Original Research Article

AMELIORATIVE EFFECT OF *ALLIUM CEPA* ON OXIDATIVE STRESS AND NEURONAL DAMAGE AFTER ISCHEMIA AND REPERFUSION-INDUCED CEREBRAL INJURY

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ABSTRACT

Background: Stroke is the second most common cause of death and major cause of disability worldwide. Because of the ageing population, the burden will increase greatly during the next 20 years, especially in developing countries. Antioxidants have been the focus of studies for developing neuroprotective agents to be used in the therapy for stroke, which is an acute and progressive neurodegenerative disorder. *Allium cepa* (Linn.) has been greatly valued, commercially as well as medicinally, since ancient times. *A. cepa* is reported to be potent antioxidants and neuroprotective agent. Our previous work demonstrated that pre-treatment with methanol extract of *A. cepa* prevents ischemia-reperfusion (I/R) induced cerebral injury. **Objective**: The present study was designed to investigate the effect of methanol extract and flavonoid-rich fraction of outer scales of *A. cepa* bulbs post-cerebral injury. **Materials and methods**: Global cerebral ischemia was induced in mice by bilateral carotid artery occlusion followed by reperfusion. Treatment with extracts of *A. cepa,* was carried out for 28 days after I/R. Cerebral infract size was estimated using triphenyltetrazolium chloride staining. TBARS assay was employed to measure oxidative stress. Morris water maze was employed to assess memory, and inclined-beam walking test was employed to evaluate motor coordination. Phytochemical screening tests showed the presence of flavonoids in the bioactive extract, hence flavonoid-rich fraction was prepared and biological studies were carried out. **Results**: The flavonoid-rich fraction of the outer scales of *A. cepa* demonstrated the most significant reduction in cerebral damage and oxidative stress. It also ameliorated the damage to memory and motor coordination. This bioactive fraction found to contain high amount of total phenolics and total flavonoid content. **Conclusion**: The standardized flavonoid-rich fraction of the outer scales of *allium cepa* may be a potential candidate for the treatment of post-cerebral damage.

Keywords: *Allium cepa*, cerebral injury, antioxidants, neuroprotection, motor coordination

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INTRODUCTION

Stroke is the world's second leading cause of mortality, resulting around 60,00,000 deaths annually, and considered the most common cause of disability in adults [1]. In western countries, stroke causes 10-12% of all deaths [2]. Currently there are approximately 2 million survivors of stroke living in the US with prolonged disability, many unable to work or resume personal relationships. In India stroke is responsible for about 1,02,000 deaths annually, which represent about 2% of the total deaths in the country [3]. In ischemic stroke, blood flow to the brain becomes blocked either by a clot or ruptured atherosclerotic

plaque and triggers a time-dependent cascade of molecular events [4]. Reperfusion or the restoration of blood flow to the ischemic tissue may lead to the formation of reactive oxygen species that paradoxically promote further tissue injury and is referred to as reperfusion injury [5]. Evidences suggest that the excessive generation of oxygen free radicals such as superoxide anions, hydroxyl radicals, and hydrogen peroxide during reperfusion plays a major role in brain injury associated with stroke [6]. Neuroprotective agents, that prevents the multiple neurochemical cascades which cause cerebral damage and result in immense morbidity and mortality in human beings, are therefore urgently required. A recent approach is the use of antioxidants for neuroprotection. Many synthetic and natural antioxidants have shown neuroprotective effects in ischemia and reperfusion induced cerebral injury [7]. A number of plants/plant constituents with antioxidant activity have shown beneficial effects in ischemia and reperfusion (I/R)-induced cerebral injury [8].

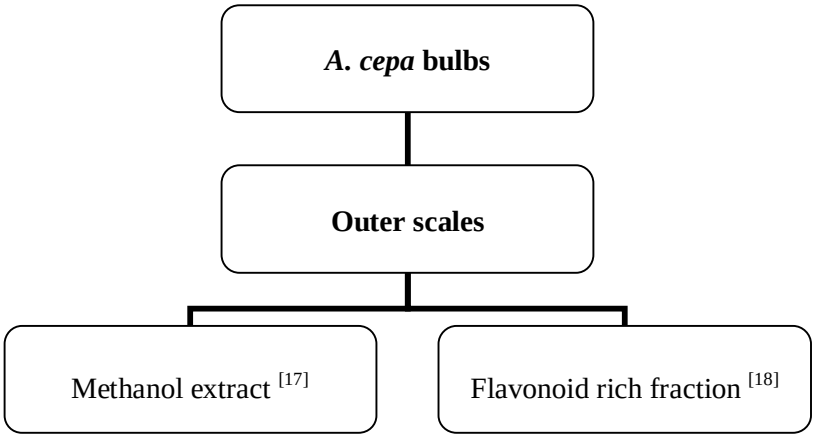
Allium cepa Linn. (Family: Alliaceae) commonly called as onion is cultivated extensively worldwide [9]. The plant has been used widely in various traditional systems of medicines. It has demonstrated significant antiallergic and anti-inflammatory activity [10]; antidiabetic activity [11]; hypolipidemic and cardioprotective effect [12]; prevention of atherosclerosis [13]. *A. cepa* has potent antioxidant effect [14] that is attributed to the phenolic and sulfur-rich compounds present in the bulbs [15]. It has been previously reported that pre-treatment with methanol extract of *A. cepa* showed neuroprotective effect due to its ability to scavenge reactive oxygen species and prevent I/R induced cerebral injury [16].

The present study was designed to investigate the effect of methanol extract and flavonoid-rich fraction of outer scales of *A. cepa* bulbs against **post-cerebral injury** in mice.

MATERIALS AND METHODS

Plant material

The bulbs of *Allium cepa* var. Agrifound Dark Red were procured from a cultivated source-"National Horticulture Research and Development Foundation" (NHRDF), Karnal (Haryana), India. This variety was identified and authenticated by Dr L.R. Verma, Director NHRDF, Karnal (Haryana), India (Ref. No. 13748, November, 2011).



Scheme 1: Various extracts and fractions prepared from *Allium cepa* bulbs

Preparation of extracts

Extract/fraction of *A. cepa* bulbs used in the present study was prepared according to scheme 1.

Standardization of extract

Total phenol content analysis

The total phenolics of the extract were determined by Folin-Ciocalteu procedure [19]. The amount of total phenols was calculated as gallic acid equivalent from the calibration curve of gallic standard solutions and expressed as mg gallic/g dry extract. All measurements were done in triplicate.

Total flavonoid content analysis

The favonoid contents were determined according to the method of Jay *et al*. [20]. The flavonoid content was determined as rutin equivalent from the calibration curve of rutin standard solutions and expressed as mg rutin/g dry extract. All measurements were done in triplicate.

Experimental animals

Swiss albino mice of either sex weighing 20-30 g were used. They were maintained on standard environmental conditions. They were fed with standard rodent diet (Kissan Feeds Ltd., Mumbai, India) and tap water ad libitum. They were housed in the departmental animal house and were exposed to natural photoperiod. The experimental protocol was duly approved by Institutional Animal Ethical Committee (IAEC). The animals were taken care according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No. 107/99/CPCSEA-2011-12).

Experimental protocol

Animals were randomly divided into six groups.

- Group I. Sham group (6 animals): Mice were subjected to surgical procedure under chloral hydrate anesthesia (400 mg/kg, i.p.). A thread was passed below carotid arteries without occlusion. After 10 min, thread was removed and animal was sutured back and allowed to recover for 24 h. after which behavioral studies were carried out, then animals were sacrificed for biochemical estimations.
- Group II. Control group (18 animals). Mice were subjected to 10 min global cerebral ischemia by carotid arteries occlusion followed by reperfusion for 24 h. after which behavioral studies were carried out on 6 animals, and then the animals were sacrificed for biochemical estimations. Remaining animals were continued on standard diet. On day 14, six animals were assessed for behavioral changes and biochemical estimations. On day 28 the remaining six animals were assessed for behavioral changes and biochemical estimations.
- **Test groups:**
- Groups III and IV. **Methanol extract of outer scales** of *A. cepa,* treated groups (12 animals). Mice were subjected to global cerebral ischemia and reperfusion. After which treatment with methanol extract of outer scales of *A. cepa* at dose of 100 mg/kg and 200 mg/kg, p.o. once daily, was started. Six animals were assessed for behavioral changes and biochemical estimations after 14 days and the remaining were assessed after 28 days of treatment with test extracts.
- Groups V and VI. **Flavonoid-rich fraction of outer scales** of *A. cepa,* treated groups (12 animals). Mice were subjected to global cerebral ischemia and reperfusion. After which treatment

with flavonoid-rich fraction of outer scales of *A. cepa* at dose of 50 mg/kg and 100 mg/kg, p.o. once daily, was started. Six animals were assessed for behavioral changes and biochemical estimations after 14 days and the remaining were assessed after 28 days of treatment with test extracts.

Induction of cerebral injury and estimation of infarct size

The experimental animals were anesthetized with chloral hydrate (400 mg/kg, i.p.). A midline ventral incision was made in neck of the animals. Right and left common carotid arteries were located and freed from surrounding tissue. Both common carotid arteries were exposed over a midline incision. Each carotid artery was freed from its adventitial sheath and vagus nerve, and a cotton thread was passed below each carotid artery. The ends of thread were pulled with constant weight to induce global cerebral ischemia. After 10 min of global cerebral ischemia, weight on the thread was removed to allow the reflow of blood through carotid arteries. The incision was sutured back in layers. The sutured area was cleaned with 70% aqueous ethanol and was sprayed with antibiotic (Neosporin) dusting powder. Body temperature of mice was maintained at 37ºC by heated surgical platform [21].

After 24 h of reperfusion, animals were sacrificed by spinal dislocation and the brain was removed. The brain was kept overnight at 4ºC. Frozen brain was sliced into uniform sections of 1 mm thickness. The slices were incubated in 1% 2,3,5-Triphenyltetrazolium chloride (TTC) at 37ºC in 0.2M tris buffer (pH 7.4) for 20 min. TTC is converted to a red colored pigment-formazone, in the presence of nicotinamide adenine dinucleotide (NAD) and dehydrogenase which are present in living cells. Thus viable cells were stained deep red while the infarcted cells loose these enzymes and remained unstained. After staining, color images of the brain slices were recorded using a digital camera. The computerized image analyzer (MCID Elite) was used to calculate the size of infarcted area.

Biochemical estimation: Estimation of thiobarbituric acid reactive substances (TBARS)

For biochemical estimation in the brain tissue, the animals were sacrificed. The brains were removed, weighed, minced and suspended in a buffer containing 30mM Tris-HCl and 2.5mM $CaCl_2$ (pH 7.6). This was homogenized and the homogenate was centrifuged at 750 × *g* to separate cellular debris. The supernatant was divided into two parts. Both the portions were centrifuged at 8200 × *g* to obtain the mitochondrial fraction. One portion of the mitochondrial fraction was utilized for determination of TBARS and the other portion was employed for protein estimation. A standard curve for TBARS was plotted using 1,1,3,3-tetramethoxypropane. The extent of lipid peroxidation was expressed as nanomoles of TBARS formed per milligram of protein [22].

Assessment of learning and memory using Morris Water Maze Test

Morris Water Maze (MWM) test was employed to assess learning and memory of mice [23] The MWM procedure was based on a principle where the animal is placed in a large pool of water (150 cm in diameter, 45 cm in height, filled to a depth of 30 cm with water at 28°C); as the animals dislike swimming, they try to escape from the water by locating an escape platform in the pool. The pool water was made opaque with white colored dye. The tank was divided into four equal quadrants with the help of two threads, fixed at a right angle to each other on the rim of the pool. A submerged platform (10 cm^2), painted white, was fixed inside the target quadrants of this pool 1 cm below surface of the water. The position of the platform was unaltered throughout the training session. The animals were given acquisition trials for 4 consecutive days. Each animal was subjected to four consecutive trials on each day with a gap of 5 min. The mice were gently placed in the water of the pool between quadrants, facing the wall of the pool with drop location changing for each trial, and allowed 120 s to locate the submerged platform. It was allowed to stay on the platform for 20 s. If it failed to find the platform within 120 s, it was guided gently onto the platform and allowed to remain there for 20 s. Escape latency time (ELT) to locate the hidden platform in

water maze was noted as the index of acquisition or learning. On the fifth day, the platform was removed and each rat was allowed to explore the pool for 120 s. The mean time spent in all four quadrants was noted. The mean time spent in the target quadrant (TSTQ) by the animals searching for the hidden platform is noted as the index of retrieval. All the trials were completed between 09.00 and 18.00 h.

Evaluation of motor coordination

Inclined beam walking test was employed to evaluate fore and hind limb motor coordination [24]. Each animal was individually placed on metallic bar 55 cm long and 1.5 cm wide, inclined at angle of 60º ground. The motor performance of the mouse was on a scale ranging from 0 to 4. A grade of 0 was assigned to the animal that could readily traverse the beam, grade 1 was given to animal demonstrating mild impairment, grade 2 was assigned to animal demonstrating moderate impairment, grade 3 was assigned to animal demonstrating severe impairment and grade 4 was assigned to animal completely unable to walk on the beam. Inclined beam walking test was performed in animal after inducing 10 min of global cerebral ischemia and 24 h of reperfusion.

Statistical analysis

The results have been expressed as mean ± standard error mean (S.E.M.). The data of behavioral results was statistically analyzed by two-way analysis of variance (ANOVA) followed by Bonferonni's post test by using Graph pad prism Version-5.0 software. The data of biochemical results was statistically analyzed by one-way ANOVA followed by Turkey's multiple range tests. The *P*-value < 0.05 was considered to be statistically significant.

RESULTS

Extract yield, total phenols and total flavonoid content

The percentage yield of extracts and fractions and their total phenolic content and total flavonoid content of the extracts and fractions are reported in table 1.

Table 1: Yield and standardization of extract/fraction of *A. cepa.*

***Allium cepa* bulbs**	**Extract / fraction**	**Yield (On dry weight basis) % w/w**	**Total phenol content (mg GAE/100g DW) Mean^n ± S.D.**	**Total flavonoid content (mg rutin/100 g DW) Mean^n ± S.D.**
Outer scales	Methanol extract	10.41	9.35 ± 0.33	5.42 ± 0.10
	Flavonoid-rich fraction	6.53	17.89 ± 0.21	10.67 ± 0.19

n = 3; DW = dry weight basis

Effect of *A. cepa* methanol extract and flavonoid-rich fraction on cerebral infarct size

Global cerebral ischemia of 10 min followed by reperfusion for 24 h produced significant increase in cerebral infract size measured by volume method. Administration of methanol extract of outer scales of *A. cepa* (100 mg/kg and 200 mg/kg, p.o. once daily, for 28 days) and flavonoid-rich fraction of outer scales of *A. cepa* (50 mg/kg and 100 mg/kg, p.o. once daily, for 28 days) after I/R markedly attenuated ischemia

and reperfusion-induced cerebral infract size (Fig. 1). The mean infarct size in control group was 47.23% and the mean infarct size in the flavonoids rich fraction treated group was 12.22%. Thus showing a reduction of 25.88% in the infarct size.

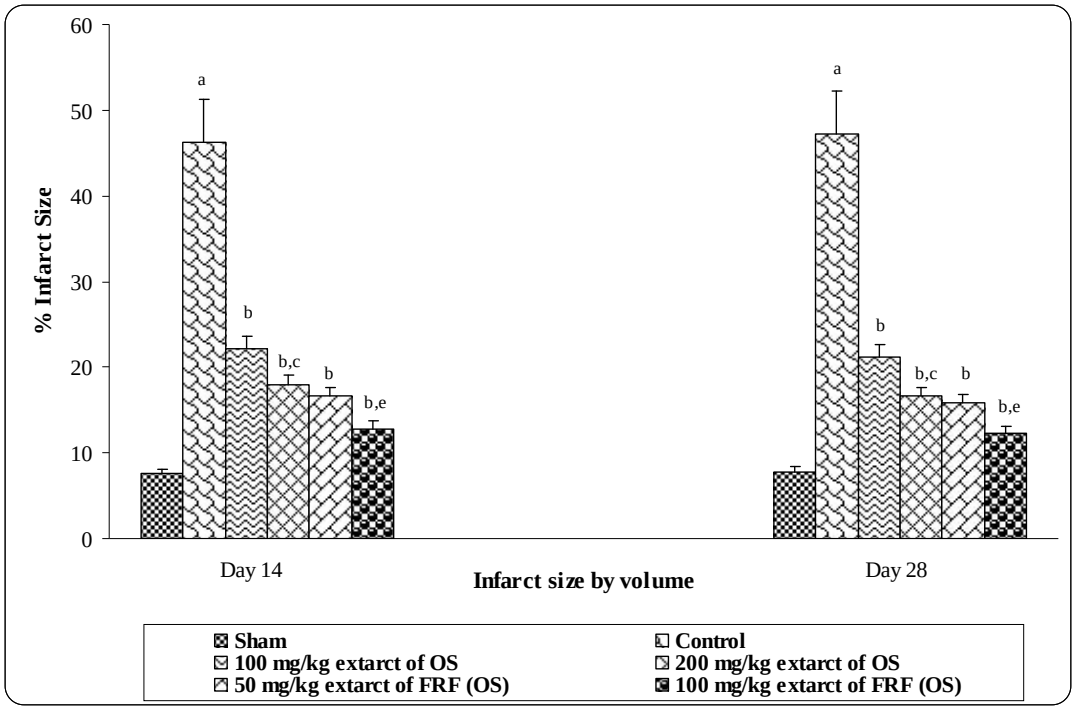


Figure 1: Effect of Methanolic Extract and Flavonoid rich fraction (FRF) of Outer scales (OS) of *Allium cepa* on Global cerebral Ischemia and Reperfusion-Induced Increase in Cerebral Infract Size. All the data are shown as mean ± SEM, n=6. a = $p < 0.05$ vs sham; b = $p < 0.05$ vs control; c = $p < 0.05$ vs 100 mg/kg methanol extract; e = $p < 0.05$ vs 50 mg/kg FRF.

Effect of *A. cepa* methanol extract and flavonoid-rich fraction on thiobarbituric acid reactive substances (TBARS) levels

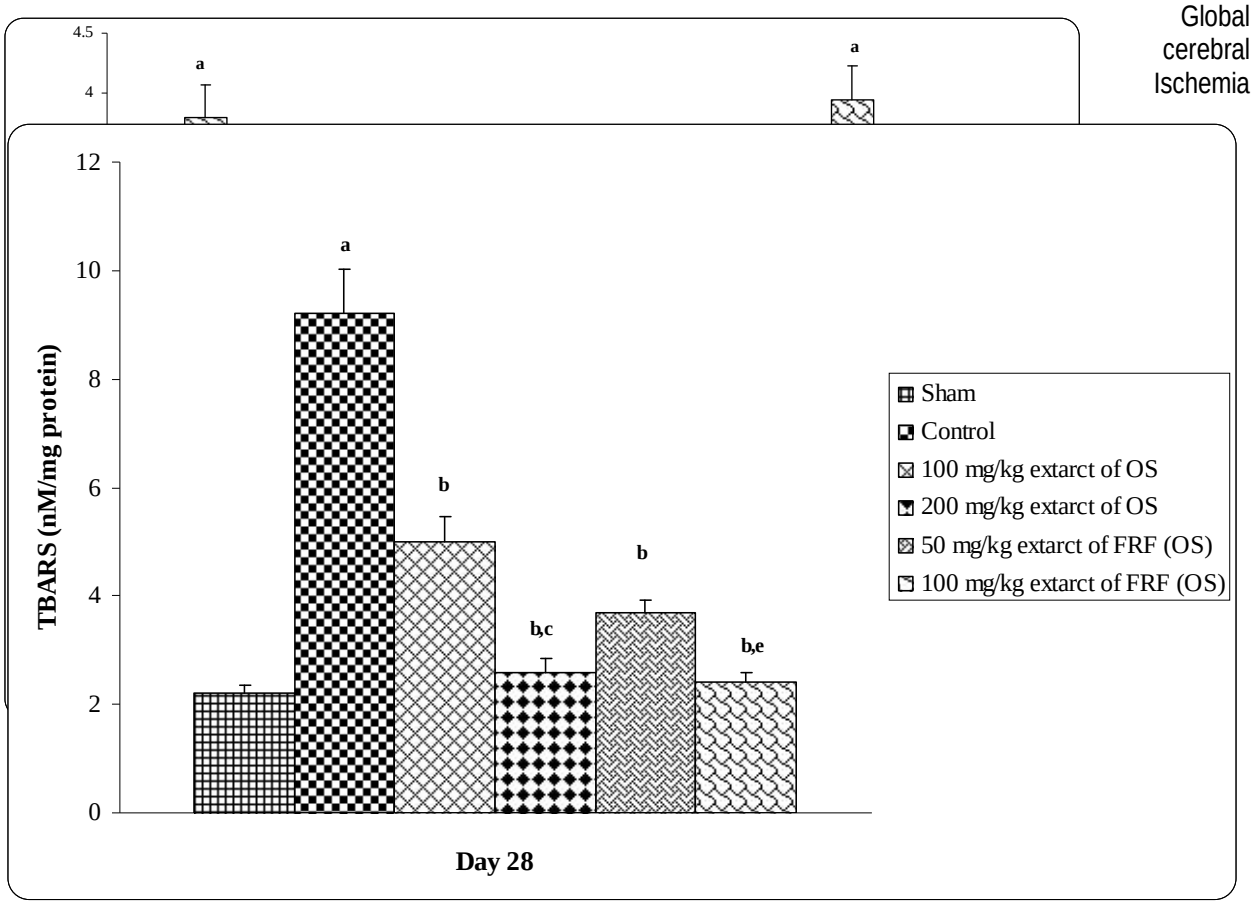
Global cerebral ischemia of 10 min followed by reperfusion for 24 h produced significant increase in TBARS concentration in brain mitochondria and supernatant fractions.

Administrations of methanol extract of outer scales of *A. cepa* (100 mg/kg and 200 mg/kg, p.o. once daily, for 28 days) and flavonoid-rich fraction of outer scales of *A. cepa* (50 mg/kg and 100 mg/kg, p.o. once daily, for 28 days) after I/R significantly decreased ischemia and reperfusion-induced increase in TBARS concentration in brain mitochondria and supernatant fractions (Fig. 2). The standardized flavonoid-rich fraction reduced oxidative stress by 23.78%

Effect of *A. cepa* methanol extract and flavonoid-rich fraction on motor performance

Global cerebral ischemia of 10 min followed by reperfusion for 24 h produced a marked impairment of motor performance as tested on the inclined-beam model. Administrations of methanol extract of outer scales of *A. cepa* (100 mg/kg and 200 mg/kg, p.o. once daily, for 28 days) and flavonoid-rich fraction of outer scales of onion bulbs (50 mg/kg and 100 mg/kg, p.o. once daily, for 28 days) after I/R significantly improved motor performance on the inclined beam (Fig. 3). Significant improvement of motor coordination was observed in the flavonoid-rich fraction treated group as compared to control group.

Figure 2: Effect of Methanolic Extract and Flavonoid rich fraction (FRF) of Outer scales (OS) of *Allium cepa* on Global cerebral Ischemia and Reperfusion-Induced Increase in Thiobarbituric Acid Reactive Substances (TBARS). All the data are shown as mean ± SEM, n=6. a = p < 0.05 vs sham; b = p < 0.05 vs control; c = p < 0.05 vs 100 mg/kg methanol extract; e = p < 0.05 vs 50 mg/kg FRF.



Effect of *A. cepa* methanol extracts and flavonoid-rich fraction on memory

The control group animals showed a significant increase in day 4 ELT, when compared to day 4 ELT of sham group mice, indicating impairment of acquisition. Further these animals showed a significant reduction in day 5 TSTQ when compared to day 5 TSTQ of sham group mice, indicating impairment of memory as well.

Administration of *A. cepa* methanol extract prevented rise in day 4 ELT, indicating reversal of I/R induced impairment of acquisition. Further the same extract also improved the day 5 TSTQ indicating improvement of memory (Fig. 4a & b). The flavonoid-rich fraction of outer scales showed significant increase in the TSTQ thereby indicating improvement of cerebral ischemia and reperfusion induced memory loss.

Figure 3: Effect of Methanolic Extract and Flavonoid rich fraction (FRF) of Outer scales (OS) of *Allium cepa* on Global cerebral Ischemia and Reperfusion-Induced Impairment of Motor Performance. All the data are shown as mean ± SEM, n=6. a = p < 0.05 vs sham; b = p < 0.05 vs control; c = p < 0.05 vs 100 mg/kg methanol extract; e = p < 0.05 vs 50 mg/kg FRF.

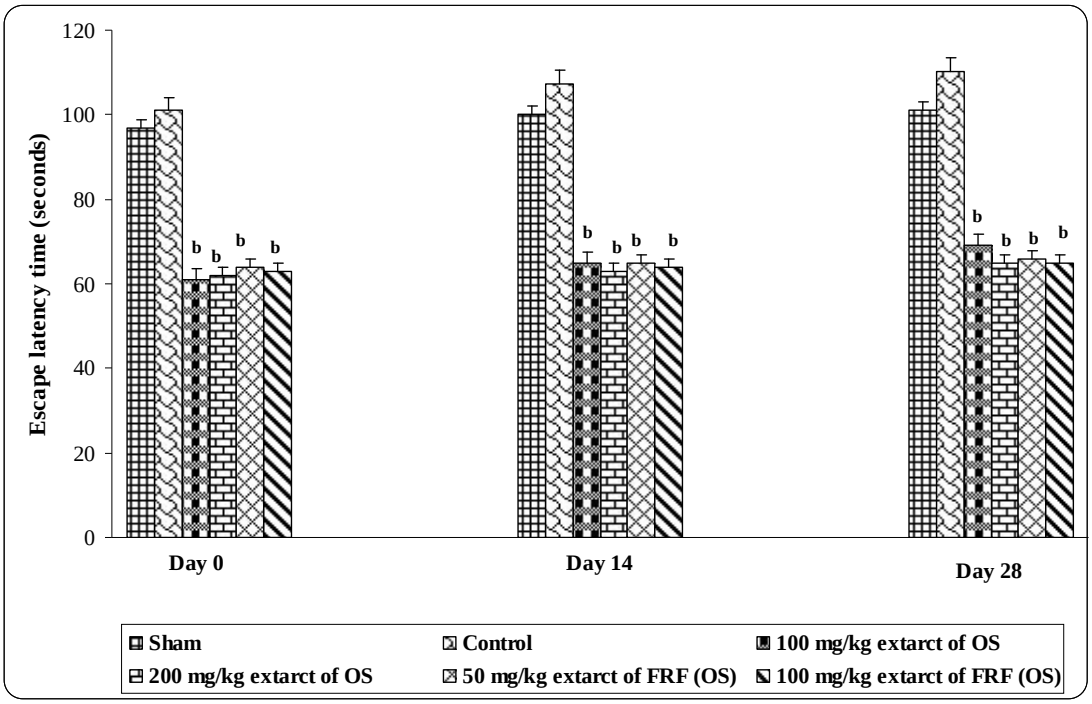


Figure 4a: Effect of Methanolic Extract and Flavonoid rich fraction (FRF) of Outer scales (O.S.) of *Allium cepa* on Global cerebral Ischemia and Reperfusion-Induced Escape Latency Time (ELT). All the data are shown as mean ± SEM, n=6. a = $p < 0.05$ vs sham; b = $p < 0.05$ vs control.

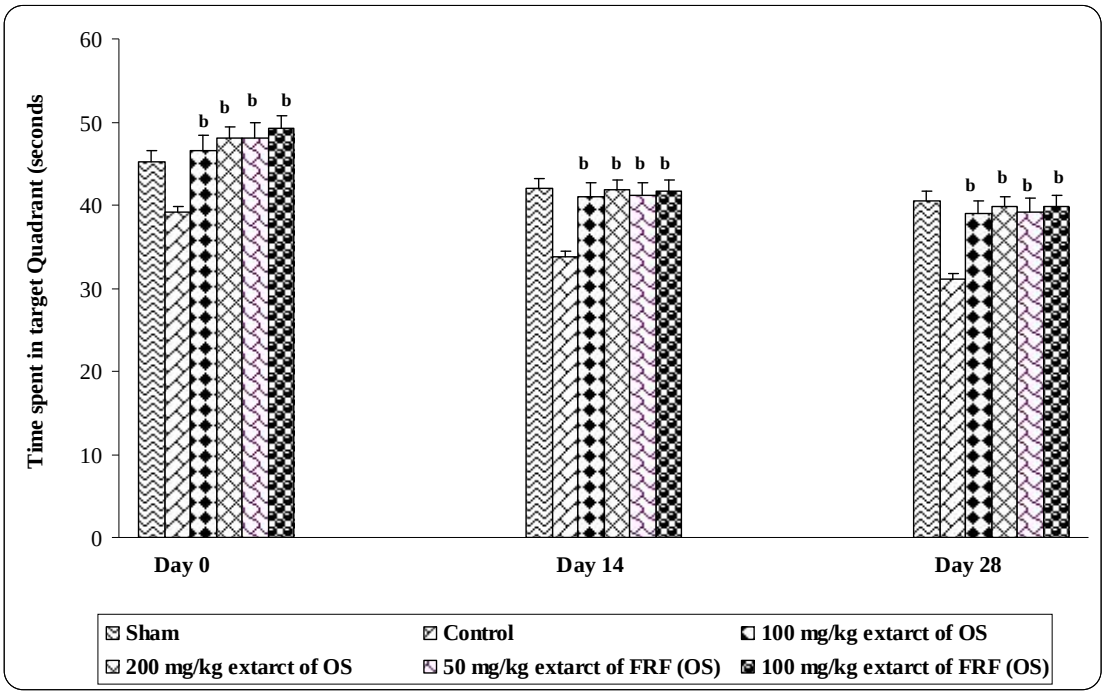


Figure 4b: Effect of Methanolic Extract and Flavonoid rich fraction (FRF) of Outer scales (O.S.) of *Allium cepa* on Global cerebral Ischemia and Reperfusion-Induced Time Spent in Target Quadrant (TSTQ). All the data are shown as mean ± SEM, n=6. a = $p < 0.05$ vs sham; b = $p < 0.05$ vs control.

DISCUSSION

A. cepa has been shown to have neuroprotective activity in pre-treatment of global cerebral ischemia and reperfusion injury [25]. Phytochemical screening tests on the bioactive methanol extract revealed the presence of flavonoids; hence in the present study methanol extract and their flavonoid-rich fraction was examined to evaluate their neuroprotective potential in mice for the treatment of I/R induced cerebral injury. Methanol extract and flavonoid-rich fraction of outer scales of *A. cepa*, standardized on the basis of total phenolic and flavonoid content, were used in the present investigations. Global cerebral ischemia was induced by BCAO for 10 min followed by reperfusion for 24 h. It is well documented that transient global ischemia causes neurological abnormality and oxidative stress [26]. Triphenyltetrazolium chloride (TTC) staining has been employed in present study to determine the area of infarction in brain tissue. TTC is a water-soluble dye that is reduced to formazone by the enzyme succinate dehydrogenase and cofactor NAD, present in mitochondria and stain viable tissue deep red in color. Ischemic tissue with damaged mitochondria remains unstained [27]. Present investigations revealed that treatment with methanol extract and flavonoid rich-fraction of *A. cepa* offered effective protection against neuronal damage induced by BCAO as it markedly reduced the infarct size.

Free radicals have been implicated in cerebral ischemia and reperfusion-induced neuronal injury [28]. Free radicals promote lipid peroxidation [29] which results in the alteration in permeability and fluidity of membrane [30]. Reactive oxygen species (ROS) produces malondialdehyde (MDA), an end product of lipid peroxidation. MDA react with thiobarbituric acid and is thus estimated as thiobarbituric acid reactive substances (TBARS). Therefore in the present study MDA was estimated using TBARS assay to estimate extent of ROS formation. In the present investigation treatment with flavonoid-rich fraction of *A. cepa* produced the most significant decrease in the global I/R-induced increase in the level of TBARS as compared to control.

The hippocampus is involved in the regulation of memory. Hippocampal neurons are highly susceptible to ischemia and reperfusion-induced injury [31]. Therefore, Morris water maze has been employed in present study to evaluate impairment of long-term memory as a result of cerebral ischemia and reperfusion. Cerebral ischemia is also documented to impair sensory motor ability [31], and thus inclined beam walking test has been used in the present study to investigate the effect of cerebral ischemia and reperfusion on motor performance. Administration of methanol extract and flavonoid-rich fraction of *A. cepa* after inducing global cerebral ischemia has prevented cerebral ischemia-induced motor incoordination, and impairment of long-term memory.

In order to ensure reproducibility of preparation of bioactive extract/fractions employed in the present study, these were standardized with reference to total phenolic content and total flavonoid content. It is very well documented that phenolic compounds are cytoprotective, neuroprotective and improve cognitive functions. [32] These protective effects are strongly correlated with the potent anti-oxidant activity [33]. While working with plants, researchers have reported that flavonoid-rich fractions are responsible for neuroprotection, anti-neuroinflammatory effect, anti-apoptotic activity and cognitive enhancement in ischemic brain injury [34]. Flavonoids have been credited for multifaceted activities for the management of I/R induced injury [35]. Flavonoids such as quercetin, kaemferol, apigenin are neuroprotective due to multi-target anti-ischemic effects [36]. In the present study also the neuroprotective effects may be attributed to the presence of phenolic compounds and flavonoids in the bioactive extract and fraction, and their property to scavenge reactive oxygen species. This is evident from the fact that the fraction with the highest quantity of total phenols and flavonoids, i.e. the flavonoid-rich fraction of outer scales of *A. cepa* bulbs, produced the most significant improvement in the I/R induced cerebral injury, reduced oxidative

stress, improved long-term memory and motor coordination. The results of present work on methanol extract and flavonoid-rich fraction of *A. cepa* bulbs show that *A. cepa* has neuroprotective and antioxidant activity and is useful for both prevention as well as treatment of I/R induced cerebral damage. Thus we may conclude that flavonoid-rich fraction of *A. cepa* may be developed for clinical use in the management of cerebral stroke.

CONCLUSION:

The present study was designed to investigate the effect of methanol extract and flavonoid-rich fraction of outer scales of *A. cepa* bulbs against post-cerebral injury in mice. The findings of the current investigations indicated that flavonoid-rich fraction of the outer scales of *A. cepa* demonstrated the most significant reduction in cerebral damage and oxidative stress. It also ameliorated the damage to memory and motor coordination. This bioactive fraction found to contain high amount of total phenolics and total flavonoid content. Thus the standardized flavonoid-rich fraction of the outer scales of *allium cepa* may be a potential candidate for the treatment of post-cerebral damage.

Declaration: There is no conflict of interest

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