compared to the control group. Treatment with the extract in doses of 100 and 200 mg/kg caused a significant increase in the antioxidant capacity of the hippocampus of ischemic animals (Figure 3b).

The effect of Beta vulgaris extract on brain cortex antioxidant capacity

As you can see in the figure below, the decrease in the antioxidant capacity of the brain cortex in the ischemia group is very significant compared to the control group. In the ischemia groups, the plant extract increased the level of antioxidant capacity by a considerable degree (Figure 3c).

The effect of Beta vulgaris extract on brain sub-cortex antioxidant capacity

In this figure, the antioxidant capacity of the brain's sub-cortex is significantly reduced in all ischemia groups compared to the control group. However, there is no significant difference between the extract treatment groups and the ischemia group (Figure 3d).

The effect of Beta vulgaris extract on serum MDA level

As this figure shows, although the amount of serum MDA increased in the ischemic groups, this increase was not significant. Also, the plant extract could not significantly reduce it (Figure 4a).

The effect of Beta vulgaris extract on hippocampus MDA level

The amount of hippocampus MDA increased significantly in all ischemia groups compared to the control group. In the treatment groups, the plant extract could not significantly reduce it compared to the ischemic group (Figure 4b).

The effect of *Beta vulgaris* extract on brain cortex MDA Level

As seen in the figure, although the level of MDA in the

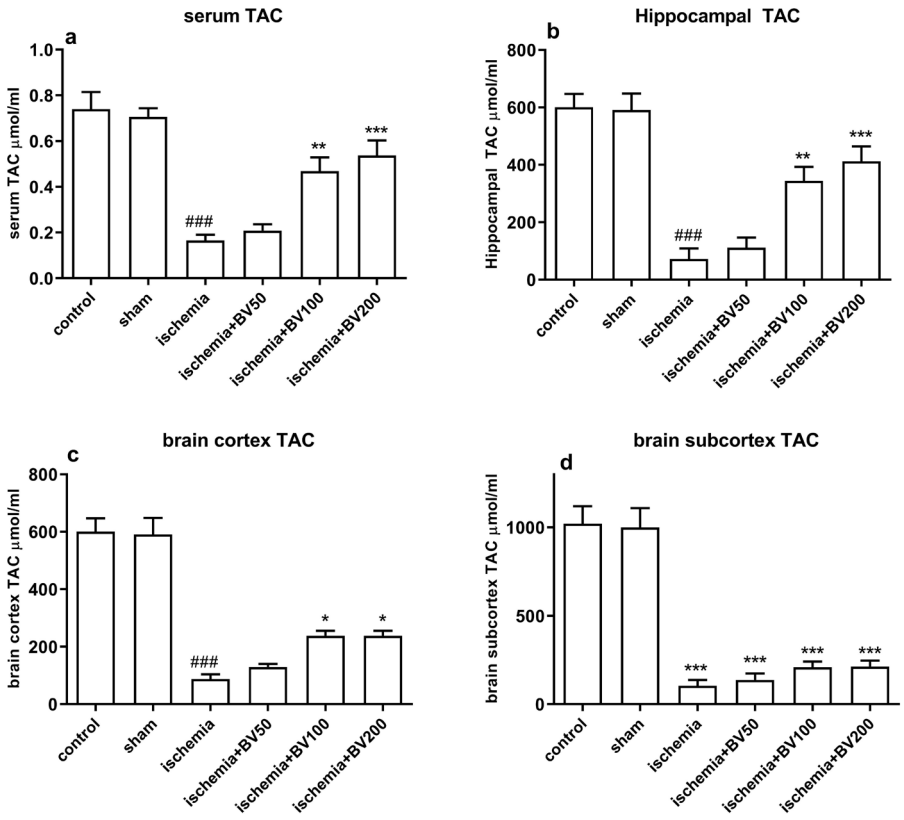

Figure 3. a. Antioxidant capacity of serum. **b.** Antioxidant capacity of the hippocampus. **c.** Antioxidant capacity of the brain cortex. **d.** antioxidant capacity of the brain sub-cortex. ### $P<0.001$ compared to control, ** $P<0.01$ and *** $P<0.001$ compared to ischemia

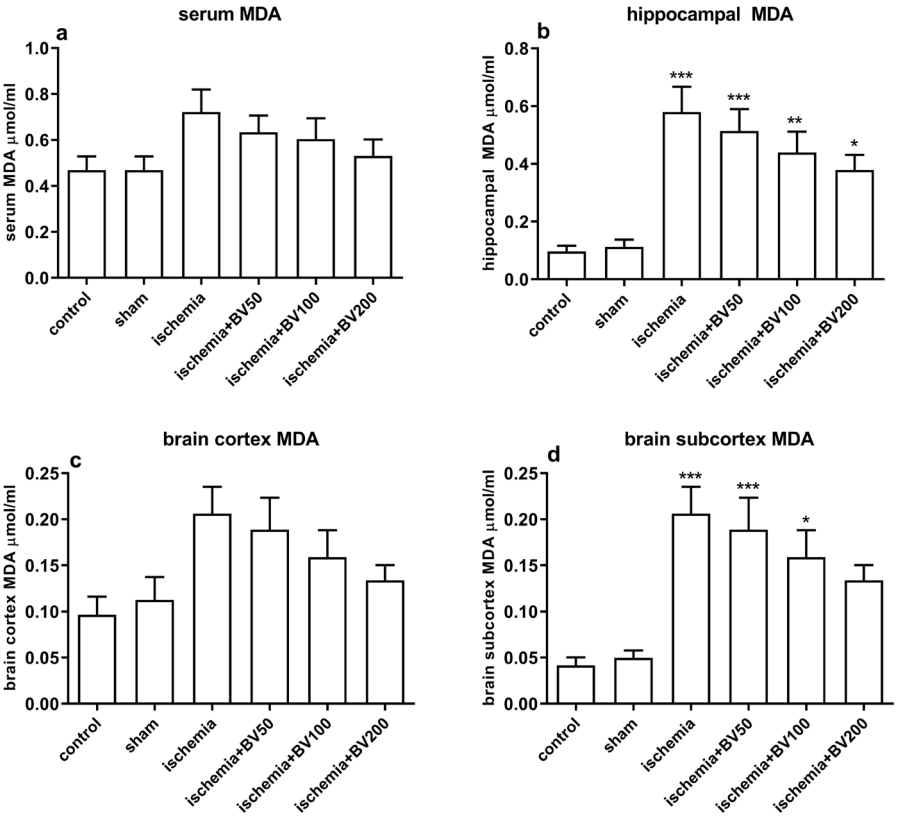

Figure 4. a. MDA of serum. **b.** Hippocampal MDA level. **c.** MDA in the brain cortex. **d.** MDA in the brain sub-cortex. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to control

cerebral cortex is lower than in the control group, this difference is insignificant. Also, the plant extract could not significantly reduce the amount of MDA compared to the ischemia group (Figure 4c).

The effect of Beta vulgaris extract on brain sub cortex MDA level

Figures 4a and 4c showed no significant difference between the groups. However, the increase of MDA in the subcortical brain of ischemia groups treated with doses of 50 and 100 mg/kg is significant compared to the control group (Figure 4d).

Discussion

The antioxidant capacity of *the B. vulgaris* plant extract used in this study, which was investigated by the DPPH method, was reported to be 160.58 µg /mL. Several reports have shown that in addition to the protective effect against tissue damage caused by hyperglycemia, Beet leaves also have antioxidant properties and memory enhancement. The impact of *B. vulgaris* extract on the nervous system, such as analgesic, anticonvulsant, and improving memory and learning, has also been reported (8).

In this study, the duration of standing on the rotating rod in the rotarod test, which indicates the animal's movement balance and cerebellum's function, was significantly reduced in the transient cerebral ischemia group compared to the control group. The administration of *B. vulgaris* extract increased the duration of standing on the rotarod, which indicates the positive effect of the plant extract on the animal's movement balance.

In our study, transient cerebral ischemia caused a significant increase in the level of MDA in the hippocampus and subcortical brain. However, it had no significant effect on the levels of MDA in the serum and cortical tissue. The researchers found that induction of transient cerebral ischemia did not cause significant oxidative stress in rat serum and cortical tissue. Therefore, the movement disorder observed in our study may be related to increased oxidative stress in the sub-cortex and hippocampus of the brain. It has been reported that the deterioration of motor function after ischemia is mainly due to disruption of the blood-spinal cord barrier and inflammation of the gray and white matter of the brain (21).

The duration of staying in the target quadrant in the Morris water maze test was significantly reduced in the transient cerebral ischemia group compared to the control group. The administration of *B. vulgaris* extract caused a significant improvement in the groups receiving the extract. This indicates an increase in spatial memory in these groups. The delay in entering the dark compartment of the shuttle box test was significantly reduced in the transient cerebral ischemia group compared to the control group. However, the administration of *B. vulgaris* extract significantly increased this delay, indicating increased learning in these groups. In this study, the destruction of avoidance and spatial memory following transient cerebral ischemia can be caused by increased oxidative stress in the brain. The plant extract improved spatial and avoidance memory in rats by reducing oxidative stress. Previous studies showed that transient cerebral ischemia increases lipid peroxidation and decreases the brain's and serum's antioxidant capacity (22).

In our study, transient cerebral ischemia significantly increased hippocampal and subcortical MDA. *B. vulgaris* extract significantly increased the antioxidant capacity of serum, hippocampus, and cerebral cortex compared to the transient cerebral ischemia group. The antioxidant capacity of the brain's serum, hippocampus, cortex, and sub-cortex decreased drastically after transient cerebral ischemia, and the plant extract could increase this capacity except in the sub-cortex of the brain. It can be concluded that the antioxidant effects of the plant extract caused these changes.

Researchers have described various mechanisms for developing ischemic nerve damage, including oxidative stress, DNA oxidation, and increased production of inflammatory eicosanoids (23). Research studies have previously shown that transient cerebral ischemia causes lipid peroxidation and a decrease in the antioxidant capacity of the brain and serum (24). The presence of fatty acids (palmitic, stearic, oleic, linoleic), phospholipids, glycolipids, folic acid, ascorbic acid, pectin, as well as saponins and flavonoids has been determined in beta vulgaris. Also, folic acid, iron, calcium, phosphorus, zinc, vitamins B, A, and C can be mentioned as other essential substances found in the plant. Betalain and non-phenolic antioxidant compounds, including two groups of red betacyanin (mainly betanin) and yellow betaxanthin, have been identified in beets (25). The neuroprotective and therapeutic effects of *B. vulgaris* extract may be related to its bioactive components.

Considering the very positive effects of *B. vulgaris* plant extract in reducing oxidative damage, it is suggested that its active ingredient be discovered by specifically extracting the effective ingredients of this plant. By studying the human community, if there are no side effects, he recommended using this plant to prevent the side effects of cerebral ischemia.

Conclusion

Consumption of beet leaves helps treat complications caused by transient cerebral ischemia.

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Authors' Contribution

Conceptualization: Mehrdad Shahrani Korrani.
Data curation: Najmeh Asgharzadeh.
Formal analysis: Zahra Lorigooini.
Funding acquisition: Kobra Mirahmadi.
Investigation: Kobra Mirahmadi, Diana Shahrani, Mohamad Shahrani.
Methodology: Marzieh Mardani.

Project administration: Zahra Rabiei.
Resources: Mehrdad Shahrani Korrani.
Software: Najmeh Asgharzadeh.
Supervision: Mehrdad Shahrani Korrani.
Validation: Najmeh Asgharzadeh.
Visualization: Najmeh Asgharzadeh.
Writing–original draft: Mehrdad Shahrani Korrani.
Writing–review & editing: Mehrdad Shahrani Korrani.

Competing Interests

The authors declare that they have no conflicts of interest regarding the publication of this paper. Besides, although one of the authors in this article is the journal's editor-in-chief, the whole process of reviewing and publishing the article is like that of other articles in the journal, and there is no difference in its review from other ones.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Ethical Approval

This project was designed and implemented based on the license number IR.SKUMS.REC.1394.53 of the Ethics Committee of Shahrekord University of Medical Sciences (Shahrekord, Iran).

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