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Neuroprotective effect of *Ziziphus spina-christi* on brain injury induced by transient global cerebral ischemia and reperfusion in rat

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Abstract

This study evaluated the protective effect of *Ziziphus spina-christi* on the cerebral oxidative stress and damage induced by ischemia. Male Wistar rats were divided randomly into six groups (seven in each group): Control group did not undergo surgery and received distilled water; shame group underwent surgery without ischemia; ischemic group underwent ischemia without any medication; extract-treated groups underwent ischemia and orally received 50, 100, 200 mg/kg/day doses *Z. spina-christi* extract. After behavioral tests, anti-oxidant capacity and malondialdehyde level of brain and serum were determined. Treatment of ischemic rats with extract significantly increased the frequency of passes through the hidden platform. *Z. spina-christi* improved motor coordination and balance. Administration of the extract into the ischemic rats prolonged the shortened step-through latency. *Z. spina-christi* extract significantly reduced the malondialdehyde level of brain and serum and improved serum and brain anti-oxidant capacity.

Introduction

Cerebral ischemia occurs when the blood supply to the brain is halted or reduced (Bhuiyan and Kim, 2010). Several factors such as thrombosis, embolism, and systemic hypoperfusion can lead to ischemia (Lakhan et al., 2009). Ischemia reduces the delivery of oxygen and nutrients to the cell which eventually leads to organ dys-function. A reduction of oxygen supply reduces cellular ATP. Required ATP is produced through anaerobic respiration. This leads to an accumulation of lactate, acidosis and finally cell death (Wells et al., 2010).

The hippocampus, which plays important roles in learning, memory and emotion, is highly vulnerable to ischemic insults. Hypoxia in this region can lead to inhibition of synaptic potential which is a key mechanism to reduce energy consumption (Meilandt et al., 2004). I/R-induced generation of reactive oxygen species can cause oxidative damage to biomolecules such as lipids, proteins and DNA (Chan, 2001). Increased production of ROS during cerebral ischemia has been considered as a major factor in delayed neuro-nal cell death, especially in hippocampal CA1 pyrami-dal neurons (Hara and Mori, 2000). Anti-oxidant agents may be effective in the treatment of memory and learning deficits. Recently, natural anti-oxidants from plant materials have been reported to reduce neuronal damage caused by free radical (Pham-Huy et al., 2008).

Sidr tree (*Ziziphus spina-christi*, Family Rhamnaceae) is an evergreen tree native to the South of Iran. It has pulpy edible fruits. Traditionally, its leaves are used to treat sores, pneumonia, dysentery, ulcer and as an antiseptics. The leaves contain phytosterols such as β-sitosterol, glycoside, flavonoids, saponins, tannins and lactone (Asgarpanah and Haghighat, 2012). Hydro-alcoholic extract of *Ziziphus spina-christi* was found to be effective against scopolamine-induced anxiety in rats (Setorki, 2016). There is no previous study on the learning and memory-enhancing activities of *Z. spina-*

christi. So, we aimed to determine the effect of ethanol extract of *Z. spina-christi* on memory and learning deficits induced by ischemia and also to determine its effects on the ischemia induced motor dysfunction.

Materials and Methods

Preparation of hydroalcoholic extract

Dried and finely powdered leaves of *Z. spina-christi* were macerated using 70% ethanol for 7 days. The resulting extract was then filtered, and the filtrate was concentrated to dryness using vacuum distillation at 40°C. Different concentrations of the extract were prepared using distilled water (Naseri, 2003).

Determination of anti-oxidant activity

Briefly, 2 mL of *Z. spina-christi* extract at different concentrations were mixed with 2 mL of DPPH solution (0.1 mM, in ethanol). After 15 min of incubation at room temperature in a dark, the absorbance of the reaction mixture was read at 517 nm. The mixture of ethanol (2 mL) and DPPH solution (2 mL) was considered to be control. The percentage of inhibition was calculated according to the equation below:

$$\text{Anti-oxidant activity}\% = 100 \times (A_{control} - A_{sample})/A_{control}$$

Where $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance of the sample

The concentration of extract required to inhibit 50% of DPPH radical (IC_{50} value) was calculated from the percentage of inhibition versus concentration graph (Baba and Malik, 2015).

Determination of total phenolic content

An aliquot of 0.1 mL of *Z. spina-christi* extract (1 mg/mL) was mixed with 0.5 mL of Folin-Ciocalteu reagent and 0.4 mL of 7.5% sodium carbonate solution. After incubation at room temperature for 30 min, the absorbance of the mixture was read at 750 nm. A standard calibration curve was plotted using gallic acid at different concentrations. Total phenolic content was calculated as mg of gallic acid equivalents per g of sample (Baba and Malik, 2015).

Determination of total flavonoid content

An aliquot of 0.5 ml of *Z. spina-christi* extract (1 mg/mL) was mixed with 1 mL of 2% aluminium chloride and 6 mL of 5% potassium acetate. The absorbance of the reaction mixture was read at 734 nm after 40 min incubation at room temperature. A standard curve was prepared using different concentrations of rutin solution. Total flavonoid content was expressed in mg of rutin equivalents per gram of dried extract by using a standard curve of rutin (Baba and Malik, 2015).

Animals and treatments

All experiments were conducted on adult male Wistar rats, weighing 250–300 g. Rats were kept under standard laboratory conditions (12 hours light/dark cycle and 22 ± 2°C) with freely accessed standard laboratory food and water. All experiments were conducted according to the guide for the care and use of laboratory animals. Rats were divided randomly into six groups, each group consist of seven animals. Control group did not undergo surgery and received distillated water orally; shame group underwent surgery without ischemia and only received distilled water; ischemic group underwent surgery without the administration of any drugs; extract-treated groups underwent ischemia and received *Z. spina-christi* extract at 50, 100 and 200 mg/kg/day doses administered orally.

Induction of ischemia

Animals were anesthetized by 400 mg/kg chloral hydrate intraperitoneally injected. Under deep anesthesia, a midline incision was given on the anterior region of the neck to expose carotid sheath and then common carotid arteries were identified and isolated carefully from the vagosym pathetic nerve. Acute ischemic stroke was induced by temporary occlusion of the common carotid arteries (Kabiri and Setorki, 2016). Blood flow was restored after 60 min of occlusion. During the surgical procedure, the animal's body temperature was kept at 37°C with an infrared heat lamp. This surgical technique produces adequate forebrain ischemia in rats. *Z. spina-christi* extract was administered daily after induction of ische-mia. Five days after induction of ischemia, spatial memory task was performed for 5 days and subsequently passive avoidance test was performed for 4 days. Rotarod test was conducted on the last day of study. After rotarod test, animals were put under deep anesthesia. Cardiac blood samples were collected and brain was removed. After removal of the brain, hippo-campus and cerebral cortex were separated on the ice and used for biochemical analysis. Blood was centrifuged and serum was separated and used for biochemical analysis (Rabiei et al., 2014a).

Spatial memory test

The Morris water maze was used to assess spatial memory and learning. This device consists of a black circular tank (60 cm in height and 136 cm in diameter), which is filled with water (20 ± 1°C) to 25 cm depth. A hidden circular platform (10 cm in diameter) was placed in the Southwest quadrant center and submerged 1 cm below the water surface. The maze was placed in a room with many visual cues such as refrigerator, bookshelves, and poster. Each rat received eight trials, divided into two sessions of four each per day, for five consecutive days. During any trial, the rats were individually placed in water. They were allowed to swim for a 60 sec duration to find the hidden platform. If they spotted the platform, they were let to rest on it for 30 sec. If they could not find the platform, they were gent-

ly guided to the platform. After each session, rats were returned to their home cages for resting. The time latency to reach the platform was recorded by a video tracking system. At 5th day as a probe trial, the platform was raised above the surface of the water and time spent by the animals in the target quadrant (Q1) and frequency of passing through the target platform where platform was located on days 1–4 was recorded (Rabiei et al., 2014a).

Passive avoidance test

Passive avoidance learning of animals was evaluated using shuttle box apparatus. Shuttle box consists of two equally sized sections, separated by a guillotine door. An electric light bulb illuminated one section while the other remained in dark. Mild electric shocks could be given through the grid floor and the animal remembered the shock when tested again later. This test is based on the fear and usually performs during 4 consecutive days. At first, the device was cleaned and the test began. In the first and second days, each rat was placed in the device and allowed to habituate for 5 min. On the third day, each animal was placed in the light section for 20 sec, then the guillotine door was opened and the latency of animal to enter into the dark compartment was recorded as initial latency (T1). After rat entering the dark compartment, the door was immediately closed and a mild electric shock was given (1 mA, 1 sec once). On the fourth day, each animal was placed in the light compartment and latency to enter the dark compartment was recorded as step-through latency (Rabiei et al., 2014a).

Motor coordination and balance

Motor coordination and balance were tested using the rotarod device. This device has a rotating rod with adjustable speed from 0 to 40 rpm. In the present study, the rotational speed was 10 rpm and grab was 7 rpm[2]. Rats were first trained to walk on the rotating rod. Animals in each extract treated groups or control group were placed on the rotating rod of rotarod which rotates for a maximum of 300 sec and the length of time for the rat to maintain balance was recorded as a rat resistance time. This experiment was repeated three times for each rat and their mean was calculated (Rabiei et al., 2014b).

Measurement of serum anti-oxidant capacity

Three solutions were used for this purpose. Solution 1: 1.5 mL of sodium acetate and 8 mL of concentrated acetic acid which diluted to 500 mL with distilled water; Solution 2: 270 mg of iron (III) chloride which dissolved in 50 mL of distilled water; Solution 3: 47 mg of treeazin which dissolved in 40 mL of HCL. Working solution was prepared by mixing solution 1 (10 mL), solution 2 (1 mL) and solution 3 (1 mL). Thereafter, 25 µL of serum sample was added to 5.1 mL of working solution. The resulting mixture was incubated at 37°C for 15 min and then absorbance measured at 593 nm (Rabiei et al., 2014a).

Measurement of brain anti-oxidant capacity

The anti-oxidant capacity of the brain was determined by ferric reducing anti-oxidant power (FRAP) assays. FRAP 17 working solution was prepared via mixing of 25 mL of acetate buffer, 2.5 mL of TPTZ and 2.5 mL of $FeCl_3$. Brain tissue was homogenized and the homogenate was centrifuged (1,000 xg for 10 min). 50 mL of the resulting supernatant was mixed with 5.1 mL of FRAP working solution. After 10 min of incubation, Fe^{3+} TPTZ complex was reduced to the ferrous (Fe^{2+}) form which produced an intense blue color. The absorbance of the mixture was measured at 590 nm (Rabiei et al., 2014a).

Measurement of serum MDA level

Briefly, serum (50 µL) was mixed with 0.05% BHT (50 µL), 0.44 M H_3PO_4 (400 µL) and 42 mM TBA (100 µL). The mixture was vortexed and then heated in a boiling water bath for 1 hour. After cooling at 0°C for 5 min, 250 µL of *n*-butanol was added to the mixture, vortexed, and then centrifuged at 14,000 rpm for 5 min. the absorbance of the supernatant was calculated at 532 nm (Rabiei et al., 2014a).

Measurement of brain MDA level

Brain tissue was homogenized in (1:10 wv^{-1}) pre-chilled KCL solution and transferred into a 20 mL tube. After incubation for 60 min at 37°C, the suspension was mixed with 1 mL of 5% tetrachloroacetic acids and 1 mL of 67% TBA and centrifuged for 15 min at 2,000 rpm. The resulting supernatant was transferred into a new tube and heated to 90℃ for 10 min in water bath. After cooling, its absorbance was measured at 535 nm (Rabiei et al., 2014a).

Measurement of serum nitrate and nitrite level

Serum total nitrate and nitrite level was measured by the cadmium-reduction procedure. Serum was deproteinized with sodium hydroxide-zinc sulfate mixture. After centrifugation, the supernatant was saved and mixed with glycine buffer. Cadmium granules were activated by rinsing with deionized distilled water and swirling in a $CuSO_4$ solution in glycine-NaOH buffer (Naseri, 2003). Freshly activated cadmium granules were added to the pretreated deproteinized serum. Following constant stirring for 10 min, the resulting solution was poured into a new tube and mixed with Griess reagent. Then it was incubated for 10 min in the dark at room temperature and finally, mixed with Griess 2 reagent and the absorbance was measured spectrophotometrically at 540 nm (Rabiei et al., 2014a)

Statistical analysis

The Kolmogorov–Smirnov test was used for checking

the normality assumption. The homogeneity of variances was determined using Levene's test. In the case of equal variances, one-way ANOVA followed by Duncan was used to compare the means between experimental groups while in the case of unequal variances, Dunnett's T3 was used.

Results

Table I shows the anti-oxidant capacity of *Z. spina-christi* extract according to DPPH method. The IC_{50} of *Z. spina-christi* extract was 75.8 µg/mL. Total phenolic and flavonoid contents of the *Z. spina-christi* extracts were 800.6 and 334 mg respectively.

Table I

Anti-oxidant capacity of *Z. spina-christi* extract

Concentration (µg/mL)	DPPH radical scavenging activity inhibition (%)
70	96.1
60	83.4
50	75.8 (IC_{50})
40	63.5
30	47.5
20	35.2
10	14.3

Morris water maze test

The latencies to reach the platform during 4 consecutive days are shown in Figure 1A and B. The control group showed better learning compared with the hypoxic-ischemic group. On the 4th day, the control group had significantly shorter latency time when compared with the ischemic group ($p<0.05$, Figure 1A). At 1st, 2nd and 3rd days, ischemic rats showed shorter latency than extract-treated ischemic rats. On the 4th day, the latency of extract-treated ischemic groups decreased compared to the ischemic group, however, these decreases were not statistically significant (Figure 1B). The time spent in target quadrant during the probe trial is shown in Figure 1C. As Figure 1C shows, during the probe test, control group spent significantly more time in the target quadrant (which the hidden platform was there before)

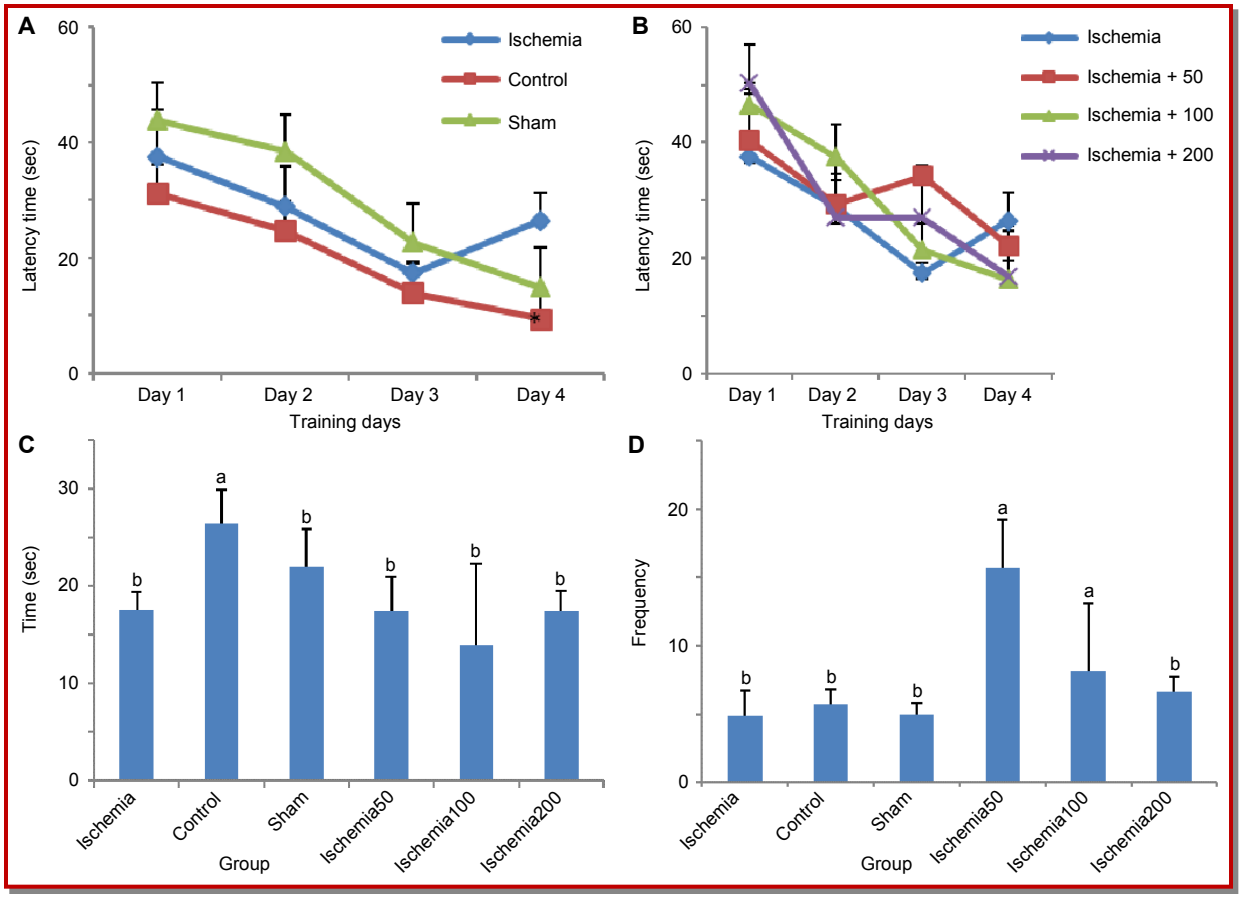

Figure 1: A) Comparison of latency time (sec) to find the platform between ischemic and control groups. B) Comparison of latency time (sec) to find the platform between ischemic and extract-treated ischemic groups. C) The time spent in target quadrant during the probe trial. D) The frequency of passing through the target platform during probe trial, All data were expressed as the mean ± SEM; a: significant differences with b at $p<0.05$

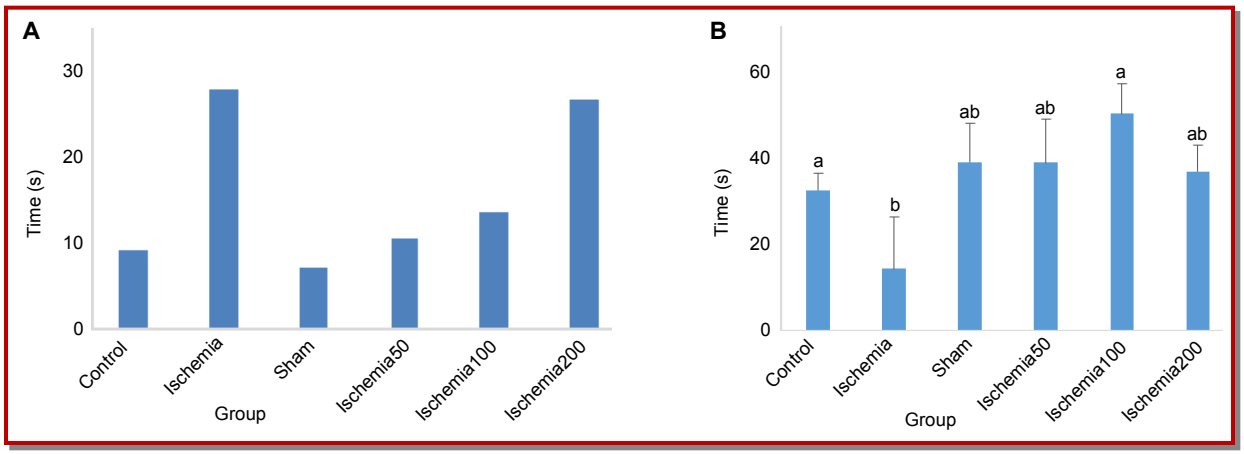

Figure 2: Comparison of: A) The initial latency and B) The step-through latency between different groups; a: significant differences with b at p<0.05 and ab: no significant differences between a and b at p<0.05

compared with the ischemia, sham and extract treated groups (p<0.05). The administration of *Z. spina-christi* leaf extract at different concentrations of 50, 100 and 200 mg/kg into ischemic rats did not cause any significant change in the time spent in the target quadrant (p>0.05). As presented in Figure 1D, there was no significant difference in the frequency of passing through the hidden platform between control and ischemia group (p>0.05). The administration of *Z. spina-christi* leaf extract increased the frequency of passing through the hidden platform (p<0.05)

Passive avoidance test

The initial latency (T1) and step-through latency (T2) in the passive avoidance test are shown in Figure 2. There was no significant difference between different groups in initial latency. The step-through latency (T2) of ischemic animals was shorter than that of the control group but not significantly. Oral administration of *Z. spina-christi* extract at a dose of 200 mg/kg into ischemic rats prolonged the shortened step-through latency.

Motor-coordination test

As shown in Table II, motor coordination and balance decreased in the ischemic group compared to the control group. Treatment of ischemic rats with hydro-alcoholic extract of *Z. spina-christi* at different doses improved motor coordination and balance.

Brain and serum anti-oxidant capacity

There was the significant difference in hippocampus, cortex and serum anti-oxidant capacity between ischemic and control groups (p<0.05; Table III). The administration of *Z. spina-christi* extract into ischemic rats didn't affect the anti-oxidant capacity of the hippocampus. *Z. spina-christi* extract at doses of 100 and 200 mg/kg significantly improved cortex anti-oxidant capacity. The administration of *Z. spina-christi* extract (100 mg/kg) into ischemic rats significantly increased the anti-

Table II

Bar descent latencies following motor coordination and balance test

Group	Bar descent latencies (sec)
Control	117.9 ± 16.0^{a}
Ischemia	48.0 ± 3.0^{b}
Sham	86.4 ± 21.0^{ab}
Ischemia plus extract (50 mg/kg/day)	89.5 ± 15.0^{ab}
Ischemia plus extract (100 mg/kg/day)	109.9 ± 12.0^{ab}
Ischemia plus extract (200 mg/kg/day)	51.4 ± 32.0^{b}

a: significant differences with b at p<0.05 and ab: no significant differences between a and b at p<0.05

Table III

Effect of *Z. spina-christi* extract on anti-oxidant level

Group	Anti-oxidant level at		
	Hippo-campus	Cortex	Serum
Control	946.2 ± 3.7^{a}	290.0 ± 1.9^{cb}	556.4 ±1.7^{ab}
Ischemia	483.4 ± 6.2^{b}	261.5 ± 2.1^{c}	97.7 ± 1.2^{c}
Sham	432.2 ± 5.8^{b}	421.0 ± 1.1^{abc}	454.3 ± 1.3^{abc}
Ischemia + extract (50 mg/kg/day)	317.0 ± 4.5^{b}	343.1 ± 3.7^{abc}	400.5 ± 1.2^{bc}
Ischemia + extract (100 mg/kg/day)	475.4 ± 8.5^{b}	534.5 ± 2.8^{ab}	809.7 ± 1.3^{a}
Ischemia + extract (200 mg/kg/day)	349.0 ± 3.2^{b}	574.0 ± 3.7^{a}	454.3 ± 1.8^{abc}

a: significant differences with b and c, b: significant differences with a and c, c: significant differences with a and b, ab: no significant differences between a and b and significant differences with c, bc: no significant differences between b and c and significant differences with a, abc: no significant differences between a, b and at p<0.05

Table IV

Effect of *Z. spina-christi* extract on MDA level

Group	Malondialdehyde (MDA) level at		
	Hippo-campus	Cortex	Serum
Control	158.3 ±1.0[b]	167.7 ± 1.1[b]	1618.0 ± 6.3[a]
Ischemia	414.2 ± 1.4[a]	306.9 ± 1.0[a]	3227.9 ± 3.1[a]
Sham	237.0 ± 1.7[b]	377.3 ± 1.2[a]	1961.4 ± 3.0[a]
Ischemia + extract (50 mg/kg/day)	261.6 ± 3.2[b]	236.9 ± 1.1[b]	1274.5 ± 6.2[b]
Ischemia + extract (100 mg/kg/day)	688.7 ± 5.7[a]	301.9 ± 8.0[a]	1685.4 ± 5.0[a]
Ischemia + extract (200 mg/kg/day)	576.9 ± 2.1[a]	601.7 ± 2.1[a]	2277.4 ± 9.0[a]

a: significant differences with b at p<0.05

oxidant capacity of the serum.

Brain and serum malondialdehyde level

As Table IV shows, there was no significant difference in brain and MDA level between ischemic and control groups. The administration of *Z. spina-christi* extract at 50 mg/kg concentration into ischemic rats significantly reduced the MDA level of brain and serum.

Serum nitric oxide level

The ischemia group had the highest serum level of nitric oxide and the treatment of ischemic rats with 50, 100 and 200 mg/kg of *Z. spina-christi* extract decreased the serum level of nitric oxide, however not significantly (p>0.05, Table V).

Discussion

In the present study, the neuroprotective effect of leaf extract of *Z. spina-christi* on brain injury induced by transient global cerebral ischemia and reperfusion was investigated in rat. In the Morris water maze test, ischemic rat had significantly higher time latency to reach the platform compared to the control group. This finding supports the previous findings that ischemia leads to spatial memory and learning deficit (Szyndler et al., 2006, Dabaghian et al., 2015). Frequency of passes through the hidden platform was significantly higher in the groups receiving 50 and 100 mg of extract compared to that of ischemic group (p<0.05). These results suggest that *Z. spina-christi* extract can reduce the ischemia-induced, cognitive impairment.

Table V

Effect of *Z. spina-christi* on serum nitric oxide level

Group	Nitric oxide level
Control	8.7 ± 8.7[a]
Ischemia	28.7 ±2.4[b]
Sham	8.1 ± 2.3[a]
Ischemia plus extract (50 mg/kg/day)	15.8 ± 1.9[ab]
Ischemia plus extract (100 mg/kg/day)	14.0 ± 5.1[ab]
Ischemia plus extract (200 mg/kg/day)	13.3 ± 5.4[ab]

a: significant differences with b at p<0.05 and ab: no significant differences between a and b at p<0.05

The shuttle box apparatus was used to assess passive avoidance learning of rats. There were no significant difference between different groups in initial latency. The step-through latency (T2) of ischemic animals was shorter than that of the control group. Oral administration of *Z. spina-christi* extract at a dose of 200 mg/kg into ischemic rats prolonged the shortened step-through latency. These findings suggested that oral administration of *Z. spina-christi* extract can improve passive avoidance memory deficit induced by cerebral ischemia.

In our study, induction of cerebral ischemia reperfusion leads to motor incoordination and further treatment with hydroalcoholic extract of *Z. spina-christi* improved motor coordination and balance.

Dangoggo et al. (2012) reported that ethanol extract of the leave of *Z. spina-christi* contains alkaloid, tannins, saponins, glycosides, steroids, flavonoids and terpeniods. A previous study showed that flavonoid compounds are able to reverse age-related spatial memory and spatial learning deficits (Rendeiro et al., 2009). Positive effects of terpenoids compounds on the memory and learning was also reported (Yoo and Park, 2012).

The results of the current study showed that the induction of transient cerebral ischemia in rats decreased the anti-oxidant power and increased the MDA levels of serum and brain tissues. It has been reported that ischemia-induced memory and learning deficits are accompanied with oxidative damage. Reactive oxygen species (ROS) are one of the most important factors that induce neuronal damage during ischemia/reperfusion (Sugawara and Chan, 2003). Damaging free radicals such as nitrogen oxide, superoxide, and peroxynitrite were shown to increase after IR (Seo et al., 2001). IR has also been found to be accompanied by reduced anti-oxidant capacity of brain and increased brain MDA level. Therefore, anti-oxidant agents may be effective in the treatment of memory and learning deficits (Ikeda et al., 2003). The administration of *Z. spina-christi* extract increased the anti-oxidant capacity and decreased serum level of MDA in comparison with control group.

The results of the current study indicated that *Z. spina-*

christi leaf extract showed neuroprotective activity against induced brain ischemia, so it could reduce the brain damage caused by ischemia and reperfusion. The mechanism involved in this phenomena may be associated with increased activity of anti-oxidant defense system and inhibition of oxidative stress in the brains of rats. Other studies also reported the anti-oxidant activity of *Z. spina-christi* extract (Abalaka et al., 2011, Michel et al., 2011). The growing trend toward the anti-oxidants is due to their ability to protect the body against damages caused by reactive free radicals that are produced during various disorders. The main phytochemical constituents of this plant are including phenolic, flavonoids, alkaloids and saponins (Asgarpanah and Haghighat, 2012). Abdel-Wahhab et al. (2007) evaluated the anti-oxidant effect of methanolic *Z. spina-christi* leaf extract on the oxidative stress of aflatoxin in rats. They reported that the treatment of rats with *Z. spina-christi* extract significantly improved all biochemical parameters and histological profiles of liver, kidney and testis. They concluded that *Z. spina-christi* extract had a markedly protective effect against a flatoxicosis. It is well documented that *Z. spina-christi* extract is enriched in flavonoid compounds, which can reduce the production of ROS and also show good scavenging activity (Abdel-Wahhab et al. (2007). These findings could explain the observed enhancement of the total anti-oxidant capacity and the reduced MDA level in rats treated with *Z. spina-christi* leaves extract.

Conclusion

The leaf extract of *Z. spina-christi* showed neuroprotective activity against induced brain ischemia. The mechanism involved in this phenomenon may be associated with increased activity of anti-oxidant defense system and inhibition of oxidative stress in the brains of rats.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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