

Protective Effect of Embelin from *Embelia ribes* Burm. Against Transient Global Ischemia-Induced Brain Damage in Rats

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Abstract *Embelia ribes* is being used in Indian traditional herbal medicine for the treatment of mental disorders and as brain tonic. The present study was designed to investigate the protective effects of embelin from *E. ribes* on global ischemia/reperfusion-induced brain injury in rats. Transient global ischemia was induced by occluding bilateral common carotid arteries for 30 min followed by 24-h reperfusion. Neurological functions were measured using sensorimotor tests. Ischemia/reperfusion-induced neuronal injury was assessed by cerebral infarct area, biochemical and histopathological examination. Pretreatment of embelin (25 and 50 mg/kg, p.o.) significantly increased locomotor activity and hanging latency time and decreased beam walking latency when compared with ischemic control. The treatment also reduced significantly the lipid peroxidation and increased the total thiol content and glutathione-S-transferase activity in brain homogenates. The decreased cerebral infarction area in embelin-treated groups and histopathological observations confirmed the above findings. These observations suggested that embelin is a neuroprotective agent and may prove to be useful adjunct in the treatment of stroke.

Keywords Embelin · Neuroprotective · Ischemia–reperfusion · Antioxidant

Introduction

Ischemic stroke accounts for 88% of all strokes and is associated with a high incidence of morbidity and mortality (Van der Worp and van Gijn 2007). Stroke has been ranked third most common cause of death worldwide and cerebrovascular diseases are considered second most frequent causes of deaths in the year 2020 (Huang and McNamara 2004). Hence, a major challenge that confronts medical researchers is the development of therapeutic agents, which prevent neuronal degeneration induced by ischemia, hypoxia and other traumatic insults to the CNS. Natural products probably represent an ideal source to develop safe and effective agents for the management of stroke and deserve scientific probe. Growing evidence supports the role of oxidative stress as one of the primary factors in brain injury mediated by cerebral ischemia and stroke (Landemmer and Harrison 2001). The brain is particularly vulnerable to oxidative stress because of its high rate of oxidative metabolic activity, intense production of reactive oxygen species (ROS) metabolites. ROS namely superoxide and hydroxyl free radicals, together with hydrogen peroxide have been proposed to cause neurotoxic effect and initiate a free radical-mediated chain reaction causing additional damage to diverse areas in the brain (Kondo et al. 1997). Therefore, oxidative injury could be one possible cellular cascade affecting all organs and tissues during ischemia.

Many antioxidants are reported to reduce ROS-mediated reactions and rescue neurons from ischemia/reperfusion-induced neuronal loss in animal models of cerebral ischemia (Gaur et al. 2009; Thiyagarajan and Sharma 2004).

Embelia ribes Burm. (Family: Myrsinaceae) is a medicinal plant used traditionally as anti-inflammatory to relieve rheumatism and fever (Kapoor et al. 1983). Its fruits

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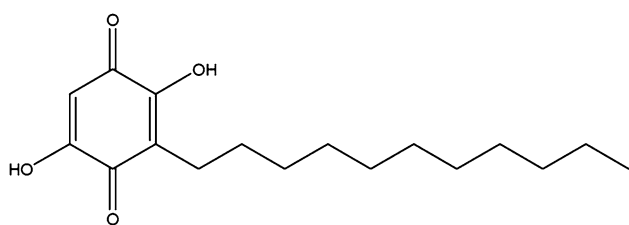


Fig. 1 Structure of embelin

are used as brain tonic, in the treatment of mental disorders, dyspnoea, diseases of the heart, etc. (Varier 2006). *E. ribes* is an important ingredient of a number of ayurvedic formulations in India (Pandey 1996; Vaidya-Arya 1999). Bhandari et al. (2002, 2007) have reported the dyslipidemic and antioxidant activity of ethanolic extract of *E. ribes* in streptozotocin-induced diabetes in rats. Its aqueous (Bhandari and Ansari 2008) and ethanolic extracts (Ansari and Bhandari 2008) were reported for their potent neuroprotective effects.

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone, Fig. 1) is a naturally occurring alkyl substituted hydroxy benzoquinone and a major constituent from all the parts of *E. ribes*. Embelin reportedly possesses anti-inflammatory, analgesic (Chitra et al. 1994), antioxidant (Joshi et al. 2007), hepatoprotective (Dharmendra et al. 2009), wound healing (Kumara Swamy et al. 2009), antibacterial (Chitra et al. 2003), anticonvulsant (Mahendran et al. 2011) and antidiabetic (Mahendran et al. 2010) properties. Many quinones, thymoquinone (Al-Majed et al. 2006), idebenone (Nagaoka et al. 1989) and pyrroloquinoline quinone (Zhang et al. 2006) are known for their beneficial effects on cerebral ischemia. However, embelin has not been screened so far for its neuroprotective activity though the plant is traditionally used and scientifically proven as a neuroprotectant and antioxidant. Hence, in the present study we were interested to investigate the effect of embelin on transient global ischemia/reperfusion-induced neuronal damage in rats.

Materials and Methods

Isolation of Embelin from *Embelia ribes*

The berries of *E. ribes* were purchased from Abirami Botanicals, Tuticorin, Tamilnadu, India, and authenticated by Medicinal Plants Survey and Collection Unit, Ootacamund, Tamil Nadu, India, where a voucher specimen is preserved for further reference. Embelin was isolated according to our previously published protocol (Mahendran et al. 2011) and purity was matched with authentic sample.

Chemicals

1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Sigma–Aldrich, St. Louis, USA. Reduced glutathione (GSH), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 2,3,5-triphenyltetrazolium chloride (TTC), thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. All the other chemicals used were of analytical grade.

Animals

Male Wistar rats (200–260 g) were obtained from the animal house of Sree Siddaganga College of Pharmacy, Tumkur, India, maintained under standard conditions (12 h light/dark cycle; $25 \pm 3^\circ\text{C}$, 45–65% humidity) and had a free access to standard rat feed and water ad libitum. All the animals were acclimatized to laboratory conditions for a week before commencement of the experiment. The experiments were performed during the light portion between 08:00 and 12:00 am to avoid circadian influences. Animal studies were performed according to the prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India.

Preparation of Suspensions

Embelin was suspended in distilled water using Tween 80 (1% v/v). Two doses of embelin (25 and 50 mg/kg) were selected for administration by oral route based on our earlier study (Mahendran et al. 2010).

Ischemic Reperfusion Injury

Animals were divided into five groups of 6–10 rats each: Group I, sham-operated control received the vehicle (Tween 80, 1% v/v) ($n = 6$). Group II received embelin alone (50 mg/kg b.w.) and was subjected to sham operation ($n = 8$). Group III received vehicle and served as ischemic control ($n = 10$). Groups IV and V received embelin at 25 and 50 mg/kg b.w. ($n = 10$). All these treatments were given for 4 days orally by using oral gavage. On the fourth day, after 2 h of the above treatments, the animals of Groups III, IV and V were subjected to cerebral ischemia/reperfusion using the following procedure.

The animals were treated with atropine sulfate (0.5 mg/kg, i.p.) as preanesthetic medication and anesthetized with chloral hydrate (350 mg/kg, i.p.). Through a midline incision, a dissection was made between the sternocleidomastoid and the sternohyoid muscles parallel to the trachea and both common carotid arteries were exposed (Jingtao et al. 1999). Each carotid artery was freed from its

adventitial sheath and vagus nerve, which were carefully separated and maintained. Ischemia was induced by occluding bilateral common carotid arteries (BCCA) with clamps for 30 min. Subsequently, both clamps were removed and both arteries were inspected for immediate reperfusion and the neck incision area was closed with stitches using suture. Sham control animals received the same surgical procedures, except the occlusion of BCCA. After 30 min of global ischemia, reperfusion was allowed for 24 h. After the completion of reperfusion period (24 h), the animals were assessed motor performance tests by the following neurological outcomes.

Locomotor Activity

The locomotor activity was recorded by using actophotometer (INCO Pvt. Ltd., Ambala, India). Before locomotor task, animals were placed individually in the activity meter for 2 min for habituation. Thereafter, locomotor activity was recorded using actophotometer for a period of 5 min (Kulkarni 1999).

Beam Walking Test

Beam walking test was used to evaluate gross vestibulo-motor function. The apparatus consisted of a rod 120 cm in length and with a diameter of 2.3 cm. A wooden box (20 cm × 20 cm × 10 cm) was set at one end of the rod as a nest for motivating the animal to cross the beam. The apparatus was suspended 50 cm above a cushion, which protected the animals against fall injury. Rats were trained twice daily for 2 days before BCCA occlusion and assessed for motor coordination after 24 h of reperfusion. The time taken to traverse the beam was recorded. The cut-off time was taken as 120 s (Song et al. 2006; Yan et al. 2007).

Hanging Wire Test

This task was used to measure forelimb grip strength of the rats. In this test, animals were suspended by the forelimbs on a wire (45 cm long and 0.3 cm diameter) stretched between two posts 40 cm above a foam pillow. The time (s) until the animal fell was recorded. The cut-off time was taken as 90 s (Hunter et al. 2000).

Biochemical Estimations

After the motor performance tests, the animals ($n = 4-8$ from each group) were killed, brains were removed and homogenized in cold phosphate-buffered saline (10% w/v) and the suspension was centrifuged at $12000\times g$ (Remi Motors Ltd., Mumbai, India) for 15 min at -4°C . The

supernatant was used for the following biochemical analysis.

Measurement of Total Protein

The protein content of the brain homogenate was determined by Lowry's method (Lowry et al. 1951) using bovine serum albumin as standard.

Measurement of Lipid Peroxidation

The extent of lipid peroxidation was measured by estimating the amount of malanodialdehyde (MDA) formed, as described by Akhtar et al. (2008). Briefly, to 0.1 ml homogenate, 1 ml of 10% (w/v) trichloroacetic acid (TCA) and 1 ml of 0.67% (w/v) thiobarbituric acid were added and placed in a boiling water bath for 30 min. Then the mixture was placed in crushed ice for 10 min followed by centrifugation at $4000\times g$ for 10 min. The absorbance of the clear pink-colored supernatant was measured at 532 nm and the results were expressed as nmol MDA/mg protein.

Measurement of Total Thiols

The total thiol content was determined by the method described by Sedlak and Lindsay (1968), with slight modifications. To a mixture of 0.2 ml of homogenate and 0.36 ml of buffer, 0.04 ml of 10 mM DTNB and 1.5 ml of methanol were added and mixed well. The mixture was centrifuged at $1500\times g$ for 5 min at -4°C . Then the intensity of the yellow color developed was measured at 412 nm and the results were expressed as nmol/mg protein.

Measurement of Glutathione-S-Transferase

Glutathione-S-transferase (GST) activity was measured as described by Habig et al. (1974). Briefly, 0.85 ml of Phosphate buffer (pH 7.4), 0.05 ml homogenate and 0.05 ml of 10 mM GSH were added, to which 0.05 ml of 1 mM CDNB was added to initiate the reaction. The rate of formation of GSH-CDNB complex was monitored for 5 min at 340 nm and the results were expressed as nmol of CDNB conjugate formed/min/mg protein.

Measurement of Brain Infarct Area

For the measurement of infarct area, two animals from each group were decapitated under deep anesthesia and the brains were removed and sliced coronally into 2-mm-thick sections, incubated in phosphate-buffered saline (pH 7.4) containing 2% of 2,3,5-triphenyltetrazolium chloride (TTC) for 30 min at 37°C and fixed in 10% neutral-buffered formalin overnight (Bederson et al. 1986; Fatehi-Hassanabad and

Tasker 2011). The images of the stained sections were acquired using a high-resolution scanner, quantitatively analyzed by using Image J 1.43u software (NIH, USA) and expressed as a percentage of the total measured brain area.

Histopathological Studies

A section of the brain was fixed with 10% formalin, embedded in paraffin wax and cut into sections of 5- μ m thickness. The sections were stained with hematoxylin and eosin dye for histopathological observations.

Statistical Analysis

The values were expressed as mean \pm SEM. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. *P* values <0.05 were considered as significant.

Results

Neurological Outcomes

In the sham control animals, the locomotor activity count and hanging latency time were found to be high and beam walking latency was low indicating the normal behavior of the animals. In the ischemic control animals, the locomotor activity and hanging latency time decreased significantly and beam walking latency was significantly increased indicating impairment of sensorimotor functions due to BCCA occlusion. The decrease in the locomotor count and hanging latency time was found to be 72.54 and 75.19%, respectively. Similarly, the increase in the beam walking latency was found to be 189.59%.

Pretreatment with different doses of embelin (25 and 50 mg/kg p.o.) for 4 days followed by ischemic reperfusion injury significantly increased (*P* < 0.05) the locomotor activity and hanging latency time and significantly decreased (*P* < 0.01) the beam walking latency periods towards the normal indicating the protective effect of

embelin. The increase in the locomotor activity, hanging latency time and the decrease in beam walking latency when compared with ischemic control for the embelin 25 mg/kg treatment were found to be 200, 254.50 and 64.76%, respectively. These values for embelin 50 mg/kg treatment were found to be 260, 279.16 and 65.42%, respectively. Embelin 50 mg/kg pretreatment exhibited almost similar results to the sham control animals indicating complete reversal of ischemic damage. Embelin, when administered to sham-operated rats, showed slight reduction in the locomotor activity as well as hanging latency time. However, these were found to be insignificant indicating its safe nature (Table 1).

Biochemical Estimations

The level of MDA in BCCA occluded ischemic control rats was found to be 3.70 ± 0.42 , a significant rise (*P* < 0.001) of about 141% when compared with 1.53 ± 0.07 of sham control animals. Pretreatment with embelin at 25 mg/kg followed by BCCA occlusion caused a significant reduction (*P* < 0.01) in the MDA levels to 1.85 ± 0.44 when compared with ischemic control animals. Embelin at 50 mg/kg significantly (*P* < 0.001) lowered this value to

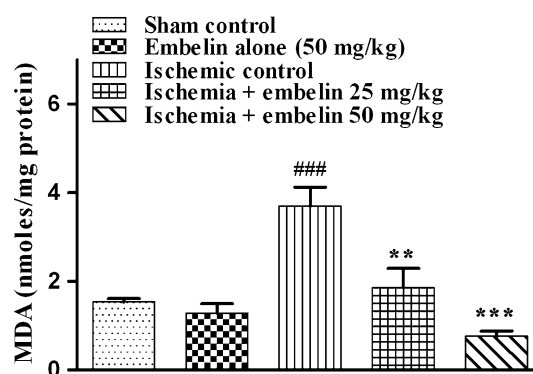


Fig. 2 Effect of embelin on lipid peroxidation after transient global ischemia in rats. Values are given as mean \pm SEM. ###*P* < 0.001 versus sham control; ***P* < 0.01, ****P* < 0.001 versus ischemic control

Table 1 Effect of embelin on behavioural parameters in BCCA occluded rats

Treatments	Locomotor activity counts/5 min	Hanging latency time (s)	Beam walking latency (s)
Sham control (<i>n</i> = 6)	174.80 \pm 35.30	65.40 \pm 11.63	14.23 \pm 5.10
Embelin alone (50 mg/kg) (<i>n</i> = 7)	157.00 \pm 28.78	42.00 \pm 10.43	16.09 \pm 2.51
Ischemic control (<i>n</i> = 8)	48.00 \pm 6.97 ^{##}	16.22 \pm 4.95 [#]	41.21 \pm 8.21 ^{##}
Ischemia + embelin 25 mg/kg (<i>n</i> = 8)	144.00 \pm 20.00*	58.50 \pm 10.59*	14.52 \pm 1.23**
Ischemia + embelin 50 mg/kg (<i>n</i> = 7)	172.80 \pm 32.62*	61.50 \pm 4.87*	14.25 \pm 3.68**

Values are given as mean \pm SEM

P < 0.05, ## *P* < 0.01 versus sham control; * *P* < 0.05, ** *P* < 0.01 versus ischemic control

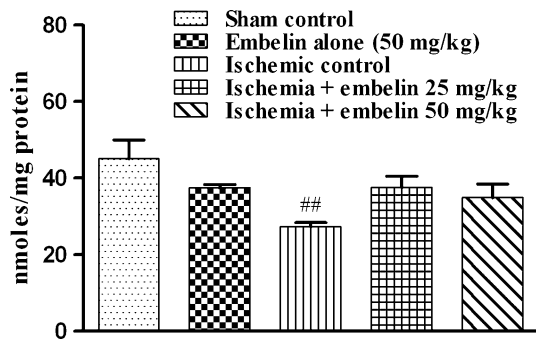


Fig. 3 Effect of embelin on total thiol levels after transient global ischemia in rats. Values are given as mean ± SEM. ##*P* < 0.01 versus sham control

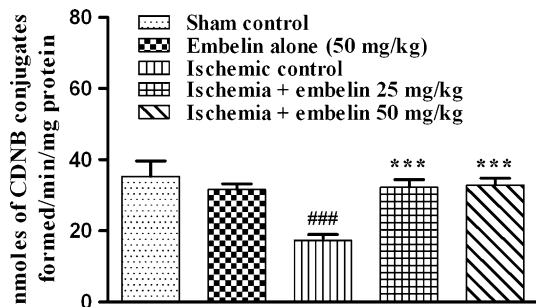


Fig. 4 Effect of embelin on glutathione-S-transferase activity after transient global ischemia in rats. Values are given as mean ± SEM. ###*P* < 0.001 versus sham control; ****P* < 0.001 versus ischemic control

0.75 ± 0.12, a value lower than the sham control indicating its potent neuroprotective effect (Fig. 2).

Ischemia for 30 min, significantly (*P* < 0.01) reduced the total thiol levels to 27.31 ± 1.15 compared with sham control (45.01 ± 4.95). The pretreatment with embelin at both the doses increased the total thiols to 37.54 ± 2.98 and 34.88 ± 3.60, respectively, when compared with ischemic control (Fig. 3). A significant reduction (*P* < 0.001) of around 51% in the GST activity was observed in ischemic

control when compared with sham control. A significant increase (*P* < 0.001) in the enzyme level was found in the pretreatment of embelin at both the tested doses when compared with ischemic control. The increase is almost equivalent to sham control indicating the reversal of ischemic insult to normal levels (Fig. 4). However, the embelin-alone treatment caused a slight decrease in all the biochemical parameters which was non-significant.

Brain Infarct Area

The brain infarct area of ischemic control animals was found to be 21.08 ± 1.22%. The pretreatment with different doses of embelin at (25 and 50 mg/kg) significantly (*P* < 0.01) reduced the brain infarct area to 11.96 ± 0.19 and 8.61 ± 0.61%, respectively (Figs. 5, 6).

Histopathological Studies

The brain histology of sham control animal has showed normal appearance. Ischemic control animal brain exhibited increased glial density, presence of lymphocytes, edema, congested blood vessels and neuronal necrosis. The pretreatment with embelin at 25 mg/kg followed by ischemia showed increased glial density and edema, along with absence of lymphocytes, decreased congestion of blood vessels and neuronal necrosis. Embelin pretreatment at 50 mg/kg showed normal glial density, decreased edema, absence of lymphocytes, congestion of blood vessels and necrosis (Fig. 7).

Discussion

Bilateral common carotid artery (BCCA) occlusion is a well-known model used to study cerebral ischemia. BCCA occlusion causes reduction in blood flow to diverse regions of brain and contributes to associated behavioral and cognitive deficits (Tsuchiya et al. 1993; Martinez et al. 1997).

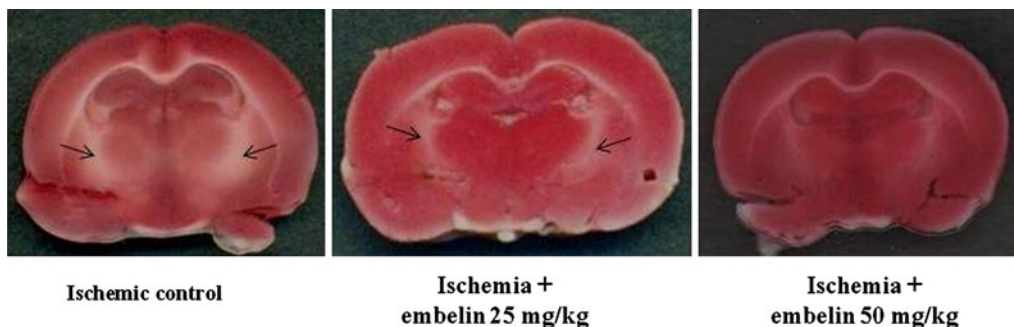


Fig. 5 A representative photograph of rat brain coronal sections after staining with TTC in experimental rats. Arrow indicates area of infarction

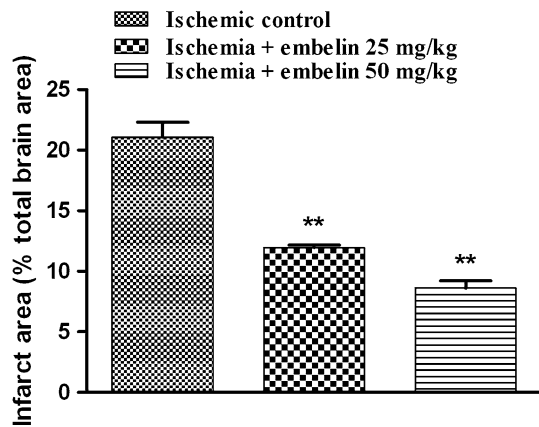
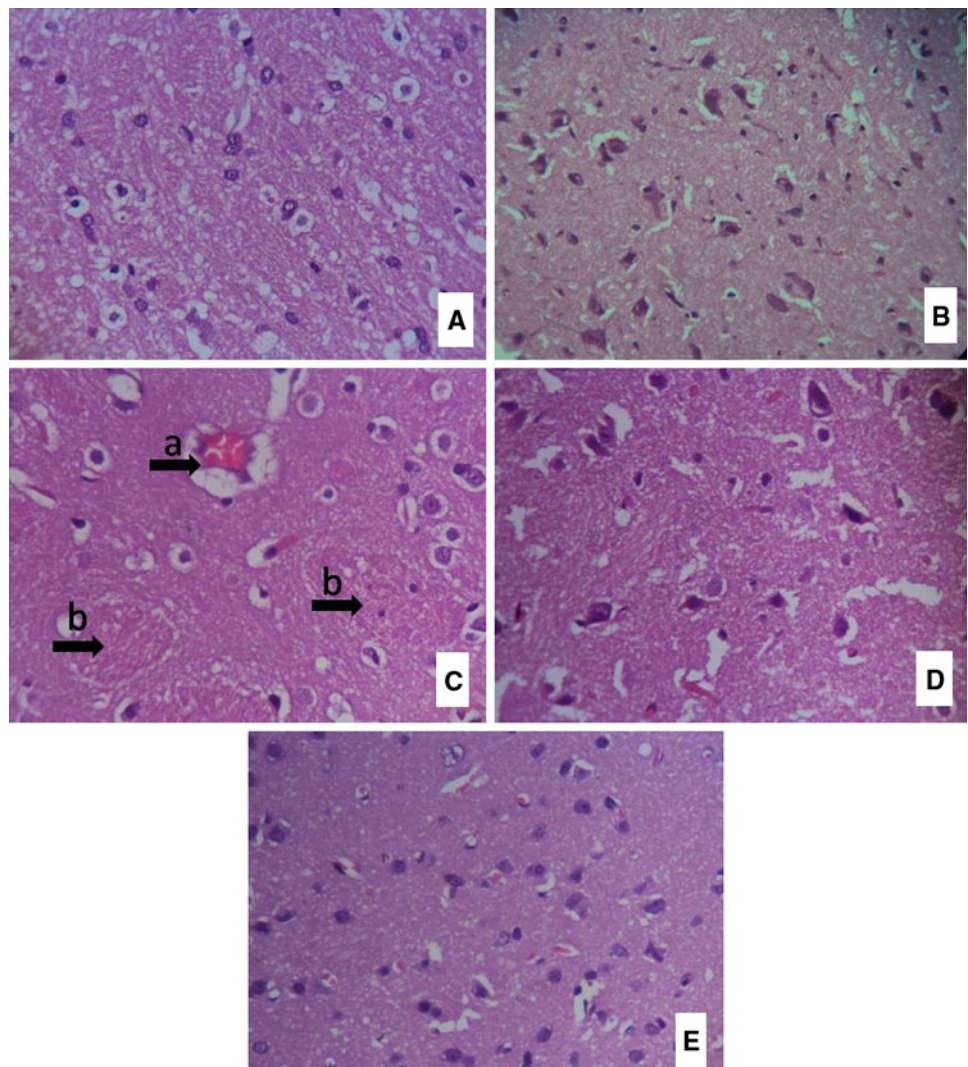


Fig. 6 Effect of embelin on brain infarction area after transient global ischemia in rats. Values are given as mean \pm SEM. ** $P < 0.01$ versus ischemic control ($n = 2$)

In the present study, a significant decrease (72.54%) in locomotor activity and an increase (189.59%) in beam walking latency have been observed after ischemia

Fig. 7 Histopathological changes in brain of experimental rats. **A, B** Sham control and embelin alone, normal brain showing normal blood vessels and brain parenchyma. **C** Ischemic control showing congested blood vessels (*a*), neuronal necrosis (*b*) and increased lymphocyte infiltration. **D** Ischemia + embelin 25 mg/kg showing reduced congested blood vessels, lymphocytes and absence of necrosis. **E** Ischemia + embelin 50 mg/kg showing absence of necrosis and blood vessel congestion



reperfusion, which was reversed by embelin pretreatment (25 and 50 mg/kg p.o.) for 4 days. Similarly, global ischemia has been reported to cause significant decrease in grip and muscle strength of the limbs (Mukherjee et al. 2007; Rehni and Singh 2007). Consistent with this report, a significant increase in muscle strength and grip has been observed in embelin pretreatment at both the tested doses, suggesting the therapeutic potential of embelin against ischemic/reperfusion injury. A complete reversal of ischemic damage caused by BCCA occlusion was observed in animals pre-treated with higher dose of embelin.

Oxidative stress has been reported to play a major role in the ischemic reperfusion injury (Gaur et al. 2009; Al-majed et al. 2006; Nagaoka et al. 1989). The elevated MDA levels observed in the ischemic rats was significantly reduced in the embelin-pretreatment indicating its free radical scavenging effects. Ischemic injury to neurons depletes the total thiol content of the brain (Chandrashekhar et al. 2010). Rats pretreated with embelin at 25 and 50 mg/kg significantly increased the total thiol content

when compared with ischemic rats. The pretreatment with embelin at both the doses restored the depleted GST activity in the brain homogenates induced by ischemia. These results are in agreement with the earlier reports of potent antioxidant activity of embelin (Dharmendra et al. 2009; Joshi et al. 2007).

TTC staining was used to assess the extent of cell death in brains subjected to ischemia/reperfusion (Fatehi-Hassanabad and Tasker 2011). The extent of infarction varies with different animal models of stroke. In the present study, approximately 20% of the total brain area was infarcted. This observation is consistent with an earlier report showing a relatively lesser (14–18%), but observable infarct area in global ischemia/reperfusion-injured animals (Fatehi-Hassanabad and Tasker 2011). The pretreatment with embelin at both the doses showed comparatively less (9–12%) infarct lesion area indicating that embelin could protect the neurons from ischemic injury. The above results were further confirmed by histopathological studies, where the brain tissue of embelin-treated rats showed a reversal of the damage caused by ischemic reperfusion injury.

Hypoxic ischemic brain injury, carotid artery pathologies, asphyxiation and shock are a few age-related disorders seen frequently during cerebral ischemia (Gaur et al. 2009). Several plants including *E. ribes* and benzoquinone comprising phytochemicals exhibited potent neuroprotective effects and found to be useful in ischemia-related disorders (Bora and Sharma. 2010; Chandrashekhar et al. 2010; Ansari and Bhandari 2008; Al-Majed et al. 2006; Nagaoka et al. 1989; Zhang et al. 2006). Neuroprotection aimed at preventing ischemia/reperfusion injuries in transient global cerebral ischemia is an effective strategy in the treatment of stroke and related pathologies. Naringin, thymoquinone, idebenone and several other known antioxidants have shown protective effects against cerebral ischemia (Gaur et al. 2009; Al-majed et al. 2006; Nagaoka et al. 1989). The anticonvulsant effect of embelin in maximal electroshock and pentylenetetrazole-induced seizure was earlier reported by us (Mahendran et al. 2011). Several anticonvulsants have shown protection against ischemic/reperfusion-induced neuronal damage (Edmonds et al. 2001). Many benzoquinones are known to possess neuroprotective property in different animal models of ischemic brain injury (Al-Majed et al. 2006; Nagaoka et al. 1989; Zhang et al. 2006). Hence, these reports further supported the evidence of embelin having neuroprotective action.

In conclusion, embelin normalized the altered behavioral, biochemical and histopathological parameters in ischemic rats, suggesting the neuroprotective action of embelin. The possible mode of protection may be due its antioxidant and anticonvulsants properties. However,

further research is warranted to elucidate the specific mechanism/s involved.

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