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Original Article

Protective effect of *Canna indica* on cerebral ischemia-reperfusion injury in rats

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ABSTRACT

The antioxidant capacity and cerebroprotective effect of *Canna indica* roots were estimated. The methanolic extract of *C. indica* roots was studied for free radical scavenging activity on superoxide, hydroxyl and 1, 1-diphenyl-2-picrylhydrazylfree radicals and compared with standard drug ascorbic acid using a pre-treatment for 1 wk at a daily oral dose, of 400 mg/kg or 800 mg/kg and then cerebral ischemia-reperfusion injury was induced by occluding bilateral common carotid arteries for 30 min, followed by 4 h reperfusion. Quercetin (20 mg/kg, by intraperitoneal injection) was used as the standard drug. At the end of the experiment, animals were sacrificed by decapitation, and the brain was removed for the estimation of various biochemical parameters—assessment of cerebral infarct size and examination of oxidative stress enzymes such as superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and myeloperoxidase (MPO). Pre-treatment with methanolic extract of *C. indica* significantly reversed the levels of biochemical parameters and significantly reduced theedema and cerebral infarct size compared to the ischemic control group. The results indicated that *C. indica* ameliorates the cerebral ischemia/reperfusion injury and enhances the antioxidant defense. Further studies should involve the complete isolation of pure, biologically active compounds from the different extracts from *C. indica* like methanol in the present research.

Introduction

Cerebral ischemia (CI) also known as brain ischemia is a condition that occurs when there is insufficient blood flow to the brain for its metabolic requirements (Raichle, 1983). An insufficient supply of blood leads to poor oxygen supply to the brain tissue cells, causing the death of brain tissue or an ischemic stroke and this condition leads to alterations in the brain metabolism, brain energy crisis and a decline in its metabolisms (White et al., 2000). CI can be divided into two types: 1) local ischemia, which is restricted to a specific region of the brain; and 2) global ischemia, which covers a wide area of brain tissue (Hinds, 1985). The occurrence of CI is related to different abnormalities in the body like sickle cell anemia, moyamoya disease, squashed blood vessels, arteries, blood clots, low blood pressure as a result of a heart attack and congenital heart defects (Raichle, 1983; Caplan, 2006). All these conditions in the life span of the living human body cause the slowdown of blood flow in different circumstances and this leads to CI (Hinds, 1985; Scott and Smith, 2009). CI causes dizziness, double vision, difficulty in speaking or slurred speech, loss of body coordination and sometimes paralysis, while untreated CI will result in unconsciousness (coma), permanent damage to the brain or death (Stapf and Mohr, 2002).

Many neuroprotective agents are designed to protect the brain from injury due to ischemia/ischemia reperfusion and drugs (such as Aspiring, Clopidogrel and Dipyridamole) have also have been used to treat different diseases which cause CI in their progress (Green, 2008; Ly et al., 2006). Different medications used in the treatment of CI and its related diseases cause different side effects (Lipton, 2007; Zhou and Zhu, 2009). Recent studies (Balkrishna and Misra, 2017; Guzman et al., 2017) have focused on natural compounds extracted from different medicinal plants to prevent neurological disorders including CI and disorders leading to CI.

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Thus, the present study was carried out to evaluate the protective effect of *Canna indica* roots on a cerebral ischemia-induced rat model based on its traditional uses (Asolkar et al., 1992). *Canna indica* also known as sakasiri or Indian shot, is a species of the *Canna* genus, belonging to the family Cannaceae and is widely cultivated as a garden plant (Gade, 1966). Parts of *C. indica* have been used in the treatment of different illnesses:the roots are used in the treatment of gonorrhea and amenorrhea, the leaves and powdered seeds are mixed and used to treat dermatoses (Defilipps et al., 2004), but there have been no reports on phytochemical analysis and scientific evidence for biological activities.

Materials and methods

Chemicals and reagents

All chemicals used were of analytical grade. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemicals, St. Louis, MO, USA, Nitrobluetetrozolium was purchased from Sisco Research Laboratories Pvt Ltd., Mumbai, India. Riboflavin was purchased from LobaChemie Pvt Ltd., Bombay, India. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and myeloperoxidase (MPO) analysis kits were purchased from Allied Scientific Products, Kolkata, India.

Plant material and preparation of extracts

The plant material was collected in the Bhmavaram region, Andhra Pradesh, India during December 2015. Authentication of plants was done by retired Professor M. Venkaih, Department of Botany, Andhra University, Visakhapatnam, India (Fig. 1). The rhizome (roots) of the plant material was shade dried and powdered (1 kg) and then extracted separately using a maceration process with methanol (5 L), after which the extracted solvent was concentrated to dryness under vacuum using a rotavapor and to produce 200 g of dried extract.

In vitro antioxidant activity

For the assessment of antioxidant activity, the extract of *C. indica* roots was dissolved in dimethyl sulphoxide (DMSO) at different concentrations of 25 μg/100 μL, 50 μg/100 μL, 100 μg/100 μL, 200 μg/100 μL or 400 μg/100 μL on hydroxyl, superoxide and DPPH free radicals. The results were shown as mean ± SEM. The percentage inhibition and 50% inhibition concentration (IC_{50}) were calculated (Mallikarjuna et al., 2012; Rao et al., 2013).

Superoxide radical scavenging activity

The superoxide scavenging activity of the selected plant extract was evaluated according to the method of McCord and Fridovich (1969) using absorption of light at 560 nm, induction of superoxide free radical generation by riboflavin and corresponding reduction by nitroblue tetrazolium.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured according to the method of Kunchandy and Rao (1990) using competition between deoxyribose and the extract's antioxidant molecules for hydroxyl radicals generated from the Fe^{+2}/EDTA/H_2O_2 system.

2,2-Diphenyl-1-picrylhydrazylradical scavenging activity

The DPPH radical scavenging activity was measured according to the method of Murali et al. (2011) and Braca et al. (2003). Based on the measurement of the color absorbance of alcoholic DPPH solution (blue color) after the addition of antioxidant solution (extract/compound). If the antioxidants are present in the test then there is color change to blue-yellow due to diphenyl-picrylhydrazine.

Fig. 1. *Canna indica* plant and its rhizome.

Calculation of percentage inhibition

The percentage inhibition of superoxide production by the extract was calculated using Equation (1):

$$\text{Inhibitory ratio} = (A_0 - A_1) \times \frac{100}{A_0} \quad (1)$$

where A_0 is the absorbance of the control and A_1 is the absorbance with the addition of plant extract/ascorbic acid.

Calculation of 50% inhibition concentration

The optical density obtained with each concentration of the extract/ascorbic acid was plotted and the graph was extrapolated to find the 50% inhibition concentration (IC_{50}) of extract/ascorbic acid.

Selection of animals

Healthy albino rats (Mahaveer Enterprises; Hyderabad, India) of either sex weighing 180–250 g aged 60–90 d were used for the study. They were maintained on a 12 h light and 12 h dark cycle and were fed with regular grain chow (Rayans Biotechnologies Pvt. Ltd.; Hyderabad, India).

Acute toxicity studies

The acute toxicity study was conducted for extract of *C. indica* roots according to OECD guidelines 420 (Organisation for Economic Co-operation and Development, 2001) and regulations of the Institutional Animal Ethics Committee (Registered number 516/01/A/CPCSEA). Albino female rats aged 60–90 d and weighing 180–250 g were separated into four groups ($n = 4$). They were maintained for 1 wk before the experiment at room temperature (22 ± 3 °C) and allowed free access to water and diet. The animals were subjected to acute toxicity study using *C. indica* extract at doses of 5 mg/kg body weight, 50 mg/kg body weight, 300 mg/kg body weight or 2,000 mg/kg body weight applied orally to the four groups at regular time intervals of 1 h, 2 h, 4 h, 8 h, 12 hand 24 h and were observed to note different conditions such as skin color changes, morbidity, aggressiveness, oral secretions, sensitivity to sound and pain, respiratory movements and finally mortality.

Induction of cerebral infarction

Induction of cerebral ischemia/reperfusion was carried out using the standard method (Farbiszewski et al., 1995). Overnight fasted rats were anaesthetized with thiopental sodium (30 mg/kg). A midline ventral incision was made in the throat. Right and left common carotid arteries were located and freed from surrounding tissue and vagus nerve. A cotton thread was passed below each artery. Global cerebral ischemia was induced by occluding the carotid arteries with a knot. After 30 min of global cerebral ischemia, the cotton threads were removed with the help of two knot releasers to allow the reperfusion of blood through carotid arteries for 4 h. Rats were maintained at 37 °C on a heated surgical platform. All surgical procedures were carried out under sterile conditions.

Wistar albino either-sex rats were divided into six groups of six rats each and fed with extract or vehicle (normal saline) for 7 d prior to the experiment and treated as follows:

Group I: Normal saline (10 mL/kg, orally), noischemia.
Group II: C. indica extract (800 mg/kg, orally), no ischemia.
Group III: ischemic control (normal saline 10 mL/kg, orally), occlusion for 30 min followed by 4 h reperfusion.
Group IV: C. indica extract (400 mg/kg, orally), occlusion for 30 min followed by 4 h reperfusion.
Group V: C. indica extract (800 mg/kg, orally), occlusion for 30 min followed by 4 h reperfusion.
Group VI: Quercetin (20 mg/kg, intraperitoneal injection), administered 30 min before occlusion, occlusion for 30 min followed by 4 h reperfusion.

Assessment of cerebral infarct size

The animals were sacrificed by decapitation at the end of 4hr reperfusion and the global cerebral ischemia and the brains were quickly removed for 2,3,5-triphenyltetrazolium chloride (TTC) staining. The brain was sliced into uniform coronal sections about 1–2 mm thickness. The slices were incubated in 2% TTC dissolved in phosphate-buffered saline (pH 7.4) at 37 °C for 30 min and fixed by immersion in 10% phosphate buffer formalin (Bederson et al., 1986; Jiang et al., 2007). TTC converts the living tissue in red zone pigment by nicotinamide adenine dinucleotide and dehydrogenase. The infarct cells having lost their enzymes thus remained unstained. Whole brain slices were weighed. The infarct unstained part was dissected out, weighed and expressed as percentage of the total weight of the brain.

Preparation of brain tissue for estimation of biochemical parameters related oxidative stress

The animals were sacrificed by decapitation at the end of 4hr reperfusion after global cerebral ischemia. The brain of each animal was removed and washed with cooled 0.9% saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized with cold phosphate buffer (0.1M, pH7.4) using a remi homogenizer. The homogenization procedure was performed as quickly as possible under completely standardized conditions. The homogenate was centrifuged at 1,000 rpm and 4 °C for 3 min and the supernatant was divided into two portions, one of which was used for measurement of Malondialdehyde (MDA). The remaining supernatant was again centrifuged at 12,000 rpm at 4 °C for 15 min and used for the measurement of superoxide dismutase (SOD) and catalase (CAT) levels by standard methods (Utley et al., 1967; Aebi, 1974; Carlberg and Mannervik, 1975; Okhawa et al., 1979; Kakkar et al., 1984; Mullane et al., 1985).

Statistical analysis

The results were expressed as mean ± SE. Differences in infarct size, MDA, SOD, catalase and myeloperoxidase were determined using factorial one-way analysis of variance. Individual groups were compared using Dunnet's test. Differences with $p < 0.01$ were considered statistically significant using the Prism software (Prism 6; GraphPad Software Inc.; La Jolla, CA, USA).

Table 1
Acute toxicity study of *C. indica* at different doses.

Group (dose)	Behavioral observation	Mortality
I (5 mg/kg)	Normal	0/4
II (50 mg/kg)	Normal	0/4
III (300 mg/kg)	Normal	0/4
IV (2000 mg/kg)	Normal	0/4

Table 2
Antioxidant activity of *C. indica* at different doses. Values shown as mean ± SE.

Concentration of *C. indica* extract (μg/mL)	% inhibition on			Concentration of ascorbic acid (μg/mL)	% Inhibition on		
	Superoxide free radical	Hydroxyl free radical	DPPH free radical		Superoxide free radical	Hydroxyl free radical	DPPH free radical
25	14.44 ± 0.53	15.84 ± 0.33	10.60 ± 0.61	10	21.16 ± 0.19	24.11 ± 0.25	21.40 ± 0.56
50	27.29 ± 0.30	29.07 ± 0.28	19.77 ± 0.23	20	36.00 ± 0.27	34.57 ± 0.48	35.54 ± 0.48
100	45.90 ± 0.17	41.42 ± 0.82	36.35 ± 0.88	40	49.55 ± 0.50	49.63 ± 0.66	46.98 ± 0.80
200	57.07 ± 0.42	59.62 ± 0.53	53.53 ± 0.47	80	64.26 ± 0.47	61.33 ± 0.68	56.85 ± 0.38
400	72.43 ± 0.52	73.76 ± 0.48	66.20 ± 0.39	160	79.44 ± 0.52	80.49 ± 0.47	73.97 ± 0.77

Results and discussion

There were no visible sign of toxicity, mortality and behavioral changes such as alertness, motor activity, breathlessness, restlessness, diarrhea, tremor, convulsion or coma at the administered doses of *C. indica*. The animals were physically active and no death was recorded even at the high dose of 2,000 mg/kg body weight (Table 1). Hence, the tested extract was considered as safe and nontoxic and further study was carried with 400 mg/kg and 800 mg/kg body weight for CI.

The methanolic extract of *C. indica* was evaluated for its antioxidant activity on different free radicals (superoxide, hydroxyl and DPPH free radicals) using *in vitro* methods and there was dose-dependent percentage inhibition on free radical formation (Table 2 and Fig. 2). As the concentration increased, the percentage of free radical inhibition capacity increased. The extract reduced the formation of superoxide free radicals from riboflavin, the extract reduced the reaction of hydroxyl free radicals from Fe^{2+}, EDTA/H_2O_2 system (Fenton reaction) with deoxyribose in the formation of thiobarbuteric acid reacting substances and it also stabilized the DPPH free radicals. The extract showed better activity on superoxide and hydroxyl radical inhibition. The IC_{50} values for the tested extract were 128 μg/mL, 154 μg/mL and 181 μg/mL and for ascorbic acid were 108 μg/mL, 103 μg/mL and 124 μg/mL on superoxide, hydroxyl and DPPH free radicals, respectively (Fig. 3).

The pre-treatment of methanolic extract of *C. indica* was evaluated after CI; it was found to have great capability in controlling the CI/reperfusion injury. The results of the present study clearly indicated that the *C. indica* therapy significantly improved/restored the changes such as increased MDA and MPO levels, and reduced SOD and CAT levels due to CI (Figs. 4–7). Ischemia is caused by the occlusion of a blood vessel that supplies a main supply of blood to the brain, causing deprivation of oxygen and glucose resulting in damage to the brain (Gund et al., 2013). Different ischemia medications target the occlusion and induce reperfusion to the blood vessel, during which oxygen is replenished to the tissue and this is very important in sustaining neural ability (White et al., 2000). However, during ischemia and after reperfusion, brain tissue damage occurs (Chan, 1996; Dröge, 2002) due to the imbalance of pro-oxidants and anti-oxidants including the over production of the oxidants, inactivation of the detoxification system and the consumption of the antioxidants (Beckman et al., 1990; Brennan et al., 2009; Chan, 2001) and after reperfusion, due to the availability of more oxygen, massive amounts of oxidants are produced which also cause damage to the brain (Kawase et al., 1999).

In the prevention of oxidative stress, SOD and CAT enzymes play very important roles. They are intracellular antioxidants, which scavenge the free radicals in the process of cell protection from oxidative damage (Beckman et al., 1990). In the present *invitro* antioxidant study, the extract of *C. indica* showed substantial inhibition of the generation of free radicals. The SOD and CAT levels represent the tissue ability to evade the toxicity of free radicals; free radicals promote lipid peroxidation, which results in changes in the integrity of cell permeability, which leads to apoptotic cell death in the brain (Levine et al., 2001; Saito et al., 2006). The end product of the lipid peroxidation was MDA. The estimation of the MDA, SOD and CAT levels directly indicates the free radical levels in the oxidative tissue damage. Under oxidative stress, the MDA levels will increase and the SOD and CAT levels will decrease. The *C. indica* extract significantly ($p < 0.01$) decreased the increased MDA levels due to ischemia and reperfusion injury in a dose-dependent manner (Fig. 4) along with the quercetin.

The SOD enzyme specifically scavenged the superoxide radicals. The superoxide radical was mainly produced after the ischemia reperfusion. Superoxide free radicals are highly damaging to the biological system of cells by producing the hydroxyl free radicals through reaction with the hydrogen peroxide (Maral et al., 1977; Janssens et al., 2000). CAT is an enzyme that functions as a catalyst for the decomposition of the hydrogen peroxide into water and oxygen when exposed to oxygen (Aebi, 1984). A low level of CAT increases the participation of hydrogen peroxide in the formation of hydroxyl free radicals, which are highly toxic to tissue cells

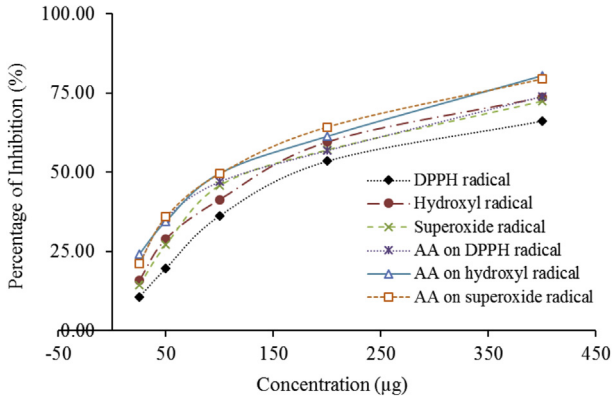

Fig. 2. Percentage inhibition of methanolic extract of *C. indica* and standard drug ascorbic acid (AA) on different free radicals (DPPH = 1, 1-diphenyl-2-picrylhydrazyl).

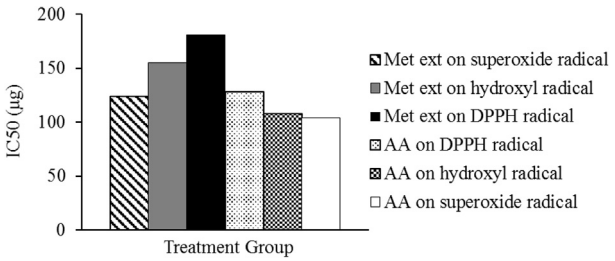

Fig. 3. Values for 50% inhibition concentration (IC_{50}) for methanolic extract (Met ext) of *C. indica* and standard drug ascorbic acid (AA) on different free radicals (DPPH = 1, 1-diphenyl-2-picrylhydrazyl).

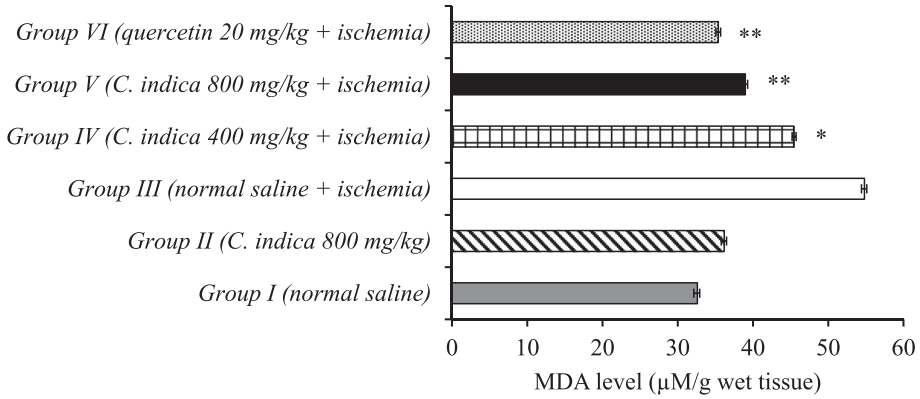

Fig. 4. Effect of methanolic extract of *C. indica* on malondialdehyde (MDA) level of rat brain to occlusion followed by 4 h reperfusion-induced ischemia, where values are expressed as mean + SEM ($N = 6$) and the results are compared with ischemic control group ($^{*} = p < 0.05$, $^{**} = p < 0.01$).

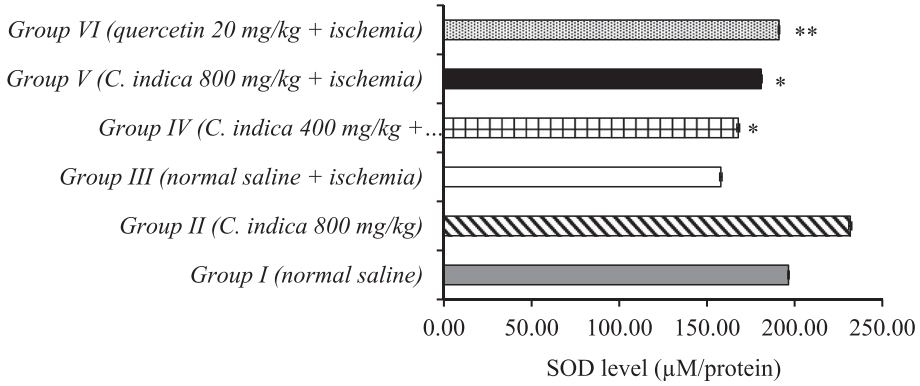

Fig. 5. Effect of methanolic extract of *C. indica* on superoxide dismutase (SOD) level of rat brain to occlusion followed by 4 h reperfusion induced ischemia, where values are expressed as mean + SEM ($N = 6$), and the results are compared with ischemic control group ($^{*} = p < 0.05$, $^{**} = p < 0.01$).

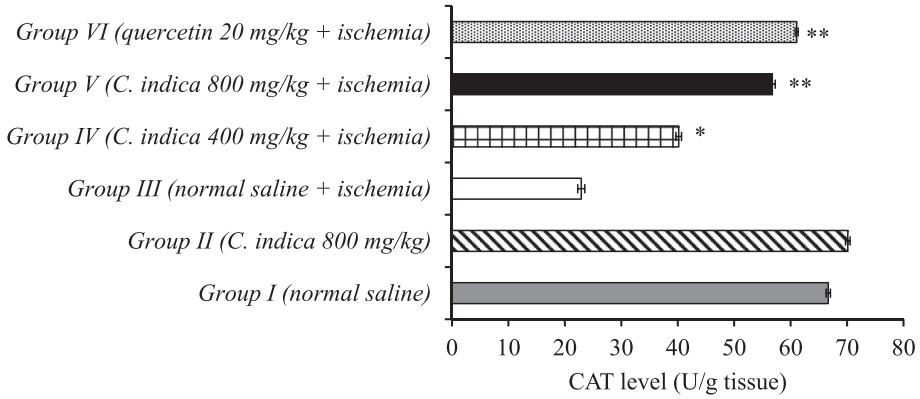

Fig. 6. Effect of methanolic extract of *C. indica* on catalase (CAT) level of rat brain to occlusion followed by 4 h reperfusion induced ischemia, where values are expressed as mean + SEM ($N = 6$), and the results are compared with ischemic control group ($^{*} = p < 0.05$, $^{**} = p < 0.01$).

(Mukherjee et al., 2007; Wong and Crack, 2008). Low levels of SOD (Fig. 5) and CAT (Fig. 6) were observed in the ischemia-induced and reperfusion group rats. The *C. indica* extract-treated group animals showed significantly increased levels of SOD and CAT along with the quercetin-treated group. The group treated with extract at 250 mg/kg body weight showed a lower significant difference ($p < 0.05$) than the 800 mg/kg body weight ($p < 0.01$) group.

CI induces a complex cascade of biochemical and molecular changes, including inflammatory reactions (Lakhan et al., 2009). The slowdown of the blood flow to the brain prevents the availability of oxygen; this leads to a reduction in aerobic metabolism in the brain cells (White et al., 2000). So, automatically they cannot perform their anaerobic metabolisms due to a lack of energy (ATP) (Xiang et al., 2010). This causes an electrolyte imbalance in the cells (inflow of Ca ions into cytosol), removal of waste slowdown, formation of reactive oxygen species (ROS/free radicals) increases due to the alteration in the anti-oxidative enzymes dysfunction, which affect permeability and the cell structure (McCord, 1985). These imbalances in a series of mechanisms cause injury to the brain tissue and inflammation (Green, 2008). MPO is

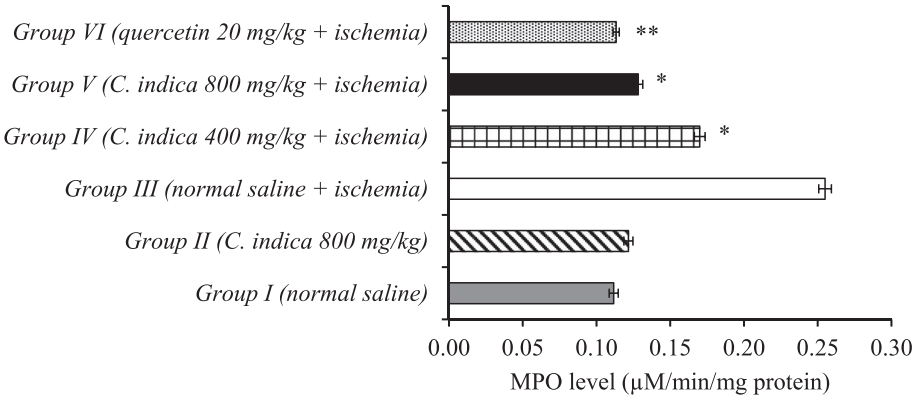

Fig. 7. Effect of methanolic extract of *C. indica* on myeloperoxidase (MPO) level of rat brain to occlusion followed by 4 h reperfusion induced ischemia, where values are expressed as mean + SEM ($N = 6$), and the results are compared with ischemic control group ($^{*} = p < 0.05$, $^{**} = p < 0.01$).

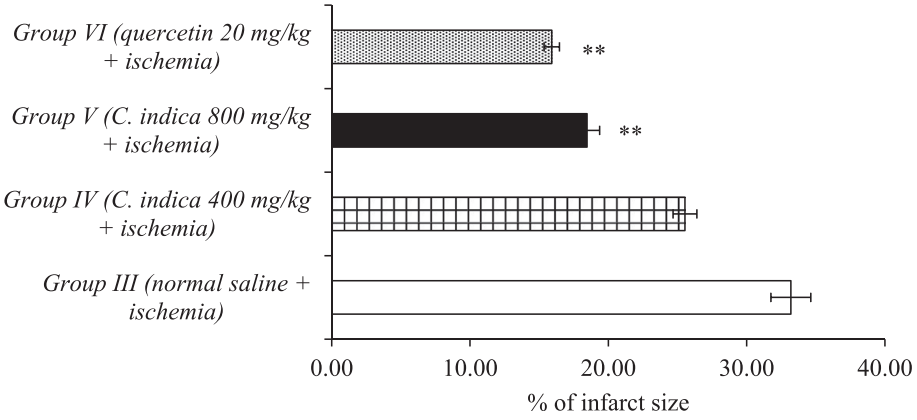

Fig. 8. Effect of methanolic extract of *C. indica* on rat brain subjected to occlusion followed by 4 h reperfusion induced ischemia, where values are expressed as mean + SEM ($N = 6$), and the results are compared with ischemic control group ($^{**} = p < 0.01$).

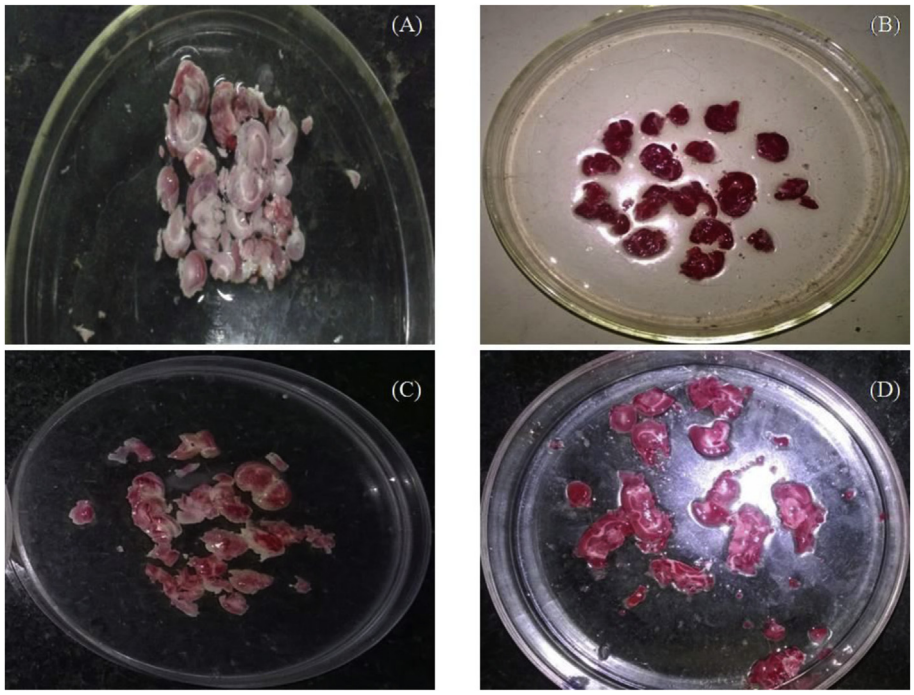

Fig. 9. Effect of ischemia and *C. indica* extract-treated groups on brain tissue: (A) damaged brain due to ischemia; (B) brain of normal animal (without ischemia). Brian tissue in Ischemia-induced animals treated with *C. indica* extract: (C) at 400 mg/kg body weight; (D) at 800 mg/kg body weight.

the most abundant component in neutrophils myeloid line, especially in monocytes and macrophages/microglia (Breckwoldt et al., 2008). MPO interacts with hydrogen peroxide to generate highly reactive species including hypochlorite (ClO^-) and radicalized oxygen species ($^\bullet O_2^-$, ONO_2^-; Jaimes et al., 2001). MPO-mediated radicalization of molecules induces apoptosis and nitrotyrosination of proteins (Lo et al., 2005; Lau and Baldus, 2006). An increased MPO level was observed in the ischemia-induced group. There was a significant reduction in the levels of MPO (Fig. 7) in the pretreatment groups with *C. indica* at 400 mg/kg and 800 mg/kg.

CI and reperfusion forms alterations in the enzyme levels of the brain and finally brain injury (Bederson et al., 1986; Chan, 1996). The reduction of enzyme activity can lead to a reduction in pH or acidosis. Ischemia forces the cells to undergo anaerobic metabolism, thereby producing lactic acid and acidosis (Schurr, 2002). Enzymes that are pH-sensitive will, therefore, be easily affected and thus, substantial alteration to the antioxidant enzyme activities during cerebral ischemia and reperfusion may be responsible for more neuro-degeneration than ischemia (Chan, 2001; Dröge, 2002). A greater infarct brain size was observed in the ischemia-induced group. However, a low infarct brain size was observed in the pretreated groups with *C. indica* (Fig. 8). The infarct size of the brain in the ischemia-induced group was 33.20 ± 1.29, the infarct size in the extract-treated groups at 400 mg/kg and 800 mg/kg were 25.54 ± 0.77 and 18.46 ± 0.81, respectively. The results for the extract at 400 mg/kg were significantly different ($p > 0.05$) compared to 800 mg/kg ($p < 0.01$) and it reduced the infarct size (Fig. 9) formation by nearly 10%.

The present investigation demonstrated the antioxidant effect of methanolic extract from *C. indica* roots on different free radicals and it was tested with cerebral ischemia and reperfusion-induced oxidative stress. The results suggested that the *C. indica* is protective against ischemia-induced oxidative stress by mechanisms involving inhibition of free radical generation, reactive oxygen species scavenging and modulation of intracellular antioxidants against ischemic reperfusion-induced decreasing. Further work is needed to isolate and characterize the responsible bioactive molecules from *C. indica*.

Conflict of interest

The authors report no conflicts of interest.

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