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Original Research Article

Neuroprotective effects of *Withania coagulans* root extract on CA1 hippocampus following cerebral ischemia in rats

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Abstract

Objective: Oxygen free radicals may be implicated in the pathogenesis of ischemia reperfusion damage. The beneficial effects of antioxidant nutrients, as well as complex plant extracts, on cerebral ischemia-reperfusion injuries are well known. This study was conducted to determine the effects of the hydro-alcoholic root extract of *Withania coagulans* on CA1 hippocampus oxidative damages following global cerebral ischemia/reperfusion in rat.

Materials and Methods: Male Wistar rats were randomly divided in five groups: control, sham operated, Ischemia/ Reperfiusion (IR), and *Withania Coagulans* Extract (WCE) 500 and 1000mg/kg + I/R groups. Ischemia was induced by ligation of bilateral common carotid arteries for 30 min after 30 days of WCE administration. Three days after, the animals were sacrificed, their brains were fixed for histological analysis (NISSL and TUNEL staining) and some samples were prepared for measurement of malondialdehyde (MDA) level and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activity in hippocampus.

Results: WCE showed neuroprotective activity by significant decrease in MDA level and increase in the SOD, CAT and GPx activity in pretreated groups as compared to I/R groups ($p<0.001$). The number of intact neurons was increased while the number of TUNEL positive neurons in CA1 hippocampal region in pretreated groups were decreased as compared to I/R group ($p<0.001$).

Conclusion: WCE showed potent neuroprotective activity against oxidative stress-induced injuries caused by global cerebral ischemia/ reperfusion in rats probably by radical scavenging and antioxidant activities.

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Introduction

Cerebral ischemia caused by decreasing or stopping the blood brain circulation and reperfusion cause over-production of reactive oxygen species (ROS) and elevation of intracellular calcium that lead to neuronal death (Doyle et al., 2008).

Oxidative stress which is resulted from ROS over-production leads to cell membrane damage by lipid peroxidation (Floyd, 2002). The brain tissue is very sensitive to oxidative damage because it has high content of polyunsaturated fatty acids and a relatively low antioxidant capacity as compared to other tissues (Awwad et al., 2011).

Among brain neurons, hippocampus CA1 neurons are particularly susceptive to ischemic insult (Bin et al., 2012; Vandresen-Filho et al., 2015) and 5-min ischemia causes death in rat hippocampus CA1 neurons which becomes visible after one day (Colbourne et al., 1999).

The antioxidant defense system including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) plays an important role in the prevention of oxidative damage (Akyol et al., 2002). Therefore, an enhancement of antioxidant activities in brain tissues may be beneficial for neuronal recovery from ischemia/ reperfusion injury.

Neuronal defense mechanisms against oxidative stress have focused on antioxidant systems. Some components of various herbs have shown free radical scavenging and neuroprotective activity in animal models of cerebral ischemia (Thiyagarajan and Sharma, 2004; Al-Omar et al., 2006).

Withania coagulans, belonging to family Solanaceae is well-known in Iran, Pakistan, Afghanistan and East India, and used in traditional medicine. It is found in Sistan and Baluchestan province of Iran and known as "Paneerbad" because its berries are used for milk coagulation (Beigomi et al., 2014).

The aqueous and hydro-alcoholic extracts from various parts of this plant have shown anti-microbial, anti-inflammatory, anti-tumor, hepatoprotective, anti-hyperglycemic, free radical scavenging and neuroprotective activities (Maurya, 2010; Budhiraja et al., 1984).

Berries extract of this plant has showed antioxidant potential against diseases which are resulted from ROS production like diabetes, ageing, and atherosclerosis (Mathur et al., 2011).

This plant contains several phyto-chemicals like steroids, alkaloids, phenolic compounds, tannins and saponins. Withaferin A, a steroidal lactone which is present in *W. coagulans* is responsible for increasing antioxidant enzyme levels (Prasad et al., 2010).

Although there are some experiments on the effects of *W. coagulans*, we did not find any histological evaluation of the effects of *W. coagulans* extract on CA1 neurons of hippocampus following cerebral ischemia. Therefore, in the present study, we have investigated the neuroprotective effects of *W. coagulans* extract against global cerebral ischemia/ reperfusion in rat hippocampus.

Materials and Methods

Plant extract

W. coagulans roots were collected from the herbarium center of Sistan and Baluchestan University in May 2014. Extraction was conducted by maceration method. Briefly, hydro-alcoholic extract was prepared by soaking of 1 g of ground root powder in a 20 ml mixture of ethanol and water (3:1) at room temperature, overnight. The extracts were filtered, the solvent was distilled off and excess solvent was completely removed by using a rotatory flash evaporator to get concentrated then, completely dried in a freeze drier and refrigerated in an airtight container.

Animals and experimental protocol

A total of 60 male Wistar rats (220-250 g) supplied by Animal Lab of Zahedan

University of Medical Sciences, Zahedan, Iran, was used. The rats were kept in standard cages; at 22°Cwith a 12:12 light/dark cycle in a controlled environment with free access to food and water. The animals fed with extract or vehicle by oral gavage for 30 days prior to ischemia except for the control group.

The animals were randomly divided into five groups (n=12): (I) control group, (II) sham group that received distilled water as vehicle and underwent surgical procedures without ischemia (III) I/R group that received distilled water as vehicle and subjected to ischemia, (IV and V) WCE+I/R groups that received *W. coagulans* extract 500 or 1000 mg/kg, respectively and underwent ischemia. The effective doses were selected accordance to previous studies (Chaudhary et al., 2003; Jaiswal et al., 2009).

Ischemia was induced by bilateral common carotid artery occlusion for 30 min (Yigitkanli et al., 2013) under anesthesia with an i.p. co-injection of ketamine (80 mg/kg) and xylazine (8 mg/kg).

Six animals from each group were decapitated under deep anesthesia 72 h after ischemia and the brains were immediately removed for biochemical assay. The other six animals were perfused transcardially and the brains were prepared for histological studies.

All animal procedures were performed according to Institutional Ethical Committee of Zahedan University of Medical Sciences (EC/93/ 8613).

Biochemical analysis

Preparation of hippocampal supernatant

For biochemical evaluation, both hippocampi from each rat were quickly isolated, weighted and homogenized (10% w/v) in cold PBS (0.1 M, pH 7.4). The homogenates were centrifuged at 1.2×10^4 g at 4°C for 20 min. The supernatants were stored at −70 ◦C for measurement of MDA level and SOD, CAT and GPx activity. The content of protein in the hippocampus homogenates was measured by the method of Bradford (Bradford, 1976).

MDA activity assay

Malondialdehyde level (an indicator of lipid peroxidation) was determined as described by Ohkawa et al. (Ohkawa et al., 1979). Briefly, in a 10 ml centrifuge tube, hippocampus homogenate (0.2 ml), sodium dodecyl sulphate (0.2 ml, 8.1%), acetic acid (1.5 ml 20%), and thiobarbituric acid (1.5 ml, 0.8%) were added. The mixture was heated at 95°C in a boiling water bath for 60 min. After cooling, 5 ml of n-butanol: pyridine (15: 1% v/v) was added. The mixture was shaken vigorously. After centrifugation, absorption of the supernatant was measured at 532 nm. MDA level was expressed as nanomoles /mg of protein.

SOD activity assay

Superoxide dismutase (SOD) activity was measured based on the method of Kakkar et al. (Kakkar et al., 1984). In brief, the assay mixture contained 0.1 ml of hippocampus supernatant, 1.2 ml of sodium pyrophosphate buffer (pH 8.3; 52 mM), 0.1 ml of phenazine methosulphate (186 μM) and 0.3 ml of nitroblue tetrazolium (300 μM). The reaction was started by addition of 0.2 ml NADH solution (750 μM). After incubation at 30 °C for 90 s, the reaction was stopped by addition of 0.1 ml of glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 2.0 ml *n*-butanol and centrifuged at 4000 g for 10 min. The absorbance of organic layer was measured spectrophotometrically at 560 nm. The activity of SOD is expressed as units/mg protein.

CAT activity assay

Catalase activity was assessed based on the ability of the enzyme to break down hydrogen peroxide (Goth, 1991). Briefly, 0.2 ml of hippocampus supernatant was mixed with 1 ml of 65 μM H_2O_2 at 37° C for 1 min. Then, 1 ml of 32.4 mM ammonium molybdate was added and the intensity of the yellow complex was read

at 450 nm. Results expressed in units/mg protein.

GPx activity assay

Glutathione peroxidase activity was assayed by the method of Paglia and Valentine (Paglia and Valentine, 1967). Briefly 10 µl of hippocampal supernatant was added to a cuvette containing potassium phosphate buffer (0.89 ml of 100 mM, pH 7.0), EDTA (1 mM), NaN_3 (1 mM), NADPH (0.2 mM), GSH reductase (1 U/ml) and GSH (1mM). The reaction was initiated by the addition of 100 µl of 2.5 mM H_2O_2 and the conversion of NADPH to NADP was monitored by a spectrophotometer at 340nm for 3 min. GPx activity is expressed as units/mg protein.

Histological studies

The brains were removed and fixed in 4% paraformaldehyde at room temperature for 24 h after being perfused transcardially. Following routine processing in paraffin, serial coronal sections of the brain were cut at 7 µm thickness for Nissl staining. Pyramidal neurons counting were done in CA1 area of the hippocampus in four sections under a light microscope at 400x magnification.

To identify the apoptotic neuronal death in the hippocampal CA1 region, sections were used for the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) staining. The assessment was done according to the Roche protocol (Roche Molecular Biochemicals kit, Germany). Briefly, sections after deparaffinization and rehydration were treated with proteinase K (20 µg/ml in 10 mM Tris/HCl, pH 7.6) for 30 min to block endogenous peroxidase activity. Then, sections were incubated with 3% H_2O_2 for 10 min, and then incubated in TUNEL reaction mixture for 1 h at 37 °C. Sections were incubated with a convertor-POD at 37°C for 30 min. Finally, the sections were incubated with diaminobenzidine substrate solution (DAB) for 15 min to visualize apoptotic cells and counterstained with hematoxylin for 30 s. Cells with deep brown-stained nuclei in four sections were counted under a light microscope at a magnification of 400x.

Statistical analysis

Data were expressed as mean ± SEM. The results were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Values of $p<0.05$ were considered significant.

Results

In the present study, we found that the hippocampus MDA levels were markedly increased in I/R group as compared to control and sham group (Figure 1A). Pretreatment with WCE (500 and 1000mg/kg) significantly reduced MDA level ($p<0.01$ and $p<0.001$, respectively) as compared to I/R group.

The results presented in Figure 1B, show SOD activity in hippocampus homogenates in all groups. Ischemia induced significant decrease in SOD activity as compared to control and sham groups ($p< 0.001$). Pretreatment with WCE (1000 mg/kg) significantly increased SOD activity nearly to control and sham groups.

Figure 1C, shows CAT activity in all groups. A significant reduction of CAT activity was observed in I/R groups as compared to control and sham groups. The rats supplemented with *W. coagulans* extract (500 and 1000mg/kg) showed a marked elevation ($p<0.01$ and $p<0.001$, respectively) in CAT activity in hippocampus region compared to I/R group.

The results indicated that GPx activity was significantly decreased in the hippocampus of I/R group in comparison with the other groups (Figure 1D). Also, a significant increase in GPx activity was observed in the WCE (500 and 1000mg/kg) groups as compared to I/R group ($p<0.01$), but it was only statistically significant in WCE500+I/R group as compared to control group.

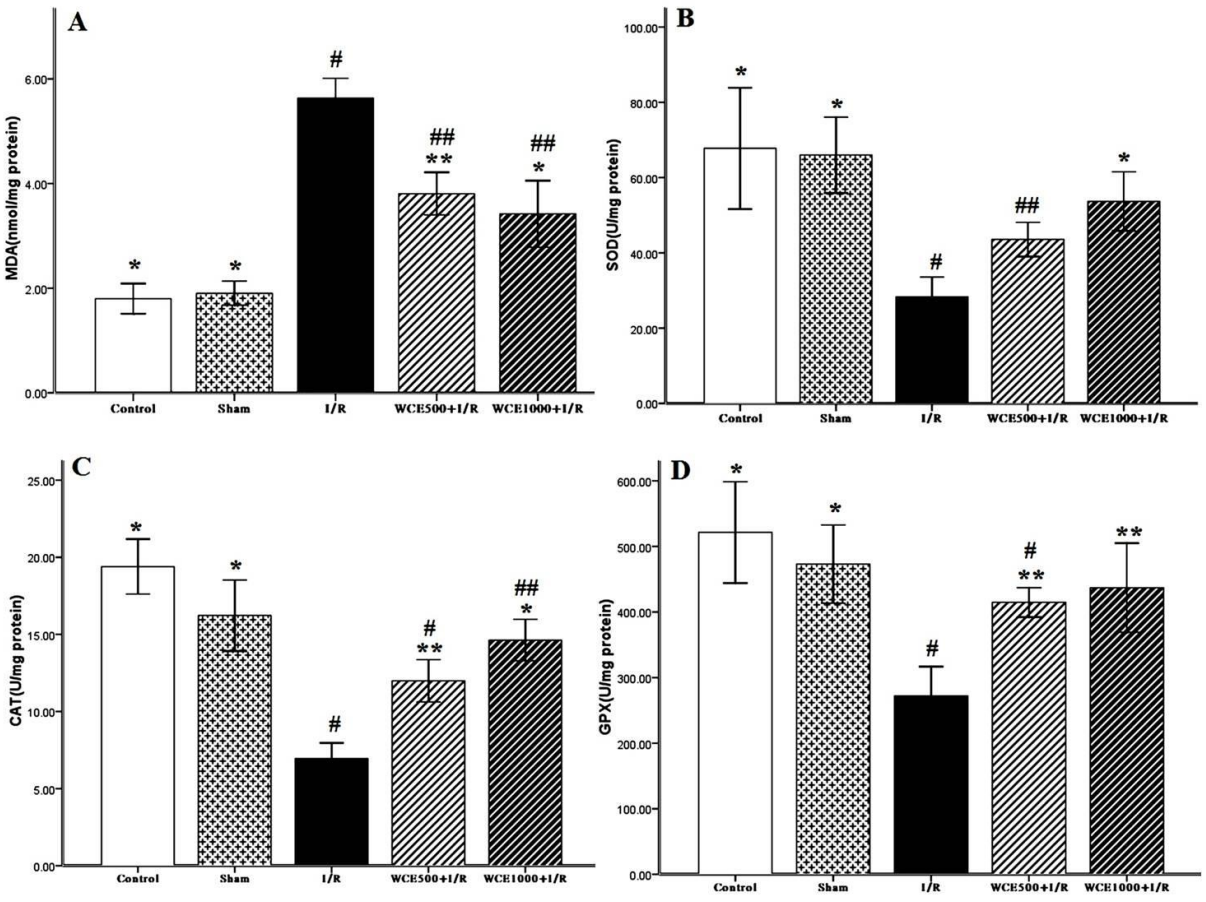

Figure 1. The effects of *Withania coagulans* extract on (A) MDA level, (B) SOD, (C) CAT and (D) GPX activity in hippocampus in different groups. Statistical analysis was done using one-way ANOVA followed by post-hoc Tukey test. Values represent means ± (SEM) of each group. *p<0.001, **p<0.01 compared to I/R group, #p<0.001, ##p<0.01 compared to control group.

Histological Assessment

The number of intact neurons in hippocampal CA1 region was counted in Nissl staininig sections in all groups. The viable neurons of control and sham groups had normal arrangement and intact nuclei (Figures 2A and B). In contrast, the most pyramidal cells in CA1 region in I/R group were completely degenerated, shrunken and had picnotic and darkly stained nucleus. The number of intact pyramidal cells was significantly lower than the control and sham groups (p<0.001) (Figure 2C).

Interestingly, the number of viable neurons in Nissl staining was significantly increased in a dose-dependent manner in the hippocampal CA1 of the pretreatment groups in comparison with I/R group (Figures 2D and E). Although the number of intact pyramidal neurons in WCE 1000+I/R group was higher than WCE 500+I/R group but the difference was not statistically significant (p>0.05).

In addition, TUNEL-positive cells were rarely detected in the hippocampal CA1 in the control and sham-operated groups (Figures 3A and B).

Ischemia/reperfusion obviously increased the number of apoptotic cells in hippocampal CA1 region when compared to control and sham groups (p< 0.001) (Figure 3C).

WCE (500 and 1000 mg/kg) pretreatment groups showed depletion in the number of TUNEL-positive cells (p<0.01 and p<0.001, respectively) in comparison with I/R group (Figures 3D and E).

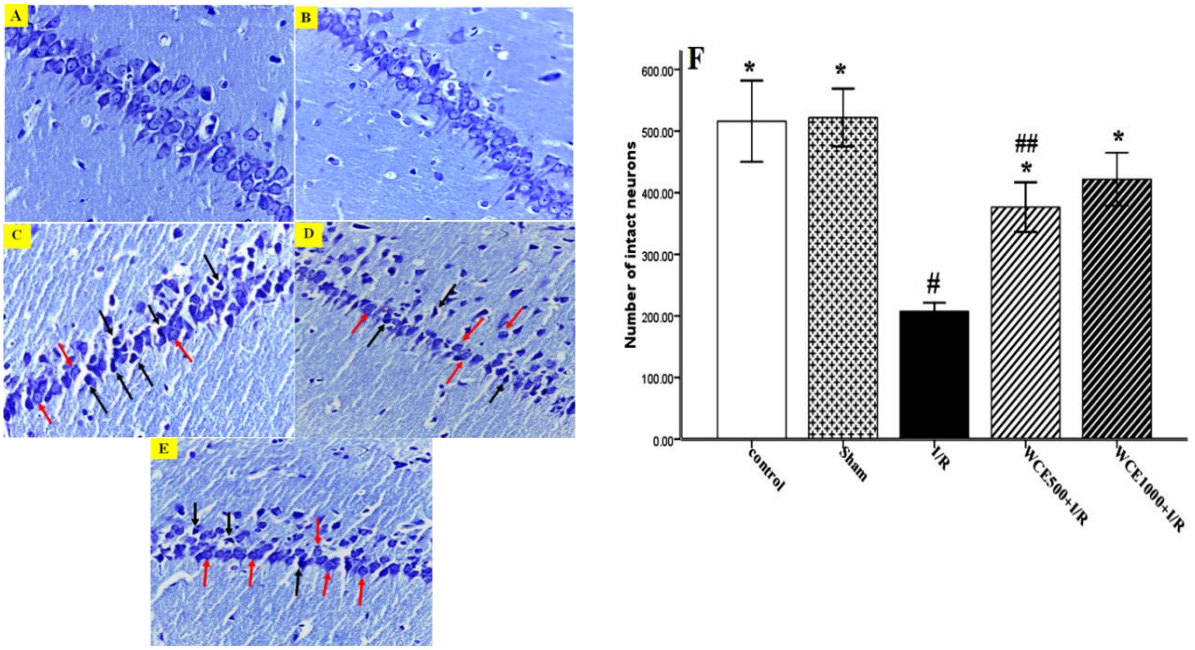

Figure 2. Nissl staining of hippocampal CA1 region in different groups. Representative pictures of Control (A), Sham (B), I/R (C), WCE500+ I/R (D), WCE1000+ I/R (E) 72 h after ischemia (400x). Red and black arrows are indicating intact cells and and necrotic cells, respectively. The graph shows the number of live hippocampal CA1 neurons. Statistical analysis was done using one-way ANOVA followed by post-hoc Tukey test. Values represent means ± (SEM) of each group. *p<0.001 compared to I/R and #p<0.001 and ##p<0.05 compared to control group.

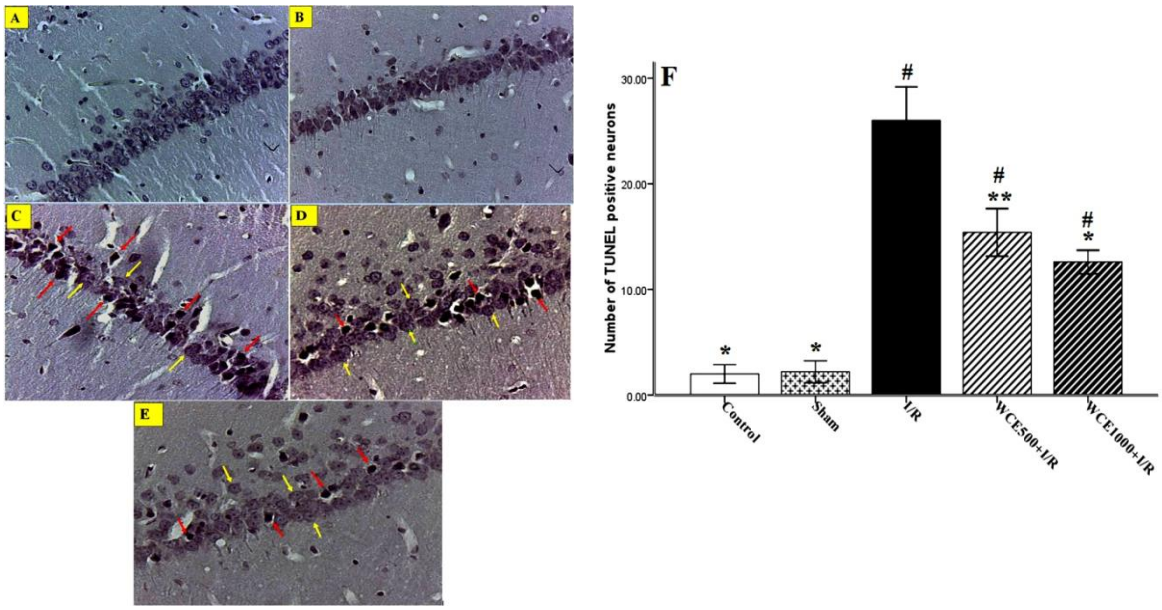

Figure 3. TUNEL staining of hippocampal CA1 region. Representative pictures of Control (A), Sham (B), I/R (C), WCE500+ I/R (D), WCE1000+ I/R (E) 72 h after ischemia (400x). Red and yellow arrows are indicating TUNEL positive cells and intact cells, respectively. The graph shows the number of TUNEL positive cells. Statistical analysis was done using one-way ANOVA followed by post-hoc Tukey test. Values represent means ± (SEM) of each group.*p<0.001 and **p<0.01 compared to I/R and #p<0.001 compared to control group.

Discussion

In the present study, we observed the potential antioxidant effects of WCE on global model of ischemia-induced oxidative stress and neuronal damage in hippocampus region. This is the first report that investigates the effect of *W. coagulans* pretreatment against global brain I/R injury in rats.

In our study, the potential neuroprotective activity of WCE was shown by reducing histological changes and MDA level in hippocampal CA1 cells and increasing the activity of antioxidant enzymes SOD, CAT, GPX during ischemia/reperfusion.

Oxidative stress which results from excessive accumulation of ROS may be implicated in cerebral ischemia. Brain is rich in polyunsaturated fatty acids that can be easily oxidized by ROS (Mohammadi et al., 2013).

MDA is a product of peroxidized polyunsaturated fatty acids (Ramana et al., 2013).

The previous studies have reported that reperfusion injury in rat brain causes significant increase in MDA level (Gupta and Sharma, 2006; Ozerol et al., 2009). In our study, elevation of MDA level clearly indicated oxidative stress in the ischemic/ reperfused brain. However, pretreatment with WCE significantly decreased the MDA level in the hippocampus region. One study showed that *W. coagulans* significantly decreased serum LPO and hepatic LPO levels in streptozotocin-induced diabetic rats (Hemalatha et al., 2004).

It was shown that pretreatment with *Withania somnifera* inhibited lipid peroxidation (LPO) in a middle cerebral artery occlusion model in rat (Chaudhary et al., 2003) which is in agreement with our results.

In the present study, reduction of MDA by WCE could be attributed to the high concentrations of whitanolides, flavonoids and other components with strong antioxidant potential, as previously stated (Salwaan et al., 2012).

Neuronal defense mechanisms against oxidative stress are exerted via antioxidant systems (Lee J and Won M. 2014). SOD is one of the enzymatic antioxidants that react with the superoxide radicals to form H_2O_2, and CAT is involved in the detoxification of H_2O_2 to form water and oxygen leading to diminish the toxic effects (Bast and Barr, 1997; Pham-Huy et al., 2008).

In the present study, SOD activity was decreased in the I/R group in comparison with the control group and the results were in agreement with previous studies (Ozerol et al., 2009; Chandrashekhar et al., 2010). Pretreatment with WCE for 30 days increased the SOD activity dose-dependently. Prasad et al. (2010) showed that withaferin-A, a steroidal lactone purified from *W. coagulans* is responsible for significant increase in the levels of SOD and CAT in streptozotocin-induced diabetic rats.

The result of several study about the effect of I/R on CAT are controversial. Some studies have demonstrated an increase expression of CAT in the brain tissue after global ischemia (Yoo et al., 2011; Yan et al., 2014). On the other hand, a few studies have shown a decrease in CAT activity in the brain that was induced by global brain ischemia (Aabdallah and Eid, 2004; Shah et al., 2005). However, in our study CAT activity showed a significant decrease in the hippocampus region of ischemic rats that is vulnerable to oxidative damage and pretreatment with WCE significantly increased CAT activity as compared to I/R group.

GPx is the most important antioxidant enzyme contributing to H_2O_2 scavenging which protects the cell membrane from lipid peroxidation and is vital for neuroprotection in the brain (Lee J and Won M. 2014). Impairment in the glutathione system is a factor in cerebral ischemia (Yabuki and Fukunaga, 2013).

In our study, the activity of this enzyme was found to be down-regulated in ischemic rats and pretreatment with WCE improved the hippocampus GPx level.

In rats, cerebral artery occlusion causes reduction of GPx level (Candelario-Jalil et al., 2001; Jung et al., 2011). Results of some studies showed that glutathione reduced the infarct volume and enhanced the neuron survival in the animal models (Anderson et al., 2004; Yabuki and Fukunaga, 2013). Some studies reported that oral administration of herbal antioxidants can restore the glutathione level in cortex and hippocampus in bilateral common carotid artery occlusion in rats (Ahmad et al., 2012; Kong et al., 2014).

Consistent with our findings, it was reported that withanolide-A, a major active constituents isolated from Withania, increases glutathione biosynthesis in neuronal cells and reduces neurodegeneration in hippocampus region (Baitharu et al., 2014). Enhancement of antioxidant capacity in acute stroke may protect against the adverse effects of free radical production during ischemia and reperfusion (Shukla et al., 2012).

In addition, the significant increase in SOD, CAT and GPX activity in the WCE-pretreated groups compared to the I/R group points to the possible neuroprotective and antioxidant effects of WCE in ischemic brain tissue.

It has been demonstrated that during brain ischemia/reperfusion, high amounts of free radicals are formed, which ultimately lead to cell death, especially in the hippocampus (Vandresen-Filho et al., 2015). In the ischemic brain, cells experience glutamate-receptors over-stimulation, cellular Ca^{2+} overload, oxygen radicals production and DNA damage which result in a neuronal cell death cascade (Jimenez-Jimenez et al., 1996; Rossi, 2000). In this study, we observed significant morphological changes in hippocampus CA1 neurons in the I/R group. The number of intact neurons was significantly reduced in the ischemia model group.

WCE administration significantly preserved neurons in CA1 region. Bhatnagar et al, showed that pretreatment with Withania can decreased cell loss *in vivo* by inhibition of Ca^{2+} influx in to neuronal cells (Bhatnagar et al., 2013).Some studies reported that pro-inflammatory cytokines such as TNF-α that have important roles in the pathogenesis of ischemic brain damage are up-regulated 30 min after brain ischemia (Bémeur et al., 2010; Rao KVR, 2010). Several studies indicated that wihtaferin-A has anti-inflammatory and neuroprotective effects and can reduce TNF-α and inhibit NF-κB activity (Oh and Kwon, 2009; Lalsare and Chutervedi, 2010).

Previously, it was shown that Bcl-2 and Bax genes are expressed at higher levels and activated in the hippocampus CAl neurons after transient global ischemia (Ajami et al., 2011).

Mohanty et al. demonstrated that Withania pretreatment in myocardial reperfusion, upregulated the expression of anti-apoptotic protein Bcl-2 and downregulated the expression of pro-apoptotic protein Bax which prevents cell loss (Mohanty et al., 2004).

Our results showed that TUNEL-positive cells were markedly reduced in the hippocampal CA1 region of ischemic rats when pretreated with WCE. Several experiments have shown significant neuroprotection following pretreatment with antioxidants (Mohagheghi et al., 2011; Sarbishegi et al., 2014). All together, the neuroprotective effects of WCE that were observed in the current study may be related to inhibition of TNF-α and NF-κB, reducing ROS generation, inhibition of excessive Ca^{2+} influx and blocking programmed cell death. Further experiments are needed to examine these possibilities.

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Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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