Research Article

Anti-oxidant and anti-inflammatory effects of *Urtica dioica* against cerebral ischemia-reperfusion injury in rats

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Abstract

Objective: In our previous study, *Urtica dioica* (UD) was found to protect against cerebral ischemia reperfusion injury (CIRI) in a rat model of bicommon carotid artery occlusion (BCCAO). The mechanisms behind the protective effects of UD on BCCAO-induced rats, however, are still unknown. Using BCCAO rats, the goal of this study was to see if UD reduced BCCAO-induced CIRI through anti-oxidant and anti-inflammatory pathways. **Material and Methods:** The following parameters were measured: (1) percentage (%) area of brain infarction; (2) Brain levels of antioxidant enzymes (3) Brain levels of inflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-β). **Results:** BCCAO considerably raised the percent area of brain infarction, whereas UD administration significantly reduced the percent area of brain infarction in these animals. With BCCAO, there was a considerable increase in brain TNF- and IL- levels, which was significantly reduced by UD. Furthermore, BCCAO reduced anti-oxidant enzymes significantly. UD, on the other hand, significantly reduced the activity of anti-oxidant enzymes. **Conclusion:** The data suggests that, inhibition of oxidative stress and inflammatory mediator release may be involved in the protective benefits of UD in BCCAO model rats.

Keywords: Cerebral ischemia, reperfusion injury, *Urtica dioica*, inflammatory markers

Introduction

Stroke is defined by a rapid loss of brain function caused by disruptions in the brain's blood flow. Either vascular insufficiency (ischemic stroke) or bleeding cause it (hemorrhagic stroke). It causes insufficient oxygen and glucose delivery to maintain cellular homeostasis, leading to a variety of cellular pathologies. Longer ischemic insults result in irreversible neuronal damage if they are not restored rapidly (Beal, 2010). The state of ischemia is reversed by reperfusion, restoration of blood flow to an ischemic area, supplying oxygen and metabolic substrates to starved tissues. Therapeutically reperfusion can be achieved using thrombolytics (tPA) or by surgery. Unfortunately, reperfusion has got further attention for its dual role. Apart from causing beneficial effects, it is identified to cause secondary injury to the penumbra. This secondary injury is called reperfusion injury, resulting in progression of vasogenic edema, hemorrhagic transformation, and an increase in stroke volume. So, the successful treatment of acute ischemic stroke remains one of the major challenges in clinical medicine. Therefore, minimizing reperfusion injury (neuroprotection) along with revascularization therapy may have more beneficial effects to minimize adverse outcomes after stroke. This has led to the investigation of neuroprotective approaches (Lipton, 1999; Leira et al., 2006).

Two important pathophysiological mechanisms involved in ischemia-reperfusion injury are oxidative stress and inflammation. Inflammation and oxidative stress, being a natural responses to injury, are increasingly recognized as the key elements in pathological progression of ischemia-reperfusion injury (Barone et al., 1997; Barone and Feuerstein, 1999; Amantea et al., 2009). Therefore, reducing oxidative stress and down regulating the inflammatory response are options that merit consideration as potential neuroprotective therapies.

Several neuroprotective approaches, like antioxidant and anti-inflammatory therapies have been studied and proved effective in reducing reperfusion injury of the ischemic brain. In this line herbal drugs have received more attention. Herbal

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drugs have been described in ancient systems of medicine for the treatment of various ailments associated with stroke and have more recently been reported to be beneficial in treating stroke. A number of traditional medicines have been studied both in animal models of stroke and human patients and revealed that neuroprotective effects are mediated by virtue of their antioxidant and anti-inflammatory properties. *Nigella sativa, Ginkgo biloba*, Garlic, Tanshinone, *Panax ginseng, Bacopa monnieri, Withania somnifera, Centella asiatica, Curcumin, Ocimum sanctum* are some examples of plants that showed protective effects against ischemia-reperfusion induced brain damage in experimental stroke models (Wu et al., 2010).

Urtica dioica, also known as common stinging nettle, is a well-known ethnomedicinal plant. Its use in the Indian traditional folk medicine is also well documented. It is reported to have potent antioxidant and anti-inflammatory activities (Mamta and Preeti, 2014a). *Urtica dioica* is also reported to have significant antioxidant activity comparable to standard antioxidant compounds like tocopherol, ascorbic acid and BHA (Butylated hydroxyanisole). Its antioxidant effects are shown to be mediated by decreasing MDA level as well as a significant increase in SOD, catalase levels, clearly indicating inhibition of lipid peroxidation and improvement of antioxidant defense enzyme system (Upton, 2013). It has been used in Ayurveda since olden days for chronic inflammatory disorders like rheumatoid arthritis, prostitis, and colitis. Mechanistic studies strongly suggest that its anti -inflammatory actions are mediated by inhibition of key events in inflammatory process like release of prion flammatory cytokines (TNF-α and IL-1β) (Chrubasik et al., 1997; Riehemann et al., 1999).

In our previous study, *Urtica dioica* (UD) was found to protect against cerebral ischemia reperfusion injury (CIRI) in a rat model of bi-common carotid artery occlusion (BCCAO) (Padmavathi und Annapurna, 2021). However, the mechanisms underpinning UD's protective benefits in BCCAO-induced rats remain unknown. The purpose of this study, which used BCCAO rats, was to see if UD could prevent BCCAO-induced CIRI via anti-oxidant and anti-inflammatory pathways. The present study was attempted to explore the involvement of its antioxidant and anti-inflammatory properties in offering cerebro-protection against BCCAO induced cerebral ischemia reperfusion injury in rats.

Material and methods

Animals

Wistar male albino rats (230–300 g) were obtained from Srinivasa Enterprises, Hyderabad, Telangana. The animals were kept under standard laboratory conditions, maintained on 12 h light/dark cycle, had free access to food and water. Animals were acclimatized to laboratory conditions before the test. Each animal was used once in the experiment. Experimental protocol was approved by Institutional Animal Ethics Committee and was conducted according to the CPCSEA guidelines for the use and care of experimental animals (Regd. No. 516/PO/C/01/CPCSEA).

Chemicals

2,3,5-Triphenyltetrazolium chloride (TTC)	Sigma Aldrich, India
MDA colorimetric kit	Biodiagnostics, Egypt
SOD colorimetric kit	Biodiagnostics, Egypt
Catalase colorimetric kit	Biodiagnostics, Egypt
TNF-α ELISA kit	Diaclone, France
IL-1β ELISA kit	Diaclone, France

Urtica dioica (UD) extract

Ethanolic extract of UD in the form of homeopathic mother tincture was purchased from a local medical store (manufactured by 'Bhandaris Pvt. Ltd', Batch no. M2/07, manufacturing date 11/2012, with 48% ethanol as vehicle. drug strength 1/10).

UD is prescribed as a mother tincture in homeopathy. The whole plant is used to make a homoeopathic. A mother tincture is essentially an alcoholic extract prepared according to the procedure described in Homoeopathic Pharmacopoeia (Banerjee et al., 1980). Both the US Homoeopathic Pharmacopoeia and the German Homoeopathic Pharmacopoeia cover it. A mother tincture, despite being a homoeopathic formulation, is equally effective as an alcoholic extract often used by researchers in phytochemical or biological studies (Kumar and Sharma, 2005; Chandrakant Nimgulkar et al., 2011; Khuda-Bukhsh et al., 2011; Ghosh et al., 2013).

Dose Selection

In this study, 3 doses (0.2, 0.4, 0.8 ml/kg) of *Urtica dioica* were used. The doses were chosen based on previous experimental studies in animals with *Urtica dioica* and extrapolation from the doses used for clinical use. The use of UD has been reported safe in acute and chronic toxicity studies in mice, at the dose levels of 250, 500, 1000, 2000 mg/kg body weight. Typical daily dosages of UD for clinical use include, 360 mg aqueous extract, 460 mg dried extract, and 600 mg freeze-dried preparation. Tincture of the plant is taken at a dose of 1/2-1 tsp (2-5 mL) three times daily (Mamta and Preeti, 2014b).

Experimental Protocol

The rats were divided into seven groups of 6 rats each and following experimental protocol was used. Group 1:Normal control; Group 2: Sham control, received surgery, without BCCA occlusion; Group 3: I/R control, received 30 min BCCA occlusion and 24 hours reperfusion; Group 4:

Vehicle control, received 0.8 ml/kg of 24% ethanol (double dilution of tincture) for 7 days prior to induction of ischemia and reperfusion; Group 5: *Urtica dioica* (0.2 ml/kg) Group 6: *Urtica dioica* (0.4 ml/kg) Group 7 : *Urtica dioica* (0.8 ml/kg). Prior to induction of ischemia and reperfusion, the rats in the treatment groups (Group-5, 6 and 7) received respectively 3 doses of *Urtica dioica* (0.2, 0.4, 0.8 ml/kg) intraperitonealy daily for seven days.

Animals were anesthetized by giving thiopentone sodium (45 mg/kg) i.p. and were subjected to BCCA occlusion for 30 min and 24 hours reperfusion. At the end of the experimental procedure, the brains were removed quickly and the homogenate was prepared and supernant was used for estimation of oxidative stress marker (MDA), antioxidant enzymes, SOD and Catalase; inflammatory markers TNF-α and IL-1β.

Induction of cerebral ischemia/reperfusion (I/ R) injury in rats

Induction of cerebral ischemia/reperfusion injury was carried as modified method of Jingtao (Jingtao et al., 1999). Animals were anesthetized by giving thiopentone sodium (45 mg/kg, i.p). Both common carotid arteries were exposed over a midline incision, and a dissection was made between the sternocleidomastoid and the sternohyoid muscles parallel to the trachea. Each carotid artery was freed from its adventitial sheath and vagus nerve, which was carefully separated and maintained. A cotton thread was passed below each carotid artery. The induction of ischemia was performed by occluding bilateral common carotid arteries (BCCAO) for 30 min followed by 24 h reperfusion and the skin was closed with stitches using waxed silk suture. During the BCCAO, animals were observed for following criteria: maintenance of dilated pupils, absence of a corneal reflex when exposed to strong light stimulation, and maintenance of rectal temperature at (37 °C±0.5). Animals which did not match these criteria and showed seizures were excluded from study. Sham control animals received surgery, without BCCAO. After the completion of reperfusion period, animals were sacrificed after 24 h for biochemical assessment.

Preparation of Brain Tissue

After 24 h of reperfusion, animals were sacrificed by cervical dislocation and the brain was removed and was washed in ice cold saline (0.9%). Then it was kept on ice and subsequently blotted on filter paper, then weighed. A 10% (W/V) tissue homogenate was prepared in cold phosphate buffer (0.1 M, pH 7.4). The homogenate was centrifuged at 4000 rpm at 4^0C for 15 min and aliquots of supernatant was separated and used for biochemical estimation.

Biochemical Assays

MDA levels in the brain was measured by the method developed by Ohkawa et al. (1979) by using spectrophotometric kit provided by Biodiagnostics, Egypt. Superoxide dismutase (SOD) activity was determined by the method developed by Nishikimi et al. (1972) by using spectrophotometric kit provided by Biodiagnostics, Egypt. Catalase activity was determined by the method developed by Aebi, (1984) by using spectrophotometric kit provided by Biodiagnostics, Egypt. TNF-α and IL-1β were estimated by using ELISA kit provided by Diaclone (France).

Data Analysis

All the values are expressed as mean ± S.E.M. The data of all the experiments were analyzed using one way analysis of variance (ANOVA) followed by Tukey's post hoc test. In all the tests, criterion for statistical significance was $P < 0.05$

Results and discussion

The antioxidant activity of *Urtica dioica* was evaluated by quantification of MDA, SOD and Catalase in brain tissue. In the present study, MDA levels were increased significantly and SOD, Catalase levels were decreased significantly in the I/R control group of rats as compared to the sham control

Table 1. Effect of *Urtica dioica* on brain MDA, SOD and catalase levels in rats exposed to cerebral ischemia/reperfusion

Groups(n=6)	MDA (nmol/g tissue)	SOD (U/g tissue)	Catalase (U/g tissue)
Normal	48.73 ± 2.509	117.0 ± 2.347	3.29 ± 0.153
Sham control	51.35 ± 1.635	114.6 ± 2.472	3.41 ± 0.226
I/R control	99.92 ± 1.644$^{###}$	41.68 ± 1.992$^{###}$	1.32 ± 0.167$^{###}$
Vehicle control	99.58 ± 2.462	39.57 ± 2.186	1.17 ± 0.104
Urtica dioica (0.2 ml/kg)	77.75 ± 2.527***	59.98 ±1.891***	1.51 ± 0.114
Urtica dioica (0.4 ml/kg)	59.50 ± 1.423***	79.65 ± 1.845***	2.23 ± 0.088**
Urtica dioica (0.8 ml/kg)	51.50 ± 1.321***	101.8 ± 2.780***	3.03 ± 0.125***

I/R (ischemia/reperfusion). Results were represented as mean ± S.E.M. (N = 6). Results were analyzed by one way ANOVA, followed by Tukey's multiple comparison test. ###Significantly different from Sham control group at $P < 0.001$. ** Significantly different from I/R control group at $p < 0.01$. ***Significantly different from I/R control group at $P < 0.001$.

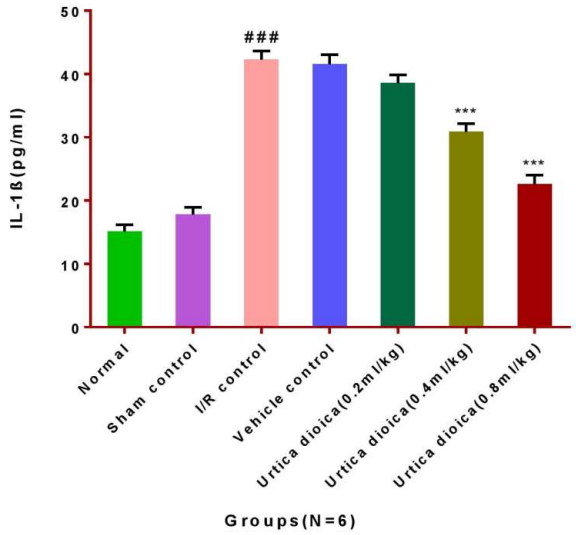

Figure 1. Effect of Urtica dioica on brain IL-1β levels in rats exposed to cerebral ischemia/reperfusion. Results were represented as mean ± S.E.M. (N = 6). ANOVA and Tukey's post hoc test was used for statistical analysis data. ### Significantly different from Sham control group at P < 0.001. *** significantly different from I/R control group at P < 0.001

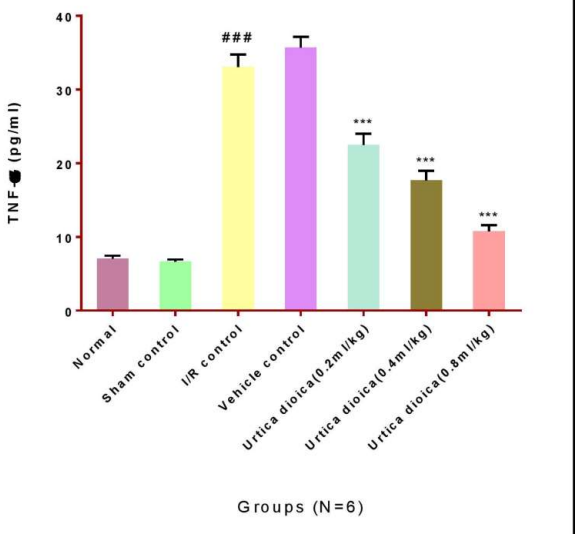

Figure 2. Effect of Urtica dioica on brain TNF-α levels in rats exposed to cerebral ischemia/reperfusion. I/R (cerebral ischemia/reperfusion). Results were represented as mean ± S.E.M. (N = 6). ANOVA and Tukey's post hoc test was used for statistical analysis data. ### Significantly different from Sham control group at P < 0.001. *** significantly different from I/R control group at P < 0.001

group. MDA levels were decreased and SOD and Catalase levels were increased significantly and dose dependently with *Urtica dioica* Treatment as compared with the I/R control group (Table 1).

Brain TNF-α, IL-1β levels were significantly elevated following ischemia and reperfusion as compared to the sham control group. Administration of *Urtica dioica* (0.2, 0.4, 0.8 ml/kg) for 7 days prior to induction of ischemia and reperfusion caused a significant reduction in brain TNF-α, IL-1β levels in a dose dependent manner as compared to the I/R control group (Figure 1 and Figure 2).

Since free radicals are extremely reactive, they have a short half life. Therefore they are difficult to measure directly. Many studies have used an indirect approach to demonstrate free radical production during cerebral ischemia/reperfusion, measuring the products of free radical reaction with other molecules, such as DNA, lipids or proteins. Brain is very rich in polyunsaturated fatty acids, which are highly susceptible to free radical attack. ROS can react with double bonds of fatty acids in lipids, produces lipid peroxides. In addition, lipid peroxides degrade to reactive aldehyde products, including malondialdehyde (MDA), 4-hydroxy nonenal (HNE), and acrolein. Malondialdehyde (MDA), a stable product of lipid peroxidation, is used as indirect marker for evaluation of oxidative damage. Higher levels of MDA indicate higher concentrations of free radicals (Adibhatla und Hatcher 2008a, 2008b). In support of this Sakamoto et al. (1991) demonstrated a positive correlation between free radical production and lipid peroxidation during ischemia-reperfusion injury in the rat brain (Sakamoto et al., 1991).

The levels of antioxidant enzymes, indirectly reflects the amount of oxidative stress according to the hypothesis that their changes are a consequence of the reaction with free radicals. Superoxide dismutase (SOD) and catalase constitute a mutually supportive team of defense against ROS. SOD, immediately dismutates superoxide to generate hydrogen peroxide, a more stable and less toxic compound. Hydrogen peroxide is a lipid soluble molecule which diffuses easily across cell membranes and exerts remote effects like peroxidation of the membrane cell lipids, induction of DNA damage, oxidation of cellular enzymes. Catalase prevents hydroxyl radical generation by dismutating hydrogen peroxide (Escobar et al., 1996). Therefore, the measurement of these endogenous antioxidants enzymes i.e. SOD and CAT has been performed to estimate the amount of oxidative stress.

In this experimental study, we tried to investigate anti-oxidant potential of UD in protecting brain against damage caused by BCCAO induced cerebral ischemia/reperfusion injury in rats. Malondialdehyde, SOD, CAT in brain tissue were detected and the results were compared before and after UD administration.

We found increased MDA levels in the brain in parallel to

significant increase in infarct size in the I/R control group when compared to the sham control group. This is indicating the induction of oxidative stress and subsequent lipid peroxidation in the present model of ischemia and reperfusion. This is also supported by earlier studies, reporting elevated levels of MDA in the similar experimental model (Gaur et al., 2009). Animals pretreated with *Urtica dioica* showed significantly less lipid peroxides due to ischemic-reperfusion injury as compared to the I/R control group.

Because of over consumption of antioxidants due to excessive production of ROS, inactivation of detoxification systems, and failure to adequately replenish antioxidants in the ischemic brain tissue, cellular antioxidant enzymes are depleted in ischemia-reperfusion condition. Therefore, the measurement of the endogenous antioxidant enzymes i.e. SOD and Catalase has been used indirectly to estimate the amount of oxidative stress (Leinonen et al., 2000).

Many experimental studies have demonstrated decreased SOD and Catalase levels in cerebral ischemia-reperfusion injury as indirect evidence of oxidative stress. It has been demonstrated that lower serum SOD levels correlate negatively with infarct volume in stroke patients (Spranger et al., 1997). Exogenous administration of different forms of SOD and catalase has been proved to reduce brain injury by ischemia-reperfusion in experimental animal models (Liu et al., 1989; Imaizumi et al., 1990; He et al., 1993; Francis et al., 1997). Further evidence for the important role of SOD in the defense of ROS damage in reperfusion injury came from transgenic animal experiments. Mice overexpressing SOD were highly resistant to reperfusion injury (Saito et al., 2003). The decrease in the infarct volume in transgenic mice correlated well with an improved neurological outcome, whereas mice with a knockout mutation for SOD had larger infarcts after focal ischemia (Sheng et al., 1999).

In the present study SOD and Catalase levels were significantly reduced in the I/R control group when compared to the sham control. This is indicating the induction of oxidative stress in the present model of ischemia-reperfusion. Pretreatment with *Urtica dioica* significantly and dose dependently increased SOD and catalase levels as compared to the I/R control group. Our results indicating that treatment with *Urtica dioica* could reduce ROS induced damage, and thus ischemia-reperfusion injury.

Our findings are also supported by earlier studies on antioxidant potential of *Urtica dioica*. Kanter et al. (2003) reported that *Urtica dioica* decreased the lipid peroxidation, and increased the antioxidant defense activity in the CCl_4 induced hepatotoxicity in rats (Kanter et al., 2005). Kandis et al. (2010) found that *Urtica dioica* increased the antioxidant ability and decreased oxygen free radicals in the early period of hepatic ischemia-reperfusion injury in rats (Kandis et al., 2010). Kataki et al. (2012) revealed that *Urtica dioica* has significant antioxidant activity comparable to standard antioxidant compounds like α-tocopherol, ascorbic acid and BHA (Butylated hydroxyanisole) (Sarma Kataki, 2012).

Moreover, some authors revealed that a positive relationship was found between total phenols and antioxidant activity in many plant species (Vinson et al. 1998). Considerably, Gulcin et al. (2004) found that pyrocatechol equivalent of phenols in water extract of *Urtica dioica* and also it is reported to contain ferulic acid, caffeoylmalic acid, which are proved as potent antioxidants (Gülçin et al., 2004).

In our study, a model of 30 min of BCCA occlusion followed by 24 hours of reperfusion significantly increased brain TNF-α and IL-1β levels when compared to sham control rats. This is demonstrating the role of inflammation in cerebral damage due to ischemia and reperfusion. Moreover, our results are in accordance with previous studies. Clausen et al. (2008) detected the expression of IL-1β and TNF- α by different subsets of microglia and macrophages after ischemic stroke in mice (Clausen et al., 2008). Lentsch et al. (1999) suggested that TNF-α and IL-1β levels does not increase during ischemia but rather just after the start of reperfusion (Lentsch et al., 1999). Increased pro-inflammatory cytokines (TNF- α, IL-1β and IL-6) have been detected in the ischemic cortex 1 hour after middle cerebral artery (MCA) occlusion in experimental models of brain ischemia.

We have studied *Urtica dioica* for its potential to modulate the brain levels of TNF- α and IL-1β in offering cerebroprotection against cerebra ischemia and reperfusion injury. It was found that pretreatment with *Urtica dioica* for 7 days before the induction of ischemia-reperfusion, significantly and dose dependently decreased the brain levels of TNF- α and IL-1β when compared to the I/R control group. Our results suggesting that cerebroprotective role of *Urtica dioica* might be due to modulation of the release of TNF- α and IL-1β.

It has been reported by many studies that modulation of TNF-α and IL-1β levels in brain protects the brain against damage due to ischemia and reperfusion (Yin et al., 2013). Intra-ventricular injection of TNF- α and IL-1β reported to enlarge infarct volume and brain edema after ischemia and reperfusion in rats. Blockade of TNF-α receptors, has been shown to reduce brain infarct volume and cerebral edema after transient focal ischemia in rats (Hosomi et al., 2005). Pretreatment with IL-1 β applied to the cortical surface reduced cerebral blood flow (CBF), increased neutrophil recruitment, and increased O^{2-} production during reperfusion (Mulcahy et al., 2003). Furthermore, inhibition of IL-1 through administration of a recombinant form of the naturally

occurring IL-1 receptor antagonist (IL-1ra) or neutralizing antibodies to IL-1, all markedly reduced ischaemic brain damage in rodents (Becker 2002; Emsley et al., 2005). The above studies supporting our finding of correlation between decreased infarct size and decreased levels of TNF- α and IL-1β with *Urtica dioica* treatment.

Our findings are also supported by many previous studies demonstrating the anti -inflammatory role of *Urtica dioica*. *Urtica dioica* significantly inhibited the lipopolysaccharide (LPS)-stimulated release of the pro-inflammatory cytokines TNF-α and IL-1β in whole human blood both in *ex vivo* and *in vivo* studies (Obertreis et al., 1996; Teucher et al., 1996). Konrad et al. (2005) showed that protection against Dextran sodium sulfate induced colitis by *Urtica dioica* was due to significant inhibition of TNF-α, and IL-1β (Konrad et al., 2005). The above studies strongly support our findings. Therefore, our results suggests that cerebroprotective effect of *Urtica dioica* might be due to modulation of proinflammatory cytokines, TNF-α, and IL-1β in the brain.

Conclusion

The results of our study, for the first time, provide clear evidence that *Urtica dioica* pretreatment ameliorates cerebral ischemia-reperfusion injury and enhances the antioxidant defense against BCCAO induced cerebral infarction in rats. The antioxidant effect obtained from our results might be explained by the fact that *Urtica dioica* contained the phenolic and flavonoid compounds, and due to these, *Urtica dioica* has scavenging ability and stabilizes the lipid peroxidation, which plays an important role in ischemia-reperfusion damage in the brain tissue.

BCCA occlusion for 30 min followed by 24 hours of reperfusion, significantly increased pro-inflammatory cytokines, TNF-α and IL-1β in the brain. Pretreatment with *Urtica dioica* showed significant inhibition of the pro-inflammatory response (TNF-α, IL-1β) after ischemia and reperfusion. Hence, the anti-inflammatory effect of *Urtica dioica* might be attributed to its cerebroprotective potential against cerebral ischemia and reperfusion injury.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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