Carthamus tinctorius L.
Extract ameliorates cerebral ischemia-reperfusion injury in rats by regulating matrix metalloproteinases and apoptosis

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Abstract:
We investigate the protective effect of Carthamus tinctorius L. (CTL, also known as Honghua in China or Safflower) on cerebral ischemia-reperfusion and explored the possible mechanisms on regulating apoptosis and matrix metalloproteinases (MMPs). High-performance liquid chromatography method with diode array detection analysis was established to analyze the components of CTL. Middle cerebral artery occlusion rats model was established to evaluate Neurological Function Score and hematoxylin-eosin staining, as well as triphenyltetrazolium was used to examine the infarction area ratio. Transferease-mediated dUTP nick-end labeling was performed for the apoptosis. Apoptosis-related factors, including B-cell lymphoma-2 (Bcl-2), Bax and Caspase3, and MMPs-related MMP2, MMP9, tissue inhibitor of metalloproteinases 1 (TIMP1) in ischemic brain, were assayed by Western blot, reverse transcription polymerase chain reaction, and immunohistochemistry. The data showed that CTL (2, 4 g crude drug/kg/d) treatment could significantly reduce the ischemic damage in brain tissue and improve a significant neurological function score. In addition, CTL could also attenuate apoptosis degree of brain tissues and regulate Bcl-2, Bax, and Caspase3 and also have a significant decrease on MMP-9 expression, followed by a significant increase of TIMP1 protein expression. These findings indicated that regulation of CTL on apoptosis and MMPs contributed to its protective effect on ischemia/reperfusion injury.

Keywords:
Apoptosis, Carthamus tinctorius L., cerebral ischemia-reperfusion, matrix metalloprotein

Introduction
Ischemic cerebrovascular disease causes various negative health consequences.[1] There is growing research interest into strategies for reducing morbidity and mortality of ischemic cerebrovascular disease, such as ischemic strokes.[2] Traditional medicine supplementation for preventing morbidity and mortality of ischemic cerebrovascular disease has been recognized as an effective intervention to attenuate cerebral ischemia-reperfusion injury in the brain.[3]

In recent years, multiple molecular mechanisms for ischemic cerebrovascular disease have been found and revealed with the development of molecular biology.[4] Mounting studies show that matrix metalloproteinases (MMPs) are engaged in pathologies associated with cerebral ischemia-reperfusion injury.[5] The evidence demonstrates that MMP-9...
expression was increased and activated by cerebral ischemia, and the attenuation of ischemic brain damage has been found to be related to an inhibition on MMP-9, at least partially. Furthermore, apoptosis, a process of cell death by the initiative of gene regulation, involved in ischemic pathological injury and was an important aspect of the development of its occurrence and significance of the outcome after ischemic injury. Therefore, regulating MMPs and reducing apoptosis may improve the damage of cerebral ischemia-reperfusion in the brain.

*Carthamus tinctorius* L. (CTL, Honghua) has been used for a long time and has functions on “removing blood stasis and promoting blood circulation.” It has been commonly used to treat many kinds of diseases such as coronary heart disease and cerebral thrombosis. Accumulating studies showed that it had a protective activity on cerebral ischemia and myocardial ischemia. Its combination with Huangqi and its main component hydroxysafflor yellow A (HSYA) could decrease the cerebral infarction with stroke rats. Furthermore, Danhong injection (DHI), one of the most popular medications of CTL, has been widely used in the clinical treatment. It has been found a remarkable reduction on cerebral infarction and caspase-3 expression, whereas a significant increase on B-cell lymphoma-2 (Bcl-2) expression by the treatment of CTL injection. There is also evidence that the representative component HSYA has been found to be related to an inhibition on MMP-9, and its anti-apoptotic effects. However, its potential mechanism of action is still unclear.

Therefore, to investigate the possible regulation of CTL on MMPs and apoptosis in I/R, the current study investigated MMP-related and apoptosis-related expression levels in the brain tissue relative to I/R.

**Experimental**

**Materials**

Antibodies for Bax, Bcl-2, caspase-3, and tissue inhibitor of metalloproteinases 1 (TIMP1) were obtained from Santa Cruz Biotechnology, Inc. Antibodies for MMP-9 and MMP-2 were ordered from Cell Signaling Technology, Inc (Shanghai, China). Triphenyltetrazolium (TTC) was ordered from Sigma. Acetonitrile 99.9% was of high-performance liquid chromatography (HPLC)-grade purchased from TEDIA (Lisbon, USA). Reference compounds HSYA and safflower yellow B (purity ≥99%) were obtained from National Standards Center of China.

**Extracts preparation**

Medicinal material CTL was purchased from Wanzhen chinese herbal medicine of Bozhou City, China, and pharmacognosy was authenticated by Dr. Xiaobin Jia. The remaining voucher specimens were deposited in the herbarium of Jiangsu Provincial Academy of Chinese Medicine (No. ACM2014061003). One hundred grams of the fine CTL (Origin: Xinjiang, China; Lot: 140715) were accurately weighed and extracted with methanol (500 mL) for 1 h in reflux extraction device (two times), centrifuged, and then evaporated at 40°C to remove methanol. The yield of extract is 2.62%. The components of CTL were analyzed by HPLC. In addition, the residue can also be redissolved in ultra-pure water for further animal experiments.

**High-performance liquid chromatography method with diode array detection analysis**

The analysis was conducted on Agilent 1200 series HPLC equipment (Waldbonn, Germany) with a diode-array detector (DAD). Components are separated on Alltima C18 column (250 mm × 4.6 mm i. d., 5 μm) with a gradient elution of the mobile phase (flow rate: 1.0 mL/min, column temperature: 30°C, and wavelength: 402 nm). The elution procedure is as follows: 5% A→45% A (HPLC-grade acetonitrile), 95% B→55% B (0.1% acetic acid in water), and 0~60 min.

**Animal and Administration**

The male Sprague Dawley rats (250 ± 20 g) used in this experiment were purchased from Animal Center of Nantong University (license number: SCXK (Su) 2014-0001), in line with clean grade experimental animal standards. The rats were housed with room temperature 22 ± 2°C, humidity 45 ± 10%, and light adaptive breeding. The rats were divided into Sham group (normal saline, NS, 10 mL/kg/d), model group (NS, 10 mL/kg/d), positive drug Nimodipine group (7.53 mg/kg/d, CTL treatment group (2 or 4 g crude drug/kg/d) with 10 rats for each group. After being modeled successfully, rats were administered orally every day for sustained two weeks. Animal experiment guidelines are used to guide this experiment.

**Middle cerebral artery occlusion model establishment and neurological function score**

Middle cerebral artery occlusion (MCAO) rat model and behavior score were performed as previously described with minor adjustment. Briefly, rats were anaesthetized with 10% chloral hydrate, and the dose was 300 mg/kg, and then the rats were fixed supinely on a mouse board. After a 2 cm midline incision in the neck, muscle and fascia were separated by blunt dissection along the inner edge of the sternocleidomastoid and then separated external carotid artery (ECA), common carotid artery (CCA), and internal carotid artery (ICA) of rats. The temporary occlusion of ICA was achieved by microartery clamp, and then, the proximal part of CCA and ECA in the
lateral side was lodged to prevent bleeding in the surgery. Next, a small incision at a distance of about 4 mm to CCA bifurcation was performed for the insert of a nylon filament (diameter of 0.26 mm coated with polylysine) into the right ICA. After lodged in the narrow proximal of ICA at the depth of 2.5 cm, the blood flow was blocked. The filament in ICA was fixed to allow permanent occlusion. The wound was stitched and treated with penicillin injection (100 000 U/rat) to prevent wound infection. Animals received identical surgery except that the ligation was less than 1cm deep was used as sham group [Figure 1]. Neurological function score was performed after MCAO 24 h on a 5-point scale: 0 indicates no symptoms of nerve damage; (1) indicates rats cannot fully extend the contralateral paws; (2) indicates circling to the opposite side of surgery; (3) indicates falling to the opposite side of surgery; and (4) indicates spontaneous walking with the disturbance of consciousness.

**Hematoxylin-eosin staining**

Brain tissue was taken for HE staining. These tissues were fixed in 4% buffered paraformaldehyde solution for paraffin embedding. Sequentially, 5 µm-thick slices were cut for hematoxylin-eosin staining. The visualization of brain tissues were examined to observe the pathological changes.

**Triphenyltetrazolium**

After being washed with 0.9% saline, brain tissues were frozen for 30 min under −20°C until tissue hardening. Coronal sections were cut from the frontal pole with 3 mm equidistant and then stained with TTC dye for 30 min in the dark, followed with 4% paraformaldehyde of 4 h. Normal tissue appears red, whereas infarction presents white. IPP software was conducted to analyze the maximum crosssection and ischemic infarct area. A percentage of infarct size was taken for the statistical analysis. The formula was shown as follows:

\[ IS = \frac{S_1 - S_2}{S_1} \times 100\% \]

\(S_1\): Maximum cross-section of the total ischemic area; \(S_2\): Infarct area).

**Immunohistochemistry**

The tissues slices (4-µm-thick) were prepared for immunohistochemistry assessment. After deparaffinization, these sections were probed with primary antibodies. After being incubated for 24 h at 4°C and washed with phosphate-buffered saline, the sections were reacted with secondary antibody streptavidin-conjugated horseradish peroxidase. Sequentially, the sections were treated with diaminobenzidine and 0.01% H₂O₂, followed by chromogen with toluidine blue and counterstain with hematoxylin. After being dehydrated and embedded, the sections were observed under microscope with × 400 for positive stains.

**Transferase-mediated dUTP nick-end labeling assay**

This experiment was operated according to the method of the reference.[15] The detailed detection procedure was conducted according to the protocols of manufacturer (Wuhan Boster Biological Technology, Ltd., Wuhan, China). Transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells and total cell nuclei were counted for semiquantitative histomorphological assessment.

**Western blot analysis**

The brain tissue was harvested and homogenized in tissue protein extraction solution as report previously.[16] The harvested extract was treated by 3000 × g centrifugation for 10 min. After centrifugation, Bradford assay was conducted to detect the concentration of contained protein in samples. Protein of 100 µg was loaded for separation by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer electrophoretically to polyvinylidenediureide membrane. Membrane was probed with primary antibodies. Sequentially, the membrane was incubated with secondary antibody in line with the operation process. The visualization was developed using enhanced chemiluminescence reagents by exposure to X-ray films. The protein band visualization is captured by the imaging system.

**Reverse transcription polymerase chain reaction**

Total RNA from the brain was extracted for the reverse transcription to cDNA with RevertAid K1622 First Strand cDNA Synthesis Kit (Fermentas, Graiciūno, Vilnius, Lithuania). The primer sequences were as follows: Bax (81bp): F, 5’-CAAGAAGCTGAGCGAGTGTC-3’;
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R, 5′-ACGTCAGCAATCATCCTCTG-3′; ]Bcl-2 (102bp): F, 5′-GAGTACCTGAACCGGCATCT-3′; R, 5′-GAAATCAAACAGAGGTCGCA-3′; Caspase-3 (126bp): F, 5′-GCAGACGAGTGCACAGGAGG-3′; R, 5′-ACAGACAGTGCCATCACAAGG-3′; Rat-GAPDH (103bp): F, 5′-GGCCTTCCGTGTTCCTACC-3′; R, 5′-CGCCTGCTTCACCACCTTC-3′. After the polymerase chain reaction (PCR) reaction, agarose gel electrophoresis was conducted for quantitative real-time. This analysis was performed with the Mx3000P System.

**Statistical analysis**
All experiments are repeated thrice to obtain the average value and SD. Statistical significance for multigroup comparisons was analyzed by the Bonferroni t-test. P < 0.05 is considered to be a statistical significant.

**Results**

**Compound analysis for Carthamus tinctorius L.**
The extract was analyzed by the high-performance liquid chromatography method with diode array detection method to identify the chemical components in CTL. As shown in Figure 2, the peaks of chromatogram were separated well. In total, two major peaks in CTL were identified as HSYA (1) and safflower yellow B.[2]

**Carthamus tinctorius L. improved neurological deficit scores of cerebral ischemic rats**
MCAO rat model was treated by CTL to evaluate its protective activity. Here, we observed MCAO rat model was successfully prepared with the typical behavioral characteristics of cerebral ischemia, namely limbs twist when upside down [Figure 3A]. The results of HE staining showed ischemia could cause unclear boundary of tissue cores membrane, edema of cell interstitial, and infiltration of inflammatory cells, as well as pyknosis of nuclear. Administration of CTL (2, 4 g crude drug/kg/d) or nimodipine (7.53 mg/kg/d) could significantly reduce ischemic damage in brain tissue sections, accompanied with a reduction of tissue necrosis range, completed cell structure, as well as mitigation of edema of cell interstitial [Figure 3B].

Neurological function scores of rats in the control and CTL-treated groups were evaluated using a modified neurological severity score scale on days 1, 7, and 14. As shown in Figure 3C, scores are basically the same among different groups on the 1st day. Interestingly, a significant decrease of neurological function score was observed on the 7th and 14th day, in CTL (2, 4 g crude drug/kg/d) groups, suggesting the improvement of CTL on the cerebral ischemic.

**Carthamus tinctorius L. reduced the infarction area ratio of cerebral ischemic rats**
As depicted in Figure 4A and B, infarction area could be remarkably observed after modeling. The infarct area ratio of the model group was up to 47.2%. However, the increased infarct area ratio was significantly reduced after being treated with CTL. This finding also indicates that CTL can improve the damage degree of cerebral ischemia of rats.

**Carthamus tinctorius L. decreased transferase-mediated dUTP nick-end labeling and apoptosis-related factor levels of cerebral-ischemic rats**
Apoptosis plays an important role in the pathogenesis of ischemic cerebrovascular disease. In this study,

![Figure 2: High-performance liquid chromatography for Carthamus tinctorius L. (a) and reference compounds (b) hydroxysafflor yellow A (1) and safflower yellow B (2)](image-url)
TUNEL assay was conducted to evaluate the degree of apoptosis. As shown in Figure 5A, a marked increase on the brown deposits was observed in the brain tissues of model rats. However, the increased deposits were decreased significantly by the treatment of CTL and nimodipine, demonstrating the attenuation of CTL on apoptosis of brain tissues of cerebral ischemic rats.

Apoptosis-related factor levels were determined to explore the underlying mechanism. It can be observed that the levels of protein expression and messenger RNA (mRNA) of Bax and Caspase3 in brain tissues were increased markedly by cerebral ischemic treatment, whereas the levels of protein expression and mRNA of Bcl-2 were decreased remarkably. Importantly, this trend was reversed to normal levels, namely CTL reduced significantly the levels of protein expression and mRNA of Bax and Caspase3 in brain tissues of cerebral ischemic rats, whereas increased Bcl-2 protein expression and mRNA levels [Figure 5B and C]. These findings suggested that CTL could inhibit cerebral ischemic-induced apoptosis through increasing Bcl-2 and decreasing Bax and Caspase3.
*Carthamus tinctorius* L. reduced matrix metalloproteinases levels of brain tissues of cerebral ischemic rats

Protein expression levels of MMPs were detected by immunohistochemistry and Western blotting. The immunoblotting analysis of MMP-2, MMP-9, and TIMP1 is depicted in Figure 6. It can be observed that the expression of MMP-9 in the brain tissue of cerebral ischemia rats is significantly increased [Figure 6A]. Interestingly, CTL of 2 g crude drug/kg/d and 4 g crude drug/kg/d treatment caused a statistically significant decrease of MMP-9 protein in cerebral ischemic mice ($P < 0.05$, $P < 0.01$). This phenomenon can also be observed in MMP 9 protein expression.

In addition, TIMP1 functions as an important endogenous inhibitor in regulating MMPs levels. There is a significant decrease on TIMP1 protein expression in the model group when compared with Sham rats. Expectedly, this decrease can be enhanced significantly by the treatment of CTL ($P < 0.05$, $P < 0.01$) [Figure 6B]. These findings strongly support the level of TIMP1 is negatively related to MMP2 and MMP9 protein expressions in the brain tissue of cerebral ischemic rats.

**Discussion**

Ischemic injury is considered to be one of the important pathogenesis of cerebrovascular disease. Traditional medicine has attracted enough interest of researchers because of its traditional application and confirmed efficacy. CTL (CTL, also known as Honghua) activates blood circulation to dissipate blood stasis.[17] In this study, the protective function of CTL on ischemia-reperfusion injury of rats was evaluated and also the underlying mechanism including its anti-apoptosis and regulating MMPs. This study provides experimental evidence for the traditional use of CTL.

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**Figure 5:** Regulation of *Carthamus tinctorius* L. on apoptosis of brain tissues of rats after cerebral ischemia. (A) Transferase-mediated dUTP nick-end labeling assay for apoptosis; (B) Western blotting for apoptosis-related factor levels including B-cell lymphoma-2, Bax, and Caspase3; (C) Reverse transcription polymerase chain reaction for messenger RNA levels of B-cell lymphoma-2, Bax and Caspase3. Nimodipine of 7.53 mg/kg/d was used as positive drug. Data are presented as means ± standard deviation ($n = 6$). *P < 0.05, **P < 0.01, versus Sham; *P < 0.05, **P < 0.01, versus model
CTL has been developed to injection for treating acute cerebral infarction in clinic.[18] The evidence showed that its prescription DHI improved cell survival which was related to hypoxia/reoxygenation and H$_2$O$_2$ injury and also reduced hypoxia/reoxygenation-induced cytochrome c release and apoptosis.[19] As an important medicinal herb, the improvement of CTL has been evaluated in MCAO rat model, and its behavior score was also examined. The results of both neurological deficit scores and infarction area ratio demonstrated that CTL could improve the damage degree of cerebral ischemic rats, providing experimental evidence for the pharmacological effects and molecular mechanisms. Multicomponent mixtures from CTL help integrate the pharmacological activity of activated blood circulation to eliminate blood stasis.[20,21] Chemical composition of CTL mainly contains flavonoids including chalcones such as safflower yellow. Existing study has found HSYA can be beneficial for the improvement of I/R injury and lipopolysaccharide-induced endothelium inflammatory injury.[22]

Apoptosis has been recognized as an important mechanism on ischemic brain injury through triggering cell death pathways. Caspase-independent cell death contributes to the severe ischemic injury.[23] Under ischemia and hypoxia conditions, the decrease of mitochondrial membrane potential and the reduction of intracellular active oxygen cytoplasmic histone-associated DNA fragmentation cause and accelerate cell apoptosis, leading to a decrease of neuronal survival. Multiple regulators are implicated in the apoptosis of ischemia reperfusion injury. Accumulated evidence shows that both cleared Caspase 3, Bax and Bcl-2 can lead to increased permeability of mitochondria in MCAO.[24,25] Our experimental data also support this conclusion.

Further evidence showed that Safflower yellow of CTL could attenuate apoptosis of I/R injury through inhibiting Bax and caspase-3 activation and enhancing Bcl-2 expression level.[20,24] Our results, whether in TUNEL assay, or in Western blot and Reverse transcription PCR, revealed that CTL extract could reduce pro-apoptotic Bax and caspase-3 protein and mRNA levels, while enhancing anti-apoptotic Bcl-2 protein and mRNA levels. On the basis of these findings, we conclude that CTL regulates Bcl-2, Bax and caspase-3 levels to inhibit apoptosis for alleviating I/R injury.

Ischemia causes increased the secretion of MMPs owing to metabolic disorders and neuronal death.[26] Under
cerebral ischemia, the activation of MMPs was enhanced to reduce the excessive accumulation of matrix. MMPs have been confirmed to be associated with various complications, including neuronal damage, apoptosis, cerebral edema, and so on. Therefore, it is easy to observe an enhanced MMP-2 and MMP-9 expression in brain tissues by I/R injury. Interestingly, CTL could significantly reduce MMP-2 and MMP-9 expression, demonstrating the inhibition of CTL on MMPs may be beneficial for the improvement of I/R injury.

TIMP1, a glycoprotein expressed in several tissues, functions as a tissue inhibitor of metalloproteinases and participates in regulating MMP. Under normal conditions, MMPs and TIMP1 maintain a relative balance state to regulate the degradation of extracellular matrix, leading to tissue repair, remodeling, cell invasion, and metastasis. Our present results show that TIMP1 has a significant decrease in an animal cerebral ischemia-reperfusion model, whereas CTL increases it. These findings suggested that the regulation of MMPs by CTL may be negatively correlated with the downregulation of TIMP1.

**Conclusion**

Taken together, this study provides new understanding on the beneficial effect of CTL on cerebral I/R injury through regulating MMPs and inhibiting apoptosis, providing new evidence for the improved effect of CTL on I/R injury.

**Acknowledgments**

Financial support was from National Natural Science Foundation of China (No. 81503265, 81473394, 81503314, 81603382), National key research projects on modernization of traditional Chinese medicine (2018YFC1706900), “Double First-Class” project from China Pharmaceutical University (CPU2018GF07, CPU2018PZQ19).

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

**References**

1. Ueno Y, Tanaka R, Yamashiro K, Miyamoto N, Hira K, Kurita N, et al. Age stratification and impact of eicosapentaenoic acid and docosahexaenoic acid to arachidonic acid ratios in ischemic stroke patients. J Atheroscler Thromb 2018;25:593-605.
2. Cho SK, Sohn J, Cho J, Noh J, Ha KH, Choi YJ, et al. Effect of socioeconomic status and underlying disease on the association between ambient temperature and ischemic stroke. Yonsei Med J 2018;59:686-92.
3. Ling C, Liang J, Zhang C, Li R, Mou Q, Qin J, et al. Synergistic effects of salvianolic acid B and puerarin on cerebral ischemia reperfusion injury. Molecules 2018;23:564.
4. Chen HS, Chen X, Li WT, Shen JG. Targeting RNS/caveolin-1/MMP signaling cascades to protect against cerebral ischemia-reperfusion injuries: Potential application for drug discovery. Acta Pharmacol Sin 2018;39:669-82.
5. Velimirović M, Jevtić Dožđučić G, Selaković V, Stojković T, Puškaš N, Zaletel I, et al. Effects of vitamin D3 on the NADPH oxidase and matrix metalloproteinase 9 in an animal model of global cerebral ischemia. Oxid Med Cell Longev 2018;2018:3273654.
6. Wu W, Zhong W, Lang B, Hu Z, He J, Tang X. Thrombopoietin could protect cerebral tissue against ischemia-reperfusion injury by suppressing NF-κB and MMP-9 expression in rats. Int J Med Sci 2018;15:1341-8.
7. Zhao Q, Wang X, Chen A, Cheng X, Zhang G, Sun J, et al. Rhein protects against cerebral ischemic-/reperfusion-induced oxidative stress and apoptosis in rats. J Int J Mol Med 2018;41:2802-12.
8. Delshad E, Yousefi M, Sasannezhad P, Rakshshandehe H, Ayati Z. Medical uses of *Carthamus tinctorius* L. (Safflower): A comprehensive review from Traditional Medicine to Modern Medicine. Electron Physician 2018;10:6672-81.
9. Wang KH, Li SF, Zhao Y, Li HX, Zhang LW. In *vitro* antiinflammatory activity and active components of safflower injection. Molecules 2018;23:170.
10. Xu H, Liu W, Liu T, Su N, Guo C, Feng X, et al. Synergistic neuroprotective effects of Danshensu and hydroxysafflor yellow A on cerebral ischemia-reperfusion injury in rats. Oncotarget 2017;8:115434-43.
11. Lyu M, Yan CL, Liu HX, Wang TY, Shi XY, Liu JP, et al. Network pharmacology exploration reveals endothelial inflammation as a common mechanism for stroke and coronary artery disease treatment of Danhong injection. Sci Rep 2017;7:15427.
12. Zhou D, Qu Z, Wang H, Su Y, Wang Y, Zhang W, et al. The effect of hydroxy safflower yellow A on coronary heart disease through Bcl-2/Bax and PPAR-γ. J Exp Ther Med 2018;15:520-6.
13. Chen L, Xiang Y, Kong L, Zhang X, Sun B, Wei X, et al. Hydroxysafflor yellow A protects against cerebral ischemia-reperfusion injury by anti-apoptotic effect through PDK/Akt/GSK3β pathway in rat. Neurochem Res 2013;38:2268-75.
14. Wang Y, Lichtman JM, Dharmarajan K, Masoudi FA, Ross JS, Dodson JA, et al. National trends in stroke after acute myocardial infarction among Medicare patients in the United States: 1999 to 2010. Am Heart J 2015;169:78-850000.
15. Liu J, Feng L, Ma D, Zhang M, Gu J, Wang S, et al. Neuroprotective effect of paenol on cognition deficits of diabetic encephalopathy in streptozotocin-induced diabetic rat. Neurosci Lett 2013;549:63-8.
16. Tan X, Gu J, Zhao B, Wang S, Yuan J, Wang C, et al. Ginseng improves cognitive deficit via the RAGE/NF-κB pathway in advanced glycation end product-induced rats. J Ginseng Res 2015;39:116-24.
17. Wang Y, Chen P, Tang C, Wang Y, Li Y, Zhang H. Antinociceptive and anti-inflammatory activities of extract and two isolated flavonoids of *Carthamus tinctorius* L. J Ethnopharmacol 2014;151:944-50.
18. Li J, Li YM, Qiao BY, Li X, Du HM, et al. The value of safflower yellow injection for the treatment of acute cerebral infarction: A randomized controlled trial. Evid Based Complement Alternat Med 2015;2015:478793.
19. Zhu HB, Zhang L, Wang ZH, Tian JW, Fu FH, Liu K, et al. Therapeutic effects of hydroxysafflor yellow A on focal cerebral ischemic injury in rats and its primary mechanisms. J Asian Nat Prod Res 2005;7:607-13.
20. Zhou D, Liu B, Xiao X, Dai P, Ma S, Huang W. The effect of safflower yellow on spinal cord ischemia reperfusion injury in rabbits. Oxid Med Cell Longev 2013;2013:692302.
21. Hong B, Wang Z, Xu T, Li C, Li W. Matrix solid-phase dispersion extraction followed by high performance liquid chromatography-diode array detection and ultra performance liquid chromatography-quadrupole-time of flight-mass
spectrometer method for the determination of the main compounds from *Carthamus tinctorius* L. (Hong-hua). J Pharm Biomed Anal 2015;107:464-72.

22. Jin M, Sun CY, Zang B. Hydroxysafflor yellow A attenuate lipopolysaccharide-induced endothelium inflammatory injury. Chin J Integr Med 2016;22:36-41.

23. Askalan R, Gabarin N, Armstrong EA, Fang Liu Y, Couchman D, Yager JY. Mechanisms of neurodegeneration after severe hypoxic-ischemic injury in the neonatal rat brain. Brain Res 2015;1629:94-103.

24. Zhang Y, Li H, Huang M, Huang M, Chu K, Xu W, *et al*. Paeoniflorin, a monoterpane glycoside, protects the brain from cerebral ischemic injury via inhibition of apoptosis. Am J Chin Med 2015;43:543-57.

25. Jung YS, Oh AY, Park HP, Hwang JW, Lim YJ, Jeon YT. Post-ischemic administration of pravastatin reduces neuronal injury by inhibiting bax protein expression after transient forebrain ischemia in rats. Neurosci Lett 2015;594:87-92.

26. Chaturvedi M, Kaczmarek L. Mmp-9 inhibition: A therapeutic strategy in ischemic stroke. Mol Neurobiol 2014;49:563-73.

27. Hill JW, Poddar R, Thompson JF, Rosenberg GA, Yang Y. Intranuclear matrix metalloproteinases promote DNA damage and apoptosis induced by oxygen-glucose deprivation in neurons. Neuroscience 2012;220:277-90.

28. Wang Y, Zhen Y, Wu X, Jiang Q, Li X, Chen Z, *et al*. Vitexin protects brain against ischemia/reperfusion injury via modulating mitogen-activated protein kinase and apoptosis signaling in mice. Phytomedicine 2015;22:379-84.

29. Blicharz-Dorniak J, Kos-Kudla B, Foltyn W, Kajdaniuk D, Marek B, Zemczak A, *et al*. Is determination of matrix metalloproteinases and their tissue inhibitors serum concentrations useful in patients with gastroenteropancreatic and bronchopulmonary neuroendocrine neoplasms? Endokrynol Pol 2012;63:470-6.

30. Borkham-Kamphorst E, Alexi P, Tihaa L, Haas U, Weiskirchen R. Platelet-derived growth factor-D modulates extracellular matrix homeostasis and remodeling through TIMP-1 induction and attenuation of MMP-2 and MMP-9 gelatinase activities. Biochem Biophys Res Commun 2015;457:307-13.