Original Article

Protective effects of *Echium amoenum* Fisch. and C.A. Mey. against cerebral ischemia in the rats

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Abstract

Background: This study was designed to evaluate the protective effect of *Echium amoenum* total anthocyanin extract (ETAE) on partial/transient cerebral ischemia in the rats.

Materials and Methods: Rats received ETAE (50, 100 and 200 mg/kg, i.p.) 30 min before the induction of cerebral ischemia. Cerebral ischemia was induced by bilateral common carotid arteries occlusion (BCCAO) for 30 min, followed by 72 h reperfusion. The neurological deficit, brain performance, and sensory motor function were assessed 48 h and 72 h after surgery. After sacrification, the brains were evaluated for myeloperoxidase (MPO) activity and histopathological changes.

Results: Our results showed that motor function significantly decreased in ischemia/reperfusion (I/R) group as compared to the sham group. Histopathological analysis exhibited the shrinkage and atrophy of the neurons in I/R group. ETAE at the dose of 200 mg/kg improved spontaneous activity and memory induced by cerebral ischemia compared to the control group and also decreased brain MPO activity following cerebral ischemia. However, it could not affect the ability to climbing, body proprioception, vibrissae touch and brain water content. In addition, pretreatment with ETAE at higher doses significantly reduced ischemia-induced neuronal loss of the brain.

Conclusion: The anthocyanin rich fraction from *E. amoenum* was found to have protective effects against some brain damages postischemic reperfusion . However, further researches are required for investigating the exact mechanisms of the effect of this plant in the prevention of cerebral ischemia in human.

Key Words: Anthocyanins, cerebral ischemia, *Echium amoenum*, rat

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INTRODUCTION

Ischemic stroke is a devastating disease and the second leading cause of death.[1] Thromboembolic occlusion of the major intracranial arteries is the most causes of the ischemic stroke. Cerebral ischemia occurs commonly in some clinical conditions including cardiac arrest, shock and asphyxia, and in complex cardiac surgery.[2-5] Neurologic deficit from brain

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injury are diverse and includes coma, seizures, ischemic stroke, delirium, and neurocognitive impairment.[6-8] The pathophysiological mechanism underlying ischemic brain injury after cardiac arrest resulted from systemic hypoperfusion that can occur with or without preexisting large-vessel occlusive disease. Recombinant tissue plasminogen activator is the only currently approved therapeutic in acute stroke, which can be infused in the selected patients within 4.5 h after the onset of ischemia to resolve the vessel-occluding thrombus and to restore cerebral blood flow. Success rates, however, are moderate at best, since even recanalization does not promise to save brain tissue, a phenomenon referred to as reperfusion injury. The pathophysiology of reperfusion induced injury during acute stroke is incompletely understood.[9-12] Restoration of blood flow, although seems to be necessary for brain survival, could lead to excessive reactive oxygen species (ROS) creation and activates nitric oxide synthase that resulting oxidative/nitrosative stress. Thus, cerebral ischemia and reperfusion can produce neuronal damage through triggering a complex series of biochemical events that affect structure and function of the brain.[13-16]

Although the pathobiological mechanisms of ischemia/reperfusion (I/R) injury are multifactorial, oxidative stress seems to represent the common final path.[16] Recently, intense interest has focused on the antioxidant properties of natural products. In particular, natural products may act by preventing the free radical generation, neutralizing free radicals by nonenzymatic mechanisms, and/or by enhancing the activity of endogenous antioxidants.[17] *Echium amoenum* Fisch and C.A. Mey (*Boraginaceae*), an important Iranian traditional remedy, is widely used as a tonic, tranquillizer, diaphoretic, and as a remedy for cough, sore throat and pneumonia.[18] It is believed that this plant possesses antibacterial, antioxidant, analgesic, anxiolytic, antidepressant and immunomodulatory properties.[19-24] Also, it has been shown that *E. amoenum* aqueous extract was effective in the treatment of obsessive-compulsive disorder.[25] Dried violet-blue petals of *E. amoenum* have been recently recognized as an important source of phenolic compounds like rosmarinic acid, cyanidin, and delphinidin.[26]

Cyanidin-3-glucoside, the most common anthocyanin, which is present in petals of *E. amoenum* inhibits activation and translocation of c-Jun and nuclear factor-κB (NF-κB) factors into nucleus so attenuates prostaglandin E2 production and cyclooxygenase-2 (COX-2) expressions.[27] Also, the neuroprotective activity of cyanidin-3-glucoside has been investigated by Min *et al*. They suggested that its beneficial effect was related to blocking apoptosis-inducing factor release in mitochondria that attenuates brain superoxide levels.[28] Delphinidin, another anthocyanin that is present in petals of *E. amoenum*, inhibits directly Fyn kinase activity so attenuates tumor necrosis factor-alpha (TNF)-induced COX-2 expression.[29]

The present study was performed to evaluate the protective effects of petals of *E. amoenum* total anthocyanin extract (ETAE) in an animal model of cerebral ischemia.

MATERIALS AND METHODS

Plant material and extraction

Echium amoenum petals (flowers) were collected from Ghazvin, Iran, in summer, and authenticated by Professor Mohammad Reza Rahiminejad, Isfahan University, Isfahan, Iran. The voucher specimen of *E. amoenum* was deposited in the Herbarium Department of our school with number 1147. Dried petals were chopped into 7 mm of particles. After that, CH_3COOH (1%) was added into 120 g of materials at a rate of 1:15, which yielded to 1800 mL of solution. The process of extraction continued for 2 h at room temperature, using magnetic blender. Extract was filtered, and 1450 mL solution was obtained. Solution was treated with dichloromethane for 3 times, using 500 mL of dichloromethane for each. Then solution was treated again with ethyl acetate 2 times, 500 mL of ethyl acetate for each. The remaining solution, 1300 mL, was dried in the lyophilisator.[30]

Determination of total anthocyanin content

Anthocyanins have a critical role in the color quality of many plants. With a change in pH, anthocyanin pigments undergo reversible structural conversions. Colored oxonium form prevails at pH 1.0; but the colorless hemiketal form exists at pH 4.5. The difference in the absorbance of oxonium and hemiketal forms at λ max of 510 nm is related to the anthocyanin content. Anthocyanin concentration was calculated as cyanidin-3-glucoside equivalent by the following equation:

Anthocyanin content (%) = ([Abs of solution A – Abs of solution B] $\times Mw \times$ DF/[$\varepsilon \times$ wt])

Where Mw is the molecular weight of cyanidin-3-glucoside (449.2) and ε is the molar absorptivity (26,900) at 510 nm in the pH = 1, DF is the dilution factor and Wt is the sample weight.[31]

Animals

Male Wistar rats weighting 200–250 g and bred in animal house (Isfahan School of Pharmacy, Isfahan, Iran) were used in this study. Animals were kept in standard condition and light/dark cycles (12/12 h), and allowed free access to pelleted rodent chow and tap water. All experiments were performed according to the international guidelines for laboratory animal use and care.

Experimental groups

In this experiment, animals were divided into five groups each having six animals. The first group served as sham. The second group was ischemia/reperfusion (I/R) induced animals, whilst the third, fourth and fifth I/R groups were pretreated with ETAE (50, 100 and 200 mg/kg i.p. respectively).

Induction of cerebral ischemia/reperfusion injury in rats

Induction of cerebral I/R was carried out by bilateral common carotid arteries occlusion (BCCAO) followed by 72 h reperfusion.[32] The animals were anesthetized with isofluran. 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. The induction of ischemia was performed by BCCAO with clamps for 30 min followed by 72 h reperfusion, and the skin was closed with stitches using waxed silk suture. Sham control animals received the same surgical procedures, except BCCAO were not performed. After the completion of the reperfusion period, the animals were assessed for neurological outcome and then sacrificed.

Behavioral assessment

Neurological deficits in I/R and ETAE treated groups were measured at the day of surgery and following after 48 and 72 h of reperfusion period by behavioral assessment including assessment of spontaneous activity, symmetry in the movement of four limbs, ability of climbing, body proprioception and response to vibrissae touch.

In spontaneous activity test, animals were observed for 5 min in its normal environment (cage). The rat's activity was assessed by its ability to approach all four walls of the cage. Scores indicate the following: (3) Rat moved around, explored the environment, and approached at least three walls of the cage; (2) slightly affected rat moved around in the cage but did not approach all sides and hesitated to move, although it eventually reached at least one upper rim of the cage; (1) severely affected rat did not rise up at all and barely moved in the cage; and (0) rat did not move at all.

For symmetry in the movement of four limbs assessment, the rat was held in the air by the tail to observe symmetry in the movement of the four limbs. Scores indicate the following: (3) All four limbs extended symmetrically; (2) limbs on left side extended less or more slowly than those on the right; (1) limbs on left side showed minimal movement; and (0) forelimb on left side did not move at all.

For climbing assessment, rat was placed on the wall of a wire cage. Normally the rat uses all four limbs to climb up the wall. When the rat was removed from the wire cage by pulling it off by the tail, the strength of attachment was noted. Scores indicate the following: (3) Rat climbed easily and gripped tightly to the wire; (2) left side was impaired while climbing or did not grip as hard as the right side; and (1) rat failed to climb or tended to circle instead of climbing.

For body proprioception evaluation, rat was touched with a blunt stick on each side of the body, and the reaction to the stimulus was observed. Scores indicate the following: (3) Rat reacted by turning head and was equally startled by the stimulus on both sides; (2) rat reacted slowly to stimulus on left side; and (1) rat did not respond to the stimulus placed on the left side.

To evaluate the response to vibrissae touch, a blunt stick was brushed against the vibrissae on each side; the stick was moved toward the whiskers from the rear of the animal to avoid entering the visual fields. Scores indicate the following: (3) Rat reacted by turning head or was equally startled by the stimulus on both sides; (2) rat reacted slowly to stimulus on left side; and (1) rat did not respond to stimulus on the left side. The score given to each rat at the completion of the evaluation is the summation of all six individual test scores.[17]

Inhibitory avoidance memory

On the 4 days after surgery, the rats were evaluated for their escape behavior in automated two-way shuttle-boxes. Rats were individually placed in a shuttle-box and allowed to habituate to the environment for 5 min, and then subjected to shuttle trials with a fixed inter trial interval of 30 s. In each trial, a mild electric shock (0.3 mA) was firstly presented for a maximum of 3 s duration; rats were allowed to avoid the electric shock by moving to

the other side of the box (avoidance response). If no avoidance response occurred within 3 s, a scrambled electric shock (0.8 mA) was applied through the grid floor for a maximum of 3 s duration; rats were allowed to escape from the shock by moving to the other side of the box (escape response). The latency to escape to the other side of the box was recorded as the index of avoidance memory performance.[32]

Histopathology examination

At the end of behavioral testing, rats were sacrificed by decapitation and the brains were removed. The intact whole brain was transferred to formalin (10% v/v). The tissue was cut into the section with 300 μm intervals, and its blocks were embedded in paraffin. The brain section (10 μm) thickness was prepared and stained with hematoxylin and eosin. For the determination of shrinkage and atrophy of the neurons, numbers of viable neurons were counted within the medial CA1 hippocampus subsectors. The average rate of neuronal cell counts from the right and left hemispheres of three sections of each rat were reported.[17]

Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity, an index of polymorphonuclear leukocyte accumulation, was measured according to the modified method of Bradley *et al*.[33] Brain tissue was homogenized in 1 mL of 50 mM potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. Then, the homogenate was sonicated in an ice bath for 10 s, freeze thawed thrice with sonication between cycles. After that, the suspensions were centrifuged at 15,000 rpm for 15 min at 4°C and then the supernatant (0.1 mL) was allowed to react with 2.9 mL of 50 mM potassium phosphate buffer (pH 6.0) containing O-dianisidine dihydrochloride (0.167 mg/mL) and 0.005% hydrogen peroxide. The absorbance of the mixture was measured at 450 nm using a ultraviolet-visible spectrophotometer. MPO activity was expressed in units (U) per gram of wet tissue weight.[32]

Myeloperoxidase activity (U/g) = X/weight of the piece of tissue taken.

Where X = 10 × change in absorbance per min/volume of supernatant taken in the final concentration.

Brain edema

The extent of brain edema was assayed by measuring tissue water content. For these latter measurements, freshly obtained blotted samples of brain were weighted on aluminum foil, dried for 12 h at 95°C, and reweighed. The difference between wet and dry tissues was calculated and expressed as a percentage of tissue wet weight.[33]

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance followed by Tukey's multiple comparison tests. Nonparametric data was analyzed by Mann–Whitney U-test. The minimal level of significance was considered at $P < 0.05$.

RESULTS

Total anthocyanin content

The total anthocyanin content of ETAE was 3.1% of cyanidin-3-glucoside equivalent.

Effects of *Echium amoenum* total anthocyanin extract on behavioral assessment

As shown in Figure 1, spontaneous activity was significantly decreased following brain ischemia. Administration of the extract at high test dose improved spontaneous activity ($P < 0.01$).

In Figure 2, it was observed that the ability of climbing in the rats significantly decreased by brain ischemia, and ETAE pretreatment could not improve it. Figure 3 shows the effects of ETAE on the body proprioception in the rats. Our results showed that the extract could not affect the body proprioception following brain ischemia. Response to vibrissae in the rats was notably decreased by ischemia, and ETAE administration could not affect this [Figure 4].

Effects of *Echium amoenum* total anthocyanin extract on inhibitory avoidance memory

Figure 5 shows the effects of ETAE pretreatment on memory. The extract at the dose of 200 mg/kg improved memory performance significantly compared to I/R group ($P < 0.01$).

Effects of *Echium amoenum* total anthocyanin extract on neuronal damages

Brain ischemia resulted in the neuronal atrophy and shrinkage that reported as a significant reduction in viable neurons in the CA1 hippocampus [Figure 6]. Administration of ETAE at the dose of 200 mg/kg significantly inhibited the neuronal damage compared to I/R group ($P < 0.01$).

The effects of *Echium amoenum* total anthocyanin extract on myeloperoxidase activity

Myeloperoxidase activity as a marker of leukocyte infiltration was obviously enhanced in the brain tissue following the ischemia. Pretreatment with ETAE at the dose of 200 mg/kg significantly reduced the MPO activity in comparison to I/R group ($P < 0.01$) [Figure 7].

The effects of *Echium amoenum* total anthocyanins extract on brain edema

Brain edema that was evaluated by brain water content assay was significant following ischemia compared to the sham group ($P < 0.01$); however, ETAE at any doses could not affect brain edema [Figure 8].

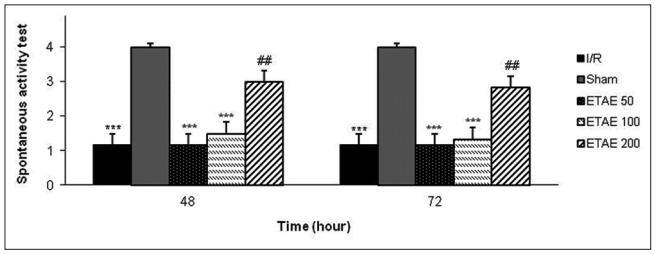

Figure 1: The effect of *Echium amoenum* total anthocyanin extract (50, 100 and 200 mg/kg i.p.) on spontaneous activity test in rats following cerebral ischemia/reperfusion. The results are expressed as mean±standard error of the mean for six rats. ***P<0.001 versus sham control group and ##P<0.01 versus ischemia/reperfusion control group

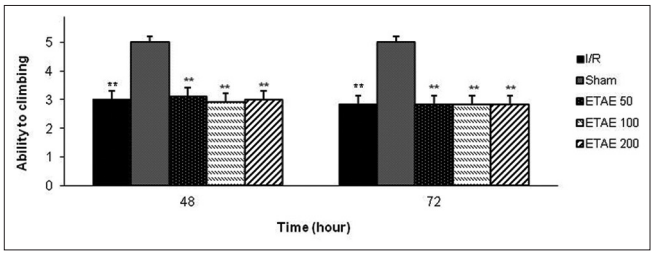

Figure 2: The effect of *Echium amoenum* total anthocyanin extract (50, 100 and 200 mg/kg i.p.) on the ability of climbing in rats following cerebral ischemia/reperfusion. The results are expressed as mean±standard error of the mean for six rats. **P<0.01 versus sham control group

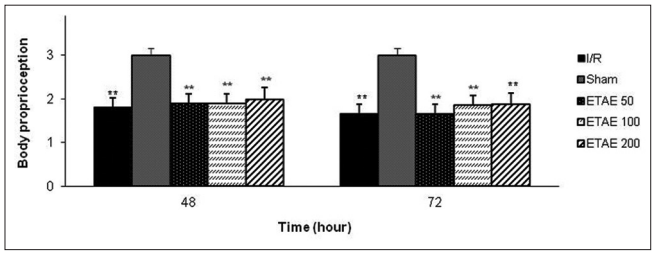

Figure 3: The effect of *Echium amoenum* total anthocyanin extract (50, 100 and 200 mg/kg i.p.) on body proprioception in rats following cerebral ischemia/reperfusion. The results are expressed as mean±standard error of the mean for six rats. **P<0.01 versus sham control group

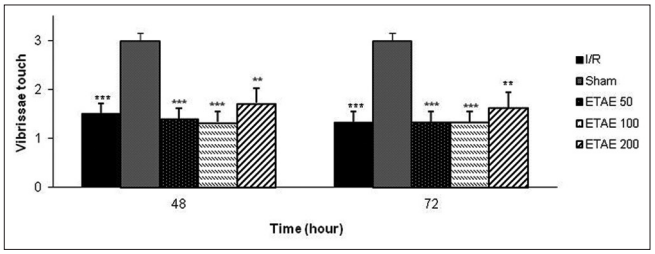

Figure 4: The effect of *Echium amoenum* total anthocyanin extract (50, 100 and 200 mg/kg i.p.) on response to vibrissae touch in rats following cerebral ischemia/reperfusion. The results are expressed as mean±standard error of the mean for six rats. *P<0.05 and **P<0.01 versus sham control group

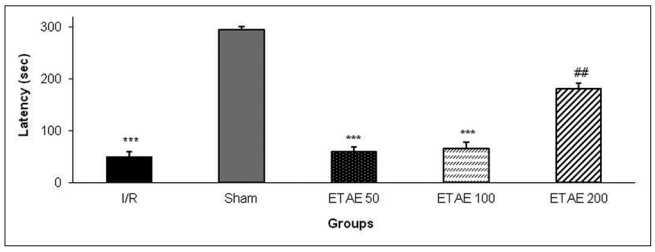

Figure 5: The effect of *Echium amoenum* total anthocyanin extract (50, 100 and 200 mg/kg i.p.) on inhibitory avoidance memory in rats following cerebral ischemia/reperfusion. The results are expressed as mean±standard error of the mean for six rats. ***P<0.001 versus sham control group and ##P<0.01 versus ischemia/reperfusion control group

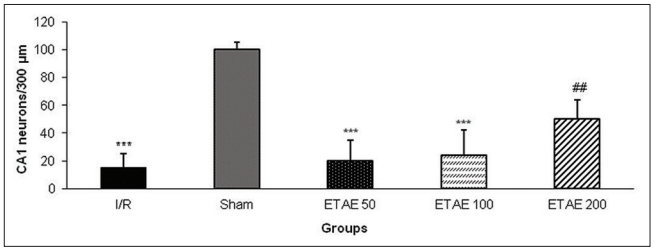

Figure 6: The effect of *Echium amoenum* total anthocyanin extract (50, 100 and 200 mg/kg i.p.) on neuronal damages in rats following cerebral ischemia/reperfusion. The results are expressed as mean±standard error of the mean for six rats. ***P<0.001 versus sham control group and ##P<0.01 versus ischemia/reperfusion control group

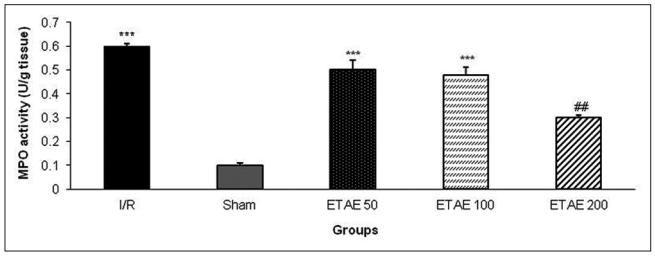

Figure 7: The effect of Echium amoenum total anthocyanin extract (50, 100 and 200 mg/kg i.p.) on myeloperoxidase activity in rats following cerebral ischemia/reperfusion. The results are expressed as mean±standard error of the mean for six rats. ***P<0.001 versus sham control group and ##P<0.01 versus ischemia/reperfusion control group

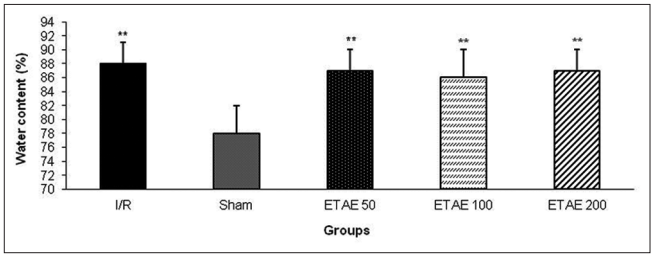

Figure 8: The effect of *Echium amoenum* total anthocyanin extract (50, 100 and 200 mg/kg i.p.) on brain edema in rats following cerebral ischemia/reperfusion. The results are expressed as mean±standard error of the mean for six rats. **P<0.01 versus sham control group

DISCUSSION

In this study, our finding showed that ETAE extract has protective effects against cerebral ischemia especially at the higher test dose. Considering our results, this extract could improve spontaneous activity and memory performance, and could reduce MPO activity and neuronal damages after brain ischemia.

Many neurological evaluations of the rats with brain ischemia were completed during the early hours after the occlusion, whereas pathological examinations were based on the measuring of neural damage 4 days later. Brain specimens in the treatment group (ETAE 200 mg/kg) had fewer necrotic neurons than those with the control group 72 h after ischemia.

It has been reported that bilateral common carotid artery occlusion model produces significant oxidative stress and mitochondrial dysfunction after 72 h.[30]

Echium amoenum is considered as a promising source of bioactive compounds with various beneficial biological activities. The antioxidant activity of Iranian *E. amoenum* flower aqueous extract has been investigated by Ranjbar *et al*. in human. Their results showed a significant reduction in blood lipid peroxidation after extract intake (7 mg/kg) for 14 days. They suggested that this antioxidant potential of *E. amoenum* may be due to its bioactive components, especially its flavonoids.[20] The presence of some bioactive compounds like rosmarinic acid with anti-inflammatory effect and cyanidin-3-glucoside that inhibits the activation and translocation of c-Jun and NF-κB factors into nucleus suggests that suppression of COX-2 expressions,[28] and reduced intracellular ROS levels via activating the glutathione antioxidant system might be involved in the protective effects of this plant. Delphinidin is one of the other active compounds of ETAE which inhibits TNF-alpha-induced COX-2 expression by directly inhibiting Fyn kinase activity,[29] and this effect may be proposed as the other mechanism involved in protective effect of ETAE against cerebral ischemia.

The protective effects of anthocyanins against heart I/R damage have been reported in some studies. Anthocyanins could reduce the microvascular impairments with protection of the endothelium and improvement of capillary perfusion.[34] The anthocyanin delphinidin could accelerate the healing of I/R injury. It has reduced the infarct size after ischemia and improved the necrosis and apoptosis of cardiomyocytes through inhibition of signal transducers and activators of transcription.[35]

Administration of medicinal herbs that possess anti-inflammatory and antioxidant properties is a new approach to attenuate inflammatory-related disorders.[30] In this regard effects of *Ginkgo biloba* extract on cerebral ischemia have been studied. The results demonstrated that *G. biloba* extract was able to decrease brain damages. The beneficial effects had attributed to the oxygen radical scavenging potential of *G. biloba* flavonoids contents.[36] Our findings indicated that various mechanisms may be involved in protective activity of ETAE on cerebral ischemia because some of the biochemical, behavioral, and histological parameters were improved.

CONCLUSION

The present study has shown the protective effects of *E. amoenum* against cerebral ischemia and may suggest a therapeutic potential for therapy or prevention in this condition. However, further experimental studies are necessary to isolate and identify the active principles present in *E. amoenum* fractions that may be responsible for the protective effects on cerebral ischemia.

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