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Research Article

Neuroprotective Activity of Water Soluble Extract from *Chorispora bungeana* against Focal Cerebral Ischemic/Reperfusion Injury in Mice

Ya-Xuan Sun,[1,2] Ting Liu,[3] Xue-Ling Dai,[1] Ya-Bo Li,[4] Yu-Yao Li,[4] Hua Zhang,[4] and Li-Zhe An[4]

[1] *Beijing Key Laboratory of Bioactive Substances and Functional Foods, Beijing Union University, Beijing 100191, China*
[2] *Cold and Arid Regions Environmental and Engineering Research Institute, Chinese Academy of Sciences, Lanzhou 730000, China*
[3] *School of Pharmacy, Shihezi University, Shihezi 832002, China*
[4] *Key Laboratory of Arid and Grassland Agroecology (Ministry Education), School of Life Sciences, Lanzhou University, Lanzhou 730000, China*

Correspondence should be addressed to Li-Zhe An; lizhean@lzu.edu.cn

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The purpose of the present study was to clarify whether the water extract of *Chorispora bungeana* was an antioxidant agent against cerebral ischemia/reperfusion (I/R). Our results showed that water extract of *Chorispora bungeana* treatment significantly reduced neurological deficit scores, infarct size, MDA and carbonyl contents, and GSH/GSSG ratio compared with the model control group. After being treated by *Chorispora bungeana*, SOD, CAT, and GSH-Px activities remarkably increased. *Chorispora bungeana* treatment also improved 8-OHdG expression and cell apoptosis. Our findings indicated that the water extract of *Chorispora bungeana* possesses neuroprotective effect which is most likely achieved by antioxidant and antiapoptotic activities.

1. Introduction

Stroke is one of the leading causes of disability and death with astronomical financial repercussions on health systems, and about 16 million strokes take place and cause a total of 5.7 million deaths in the world ever year. The global mortality is supposed to rise to 6.5 million in 2015 and to 7.8 million in 2030 [1, 2]. It is therefore urgent to develop effective therapies for stroke.

Cerebral ischemia is the most common type of stroke accounting for 88% of cases and it is reported that cerebral ischemia results in a cascade of cellular and molecular events including oxidative stress, excitotoxicity, calcium overlord, neuroinflammation, and apoptosis [3]. Reperfusion is a good measure for dealing with ischemia, but the beneficial effects of reperfusion may be in part reversed by the occurrence of reperfusion injury [4].

Oxidative stress was suggested to be implicating in the pathogenesis of cerebral ischemia/reperfusion (I/R) injury. Formation of reactive oxygen species (ROS) mainly includes the arachidonic acid pathway, the mitochondrial chain respiratory chain, the oxidation of xanthine, and hypoxanthine by xanthine oxidase and NADPH-oxidases [5, 6]. The brain is sensitive to oxidative stress because of relative low level of antioxidant enzyme and abundance of oxidizable substrates and transition metals [7].

Oxidative stress also induces neuronal apoptosis which is a feature found in cerebral ischemia/reperfusion [8–10]. Chen et al. [11] found that hydrogen peroxide, a major oxidant generated when oxidative stress occurs, leads to neuronal apoptosis in a concentration- and time-dependent manner. Wang et al. [12] suggested that hydrogen peroxide and nitric oxide synergistically induced neuronal apoptosis involving activation of p38 mitogen-activated protein kinase

and caspase-3. However, recovery of blood supply is necessary to remedy the compromised ischemic brain tissue. Antioxidants may reduce reperfusion-induced injury and extend the therapeutic window for thrombolysis [13–16].

Chorispora bungeana Fisch. And C.A. Mey (C. bungeana) is a rare alpine subnival plant species that inhabits periglacial regions in a severe low-temperature environment [17, 18]. The plant is a perennial herb belonging to the Brassicaceae-family [19]. Liu et al. [20] concluded that brassinosteroids could play the positive roles in alleviating chilling-induced oxidative damage by enhancing antioxidant defense system in suspension cultured cells of *Chorispora bungeana*. Guo et al. [21] found that a sequential and synergetic action between antioxidant enzymes such as superoxide dismutase, dehydroascorbate reductase, ascorbate peroxidase, and glutathione reductase, leading to a low antioxidation rate which contributes to retard lipid peroxidation and plays an important role in the resistance of suspension cultured cells of *Chorispora bungeana* to freezing temperatures. We presume that *Chorispora bungeana* may reduce oxidative stress induced by cerebral ischemia/reperfusion.

The present study was undertaken to evaluate the neuroprotection of water extract of *Chorispora bungeana* in cerebral ischemia/reperfusion with use of middle cerebral artery occlusion (MCAO) model and a series of oxidative stress markers.

2. Materials and Methods

2.1. Chemicals and Reagents. 2,3,5-Triphenyltetrazolium chloride, hypoxanthine, xanthine oxidase, catalase, 5,5′-dithiobis(2-nitrobenzoic acid), oxidized disulfide, and reduced glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). 1,1,3,3-Tetramethoxypropane has been obtained from Fluka Chemical Co. (Ronkonkoma, NY). All other chemicals and reagents were of analytical grade.

2.2. Animals. Male ICR (26 ± 2 g) were obtained from Vital River Laboratories (Peking, China) for this study. Animals were housed in a room at a temperature of 24 ± 1°C and 12-h dark and light cycle with free access to standard food and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of National Institute Pharmaceutical Education and Research.

2.3. Chorispora bungeana Water Extract Preparation. A wild species of *Chorispora bungeana* was obtained by the method described by Fu et al. [22].

Freshly collected *Chorispora bungeana* whole plant was dried under shade and the dried material was milled to obtain a coarse powder. The powder was immersed in water for 1 h and then was extracted at boiling temperature for 1.5 h and repeated twice. The whole water extract was freeze-dried and redissolved in water at a concentration of 0.5 g/mL.

2.4. Drug Administration. Mice were randomly divided into six groups, each consisting of eight animals. Water extract of *Chorispora bungeana*, diluted with distilled water was fed by oral gavage every day at a fixed time for 5 d in 5, 2.5, and 1.25 g/kg (equivalent to the amount of crude drug) three different doses. The model control group and sham group were treated with vehicle orally for 5 d. Edaravone group was treated with Edaravone at a dose of 3 mg/kg for 5 d and served as positive control. On day 5, 1 h after the above treatments, the mice were subjected to the middle cerebral artery occlusion.

2.5. Transient Focal Cerebral Ischemic/Reperfusion Model. All mice were subjected to 2 h transient focal cerebral ischemia followed by 22 h reperfusion, using an intraluminal suture technique described by Longa et al. [23] and Ha et al. [24] with little modification. In brief, mice were anesthetized with chloral hydrate (400 mg/kg, i. p.). Midline incision was made on ventral side of mouse neck. The left common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA) were carefully exposed and dissected away from adjacent muscles and nerves. Microvascular aneurysm clips were applied to the left CCA and the ICA. A coated filament was introduced into an arteriotomy hole, fed distally into the ICA and advanced about 12 mm from the carotid bifurcation. The ICA clamp was removed and focal cerebral ischemia started. After occlusion for 2 h, the filament was gently pulled out. The collar suture at the base of the ECA stump was tightened. The skin was closed, anesthesia discontinued, and the animals were returned to the prewarmed cages. Body temperature was maintained at 37 ± 0.5°C by lamp during the whole surgical procedures. Animals in sham control group underwent a neck dissection and coagulation of the external carotid artery, but no occlusion of middle cerebral artery.

2.6. Neurological Deficit Scores. After 22 h of reperfusion, an observer who was unaware of the identity of the groups evaluated the neurological deficits by using the method described by Longa et al. [23]. 0: no neurological deficit; 1: failure to extend the right forepaw fully; 2: circling to the right; 3: falling to the right; 4: no spontaneous walking with a depressed level of consciousness.

2.7. Infarct Size. Mice were decapitated after 22 h of reperfusion and these brains were quickly removed and sectioned coronally into five 1.5-mm-thick coronal sections. The sections were stained with 0.5% TTC at 37°C for 20 min. The stained brain sections were postfixed in 10% formalin solution and photographed with a digital camera and the infarct areas of each section were determined with the analysis of pixel counting by a computer program of Photoshop 6.0.

2.8. Biochemical Assays

2.8.1. Tissue Preparation. To measure of the contents of MDA and carbonyl, the activities of SOD, CAT, and GSH-Px, the ratio of GSH/GSSG, these mouse brains were weighed

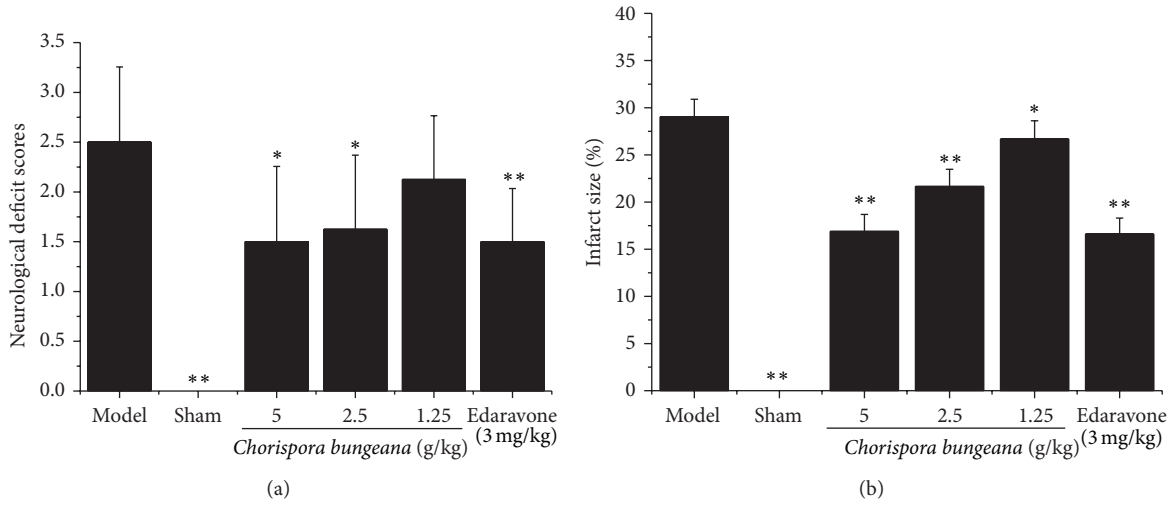


Figure 1: Effect of *Chorispora bungeana* water extract on neurological deficit scores (a) and infarct size (b) in mice subjected to cerebral ischemia/reperfusion. Data were expressed as mean ± S.M.E. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$ versus model group. $^{**}P < 0.01$ versus model group.

and homogenized with ice-cold normal saline and then centrifuged at 4624 ×g for 15 min at 4°C. The supernatant was used for bioassays. Protein content of the supernatant was determined by the method of Bradford [25] using bovine serum albumin as the protein standard.

2.8.2. GSH/GSSG Ratio. The ratio of GSH/GSSG was determined by a previously described method [26]. Briefly, T-GSH was determined basing on the 5,5-dithiobis (2-nitrobenzoic) acid (DTNB)-GSSG reductase recycling. GSSG was assayed by measuring 5-thio-2-nitrobenzoic acid (TNB) which was produced from the reaction of reduced GSH with DTNB. GSH level in brain tissue was calculated as the difference between T-GSH and GSSG.

2.8.3. Measurement of MDA Content. The MDA content was assayed in the brain tissue using a method as described by Cao et al. [27]. 1,1,3,3-Tetramethoxypropane was used as standard and the content of MDA was expressed as nmol/mg protein.

2.8.4. Measurement of Carbonyl Content. Carbonyl content was evaluated using the method according to Levine et al. [28]. The content of carbonyl was calculated by using the extinction coefficient of 22000 $M^{-1}cm^{-1}$/mg protein and expressed as nmol/mg protein.

2.8.5. Measurement of Antioxidant Enzyme Activities. SOD activity was measured as described by Jung et al. [29]. One unit of SOD activity was defined as the amount that shows 50% inhibition. SOD activity was expressed as U/mg protein.

CAT activity was evaluated according to the method of Campo et al. [30]. One unit of CAT activity was defined as the amount of CAT required to decompose 1 μmol/L of hydrogen peroxide in min. CAT activity was expressed as U/mg protein.

GSH-Px activity was determined by the method of Jagetia et al. [31]. One unit of GSH-Px activity was defined as the GSH-Px in 1 mg protein that led to the decrease of 1 μmol/L GSH in the reactive system per minute. GSH-Px activity was expressed as U/mg protein.

2.9. Immunohistochemistry Assay. Animals were overdosed with anesthetic and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS, pH = 7.4) after 22 h of reperfusion. Brains were removed and further fixed in 4% paraformaldehyde at 4°C for 24 h and then cut into equally spaced blocks. Paraffin-embedded blocks were cut into a series of 5-μm-thick slices.

8-Hydroxyl-deoxyguanosine (8-OHdG) immunohistochemistry was used to identify oxidized DNA and performed by the method of Wang et al. [32]. In brief, deparaffinized brain sections were immunostained with 5 μg/mL mouse anti-8-OHdG antibody followed by 0.5% goat anti-mouse IgG labeled with horseradish peroxidase.

Cell apoptosis was observed by the terminal deoxynucleotidyl transferase-mediated Dutp-biotin nick end labeling (TUNEL) assay. In brief, brain sections were deparaffinized and rehydrated followed by incubating with 20 μg/mL proteinase K in 0.01 M Tris-HCl (pH = 7.4) and permeabilized in a solution which contains 0.1% Triton-X 100 and 0.1% sodium citrate. Then these sections were incubated in TUNEL-reaction mixture containing terminal deoxynucleotidy transferase.

2.10. Statistical Analysis. Data were expressed as mean ± S.M.E. Statistical analysis was evaluated with one way

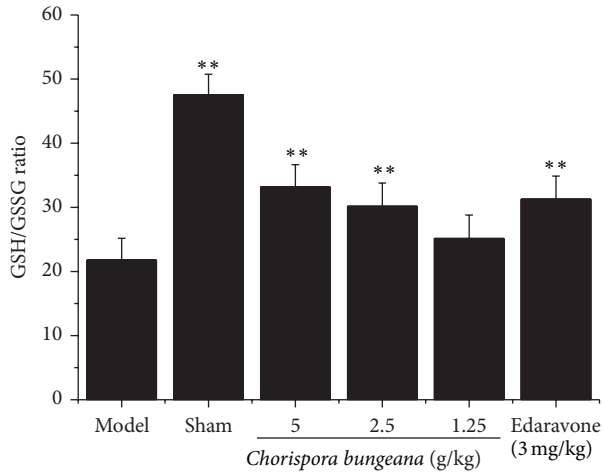


Figure 2: Effect of *Chorispora bungeana* water extract on GSH/GSSG ratio in mice subjected to cerebral ischemia/reperfusion. Data were expressed as mean ± S.M.E. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$ versus model group. $^{**}P < 0.01$ versus model group.

analysis of variance (ANOVA) and followed by a Student-Newman-Keuls (SNK) test for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Effect of Chorispora bungeana Water Extract on Neurological Deficit Scores and Infarct Size. After 22 h of reperfusion, neurological deficit scores and infarct size in ischemic/reperfusion mice were obviously higher than those of the sham control mice, indicating that we had successfully established the cerebral ischemic/reperfusion model. Treatment with *Chorispora bungeana* water extract significantly decreased neurological deficit scores and infarct size as compared to the model control group (Figures 1(a) and 1(b)).

3.2. Effect of Chorispora bungeana Water Extract on GSH/GSSG. The GSH/GSSG ratio in the mouse brain tissue, a typically of oxidative stress, was markedly lower in the model control group as compared to the sham control group. As shown in Figure 2, there was a significant increase in the ratio of GSH/GSSG in the *Chorispora bungeana* water extract treatment groups (5 and 2.5 g/kg) compared with the model control group ($P < 0.01$).

3.3. Effect of Chorispora bungeana Water Extract on MDA and Carbonyl Contents. MDA and carbonyl are the two main products of oxidative damage. Figure 3 showed that MDA and carbonyl contents were much higher in the model control group than those in the sham control group. The treatment with *Chorispora bungeana* water extract evidently reduced the contents of MDA and carbonyl in brain tissue compared with the model control group.

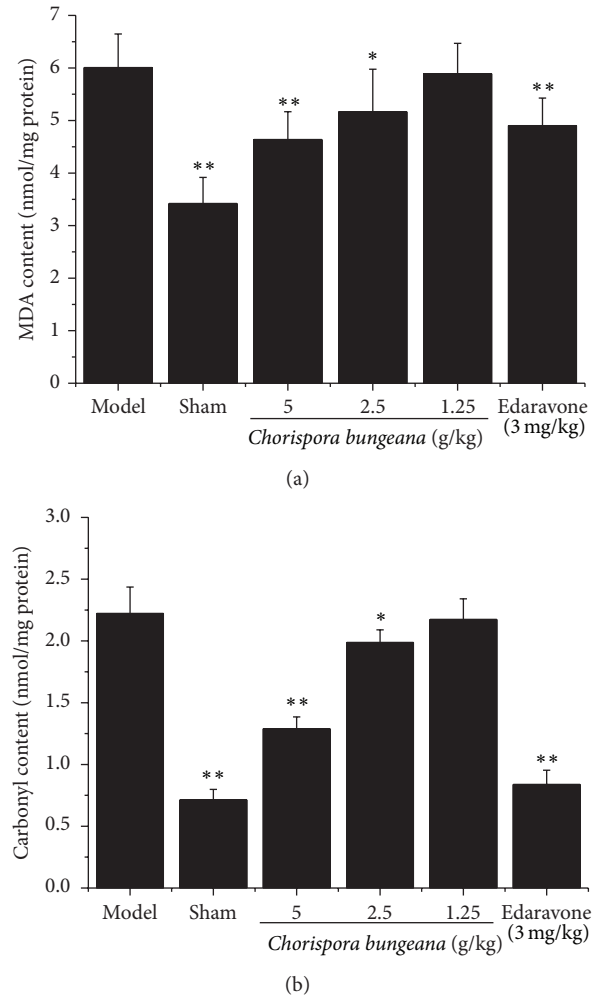


Figure 3: Effect of *Chorispora bungeana* water extract on MDA (a) and carbonyl (b) in mice subjected to cerebral ischemia/reperfusion. Data were expressed as mean ± S.M.E. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$ versus model group. $^{**}P < 0.01$ versus model group.

3.4. Effect of Chorispora bungeana Water Extract on Antioxidant Enzyme Activities. Table 1 showed that the activities of SOD, CAT, and GSH-Px were obviously decreased in model control group as compared to the sham control group and attenuated significantly by treatment of *Chorispora bungeana* water extract (5 and 2.5 g/kg) compared with the control group ($P < 0.05$).

3.5. Effect of Chorispora bungeana Water Extract on 8-OHdG Expression. The expression of 8-OHdG obviously increased in the model control group as compared to the sham control group. Treatment of *Chorispora bungeana* water extract markedly weakened 8-OHdG compared with the model control group (Figure 4, Table 2).

3.6. Effect of Chorispora bungeana Water Extract on Apoptosis. Very few TUNEL positive cells were found in normal mouse brain tissue and the number of TUNEL positive cells was

Table 1: Effect of *Chorispora bungeana* water extract on antioxidant enzyme activities in mice subjected to cerebral ischemia/reperfusion.

Group	Dose (g/kg)	SOD (U/mg protein)	CAT (U/mg protein)	GSH-Px (U/mg protein)
Model	/	62.95 ± 6.72	6.11 ± 0.61	684.53 ± 83.57
Sham	/	81.83 ± 4.09**	8.32 ± 0.47**	1054.06 ± 49.03**
Chorispora bungeana	5	79.04 ± 6.84**	7.39 ± 0.60**	961.56 ± 67.77**
Chorispora bungeana	2.5	71.51 ± 5.36*	6.95 ± 0.53*	798.45 ± 78.41*
Chorispora bungeana	1.25	63.41 ± 6.97	6.73 ± 0.58	724.03 ± 60.32
Edaravone	0.003	78.88 ± 5.23**	7.88 ± 0.57**	912.43 ± 61.37**

Data were expressed as mean ± S.M.E. Differences were considered significant at $P < 0.05$. *$P < 0.05$ versus model group. **$P < 0.01$ versus model group.

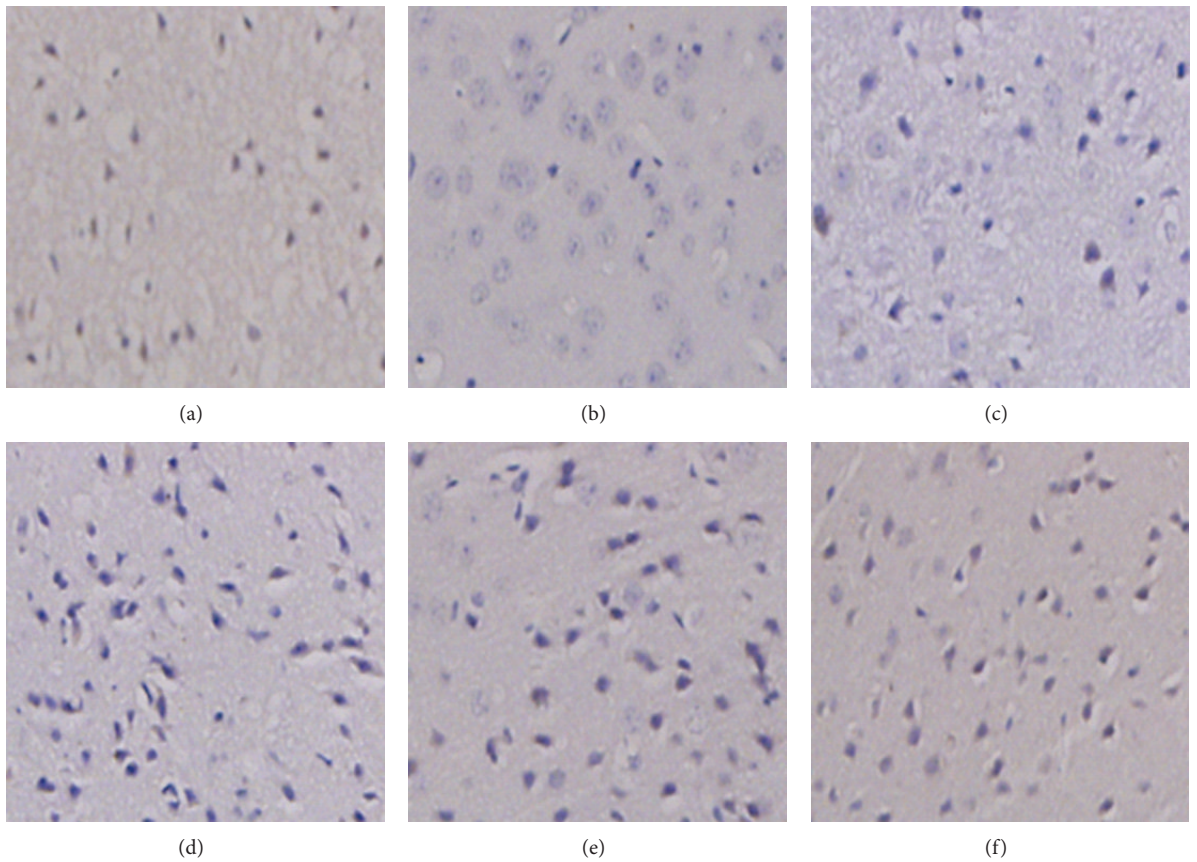


Figure 4: Effect of *Chorispora bungeana* water extract on 8-OHdG expression in mice subjected to cerebral ischemia/reperfusion. The nucleus of positive expressed cell is brown colored. (a) Model group; (b) Sham group; (c) Edaravone-treatment group (3 mg/kg); (d) *Chorispora bungeana*-treatment group (5 g/kg); (e) *Chorispora bungeana*-treatment group (2.5 g/kg); (f) *Chorispora bungeana*-treatment group (1.25 g/kg).

significantly increased by ischemia/reperfusion. Administration of *Chorispora bungeana* water extract markedly reduced TUNEL positive cells as compared to the model control group (Figure 5, Table 2).

4. Discussion

The present study reported the neuroprotective effect of *Chorispora bungeana* water extract in terms of oxidative stress markers and apoptosis in cerebral ischemic/reperfusion mouse brain for the first time. We found that water extract of *Chorispora bungeana* prevents brain from ischemic/reperfusion damage, as indicated by the improved recovery of neurological function, decreases in infarct size and oxidative stress, and increases in antioxidant enzyme activities and reduction in apoptosis.

We set up cerebral ischemic/reperfusion model with intraluminal thread insertion method in mice and tested

Table 2: Effect of *Chorispora bungeana* water extract on 8-OHdG expression and cell apoptosis in mice subjected to cerebral ischemia/reperfusion.

Group	Dose (g/kg)	8-OHdG positive cells	TUNEL positive cells
Model	/	20.88 ± 3.31	21.63 ± 4.21
Sham	/	2.13 ± 1.25**	2.25 ± 1.04**
Chorispora bungeana	5	16.25 ± 2.60**	17.13 ± 3.40*
Chorispora bungeana	2.5	17.13 ± 3.17*	18.25 ± 3.99
Chorispora bungeana	1.25	19.88 ± 3.83	19.50 ± 4.41
Edaravone	0.003	15.38 ± 3.42**	17.25 ± 3.24*

Data were expressed as mean ± S.M.E. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$ versus model group. $^{**}P < 0.01$ versus model group.

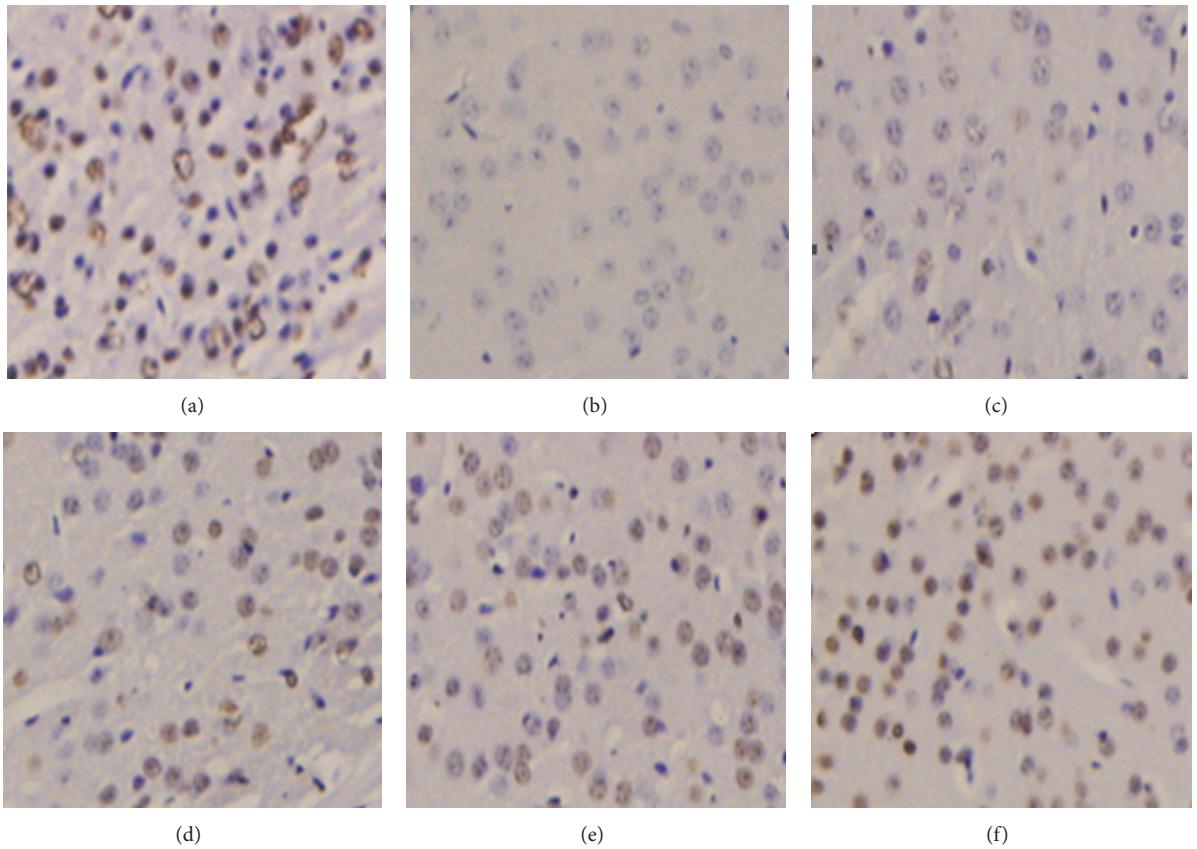


Figure 5: Effect of *Chorispora bungeana* water extract on cell apoptosis in mice subjected to cerebral ischemia/reperfusion. Nucleus has brown granules were positive cell. (a) Model group; (b) sham group; (c) Edaravone-treatment group (3 mg/kg); (d) *Chorispora bungeana*-treatment group (5 g/kg); (e) *Chorispora bungeana*-treatment group (2.5 g/kg); (f) *Chorispora bungeana*-treatment group (1.25 g/kg).

whether the model was successfully established by means of assaying the neurological deficit scores and infarct size. The mice showed visible neurological deficits and brain infarction in model control group. Treatment of *Chorispora bungeana* water extract reducing the neurological deficit scores and infarct size in a dose-dependent manner demonstrated that water extract of *Chorispora bungeana* was provided with neuroprotective activity against cerebral ischemic/reperfusion injury.

Then we determined whether brain was in oxidative stress status after 22 h of reperfusion. The ratio of GSH/GSSG is proposed to be a sensitive index of oxidative stress [33, 34]. Our data showed that the ratio of GSH/GSSG was decreased in model control group, but the decrease was attenuated by treatment of *Chorispora bungeana* water extract. That indicated that *Chorispora bungeana* water extract could release oxidative stress in ischemic/reperfusion brain tissue.

Reactive oxygen species (ROS) contain one or more unpaired electrons in their outer orbit and are highly reactive play key roles in organism physiology and pathophysiology [35–38]. Excessive production of ROS may depress cell membrane properties and cause oxidative damage to lipids, proteins, and DNA that may make them nonfunctional because of brain tissue contains large amount of unsaturated fatty

acids and catecholamines after cerebral ischemia and particularly reperfusion [6, 39, 40]. Malondialdehyde (MDA), carbonyl, and 8-hydroxy-2′-deoxyguanosine (8-OHdG) are representative markers of oxidative damage of brain [39]. We measured the MDA and carbonyl contents by using of a spectrocolorimetric assay and detected the 8-OHdG expression by immunohistochemistry. The water extract of *Chorispora bungeana* effectively reduced the content of MDA and carbonyl; the expression of 8-OHdG revealed that it could lighten oxidative damage induced by cerebral ischemia/reperfusion.

The ROS is maintained in an appropriate level by the endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) under normal physiological conditions [6, 41]. SOD catalyzes the dismutation of superoxide anions to hydrogen peroxide and molecular oxygen and CAT together with GSH-Px eliminates hydrogen and molecular oxygen [42]. But these antioxidant enzyme activities were reported to decrease during cerebral ischemia/reperfusion [43, 44]. The present study showed that the reduced activities of SOD, CAT, and GSH-Px in ischemic/reperfusion mice were significantly attenuated in *Chorispora bungeana* water extract-treated group when compared with the model control group.

Broughton et al. [45] pointed out that cerebral ischemia triggers two main pathways of apoptosis. One originates from mitochondrial release of cytochrome c and another originates from the activation of cell surface death receptors. Fujimura et al. [46] agreed that ROS mediated the mitochondrial signaling pathway that may result in apoptosis following cerebral ischemia. In order to determine the antiapoptotic activity of *Chorispora bungeana* water extract, we evaluated the apoptosis with terminal deoxynucleotidyl transferase mediated d UTP-biotin nick end labeling (TUNEL). *Chorispora bungeana* water extract was proved to be able to inhibit cell apoptosis in our study.

In summary, our study demonstrated that the water extractof *Chorispora bungeana* could effectively attenuate brain injury in focal cerebral ischemia/reperfusion. The neuroprotective activity was associated with inhibiting oxidative stress and apoptosis. Chorispora bungeana may have the potential to cure nervous system diseases as an unusual alpine subnival plant and more pharmacodynamics research work needs to be done.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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