



Research Paper: Comparative Protective Effects of *Viola Spathulata*, *Urtica Dioica*, and *Lamium Album* on Endoplasmic Reticulum (ER) Stress in Rat Stroke Model



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Running Title Protective Effects of *Viola Spathulata*, *Urtica Dioica*, and *Lamium Album* on ER Stress

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ABSTRACT

Background: Pathological conditions, including ischemic stroke, are associated with severe Endoplasmic Reticulum (ER) stress that induces apoptosis and cell death. Herbal medicines are natural treatments with few side effects in such situations.

Objectives: In the present study, we examined the probable neuroprotective effects of *Viola spathulata*, *Lamium album*, and *Urtica dioica* on splicing of ER stress mRNA gene marker (X-box Binding Protein-1 [*XBP-1*]) and Unfolded Protein Response (UPR) activation in the brain of the rat stroke model.

Materials & Methods: Sixty male Wistar albino rats (weight: 220-250 g) were selected for this study and divided into two major groups. The first major group comprised the healthy animals that are subdivided into groups number 1 to 5. The second major group comprised the Middle Cerebral Artery Occlusion (MCAO) group subdivided into groups number 6-10. The reverse transcription-polymerase chain reaction was performed after pretreatment with different herbal extracts (5, 10 mg/kg), *Viola spathulata*, and 100 mg/kg *Urtica dioica* and *Lamium album*). The results were analyzed by one-way analysis of variance in IBM SPSS v. 22.

Results: MCAO-induced ischemia caused a marked increase in *XBP-1* splicing in all rats of the MCAO group in comparison to the control groups ($P < 0.05$), and pretreatment with 3 herbal extracts dramatically decreased target gene splicing in the MCAO studied groups ($P < 0.05$).

Conclusion: All three herbal extracts of *U. dioica*, *L. album*, and *V. spathulata* had the promising potential to use as a neuroprotective agent by reducing ER stress.

Keywords: Endoplasmic Reticulum, Stroke, *Urtica dioica*, *Viola*, Herbal medicine

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Highlights

- Middle Cerebral Artery (MCA) occlusion induced ischemia and caused a marked increase in *XBP-1* splicing in all MCA occlusion animal models compared with the control animals.
- Results show that pretreatment with three herbal extracts dramatically decreased inflammatory gene activation in the MCA occlusion animal models.

Introduction

Stroke is sudden rupture or obstruction of the brain artery or vein leading to localized brain damage or clinical neurological defect [1]. It is the most prevalent nervous system disease, the main reason for mental and physical disability in adults, and the third mortality cause in industrial countries [2, 3]. According to The World Health Organization (WHO), about 15 million strokes happen worldwide every year [3]. The prevalence of ischemic stroke is higher than its hemorrhagic type [1, 2, 4, 5]. The sudden decrease or lack of blood flow to an area of the brain leads to ischemic stroke that causes loss of neurological function in that area. Ischemic stroke is caused by embolism and thrombosis [6]. As described by Sacco, the different identified risk factors for ischemic stroke include age; gender; race/ethnicity; heredity; hypertension; heart disease, particularly atrial fibrillation; diabetes mellitus; hypercholesterolemia; smoking; and alcoholic drink over consumption [7]. Stroke is followed by various complications like body paralysis, sensory difficulties, psychopathy, and death [5]. More than 50 neuroprotective agents and chemical drugs like statins or aspirin have been identified to date in clinical studies on at-risk populations; however, none of them are fully effective [8, 9]. Sometimes dual antiplatelet therapy pretreatment was not effective for acute ischemic stroke [10].

The mechanism of disease is complex, and different molecular pathways play a role in ischemic stroke incidence. Brain tissue is susceptible to ischemic injury because of its high metabolism and low oxygen reserves [2]. Neuron inflammation is one of the essential pathological events in brain damage due to ischemia. Inflammatory changes in neurons can destruct the brain-blood barrier, cause edema, and, consequently, cell death [3]. Reperfusion is critical to establish normal brain function, but it can also cause secondary injury called Ischemia-Reperfusion (I-R) injury. In this injury, oxidative stress mediators such as reactive oxygen species are produced from inflammatory cells around the I-R region [4]. Con-

sidering that oxidative stress and inflammation are the main causes of neuron damage during ischemia, as well as the ineffectiveness of existing drugs in the treatment of ischemia, efforts are made to promote antioxidant drugs. There are different signaling during stress and inflammation within intracellular organelles. The Endoplasmic Reticulum (ER) stress can lead to protein folding disturbance [5]. Aggregation of unfolded proteins results in Unfolded Protein Response (UPR) and activates ER stress sensor Inositol-Requiring Enzyme 1 (IRE-1) [5]. Ischemia and hypoxia cause ER stress and neuronal death [6]. IRE-1 sensor signaling and increased IRE-1-mediated splicing of XBP-1 mRNA exhibited a neuroprotective effect in a rat model of focal cerebral ischemia [7]. Meanwhile, the UPR activation can determine cell faith, i.e., survival (adaptive UPR) or fatal (terminal UPR). IRE-1-mediated splicing of the XBP-1 mRNA route for the inflammatory and neuroinflammatory disease have been investigated as described before [8, 9]. Searching for new neuroprotective prescriptions with anti-inflammatory and anti-neuroinflammatory effects seems to be useful [10].

The use of plants in the treatment of diseases is as old as history. However, in recent years, many researchers have paid attention to medicinal herbs and their extracted materials. One of these herbs is *L. album*, which belongs to the Lamiaceae family and is commonly known as white dead nettle. Experimental studies have reported many medicinal properties of this plant, including anti-inflammatory [11], antioxidant and free radical scavenging [12], anticancer [13], and antidiabetic effects [14-16].

Another herb is *U. dioica*, which belongs to the Urticaceae family and is commonly known as stinging nettle. It is widely grown in Iran. Studies have reported many properties of this plant, including inflammatory [17], antioxidant [18], antihyperlipidemic [19], and neuroprotective effects [20]. The other plant is *V. spathulata*, which belongs to the Violaceae family which is mainly found in temperate regions of the world. This flower contains large amounts of melatonin. Reports indicate that most plants containing melatonin are traditionally used to treat

neurological disorders or diseases associated with the production of free radicals [21]. These reports confirm the effect of melatonin as a neuroprotective agent [22]. A study has shown that melatonin has a protective role in brain ischemia induced by global ischemia [23].

This study aimed to compare the potential neuroprotective effects of *V. spathulata*, *L. album*, and *U. dioica* by considering the splicing number of *XBP-1* in the brain of the ischemic rat stroke model.

Materials and Methods

Study animals

During this experimental study, adult male Wistar albino rats (weight: 220-250 g) were used. The selected animals were housed under normal laboratory conditions (12 h light: 12 h dark at 22-24°C) with free access to their appropriate diet and water. All procedures were performed following the internationally accepted principles for laboratory animal care and use as in the US guidelines (NIH publication #85-23, revised in 1985).

Plant material and extraction

The plants *L. album* and *U. dioica* were collected from the Rasht region in Guilan Province, Iran. They were identified by Fatemeh Yousefbeyk, Department of Pharmacognosy, Guilan University of Medical Sciences, Iran (voucher Numbers 202HGUM and 156HGUM for *L. album* and *U. dioica*, respectively). For each plant, the hydroalcoholic extract was prepared by macerating the powder of stems and leaves in 50% ethanol (10 mL/g powder) for 72 h at 40°C. Then, the extracts were filtered through a 250- μ m mesh, centrifuged at 2000 RPM for 10 min, and their supernatants were dried on a water bath (40°C) [24].

V. spathulata: aerial part of *V. spathulata* was collected from Gadouk pass, Firoozkoub Road, Savadkouh City (Mazandaran Province) in May 2017. The species were authenticated at the herbarium unit of the Department of Biology, School of Sciences (Sari Payame Noor University; Herbarium number: SPNH-4727). The aerial parts of *V. spathulata* were separately dried, powdered, and extracted with 70% ethanol in a Soxhlet apparatus for 48 hours. The hydroalcoholic extracts were then concentrated in a water bath and kept at -20°C until use. All stages were conducted at the Research Institute of Medicinal Plants of Shahid Beheshti University [25].

Study design

Animals and group assignment: Sixty male Wistar albino rats (weight: 220-250 g) were selected for this study and divided into two major groups. The first major group is the healthy group that included groups number 1-5. The second major group is the Middle Cerebral Artery Occlusion (MCAO) group which are subdivided into groups number 6-10 (n=6 per each group). 1-5) Healthy+Four groups treated with normal saline, *V. spathulata* (5 and 10 mg/kg), *L. album* and *U. dioica* (100 mg/kg), respectively, 6) MCAO (middle cerebral artery occlusion) and 7-10) four MCAO groups treated by Normal Saline, *V. spathulata* (5, 10 mg/kg), *L. album*, and *U. dioica* (100 mg/kg), respectively. Pretreatment was done for 7-day before tests. The groups 6-10 had been operated on for 60 min period to establish Middle Cerebral Artery Occlusion (MCAO), and 24 hours after reperfusion, *XBP-1* splicing was studied in the brain by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Focal Cerebral Ischemia (MCAO): The rats were weighed and anesthetized with chloral hydrate (Merck, Germany) (400 mg/kg; IP). MCAO was performed as described by Longa et al. (1989) [26]. Briefly, under microscopic surgery, a 3-0 silicone-coated nylon (Nylon, homemade) suture was introduced through the external carotid artery stump. The occluder was advanced into the internal carotid artery 20-22 mm beyond the carotid bifurcation until mild resistance indicated that the tip lodged in the anterior cerebral artery and blocked the blood flow to the MCA [25]. Reperfusion started by withdrawing the suture after 60 min of ischemia. Rectal temperature was monitored (Citizen-513w, CITIZEN, United Arab Emirates) and maintained at 37°C by surface heating and cooling during surgery.

Molecular experiments: The splicing of the *XBP-1* mRNA gene was investigated in the brain of the animals. All surgical instruments were sterilized and set DNase-RNase Free/Non-Pyrogen for surgical removal and separation of animal tissue. Small 10-mg pieces were isolated and frozen immediately after aseptic transfer to DNase-RNase Free/Non-Pyrogen sterile microtube.

After brain extraction, the hippocampus was dissected under sterile-cold conditions and immediately transferred to a nitrogen tank. RNA was extracted based on acid guanidinium thiocyanate-phenol-chloroform extraction employing an RNX-Plus solution (EX 6101, Sina Clon, Iran), following the manufactures instructions. The total RNA extracted from the hippocampus

tissue was converted into cDNA using a cDNA synthesis kit (YT 4500 and YTA, Iran).

The DNA and RNA of all prepared specimens were extracted using specific kits and according to the manufacturer's guidelines. Finally, RT-PCR was performed in turn. Using the spectroscopic technique (NanoDrop-2000c Spectrophotometers, Thermo Fisher Scientific, USA), the purity of the extracted RNA was measured. Before cDNA synthesis from extracted RNAs, RNA samples were treated with DNase specific kit (Sina Clon, Iran) to eliminate possible contamination with DNA. After treatment with a DNase-specific kit, the samples were subjected to RT-PCR (Thermo Fisher Scientific, USA). For the PCR, a sensitive and specific primer pair was used for the test and control groups [27]:

XBP-1 forward primer: 5'GAACCAGGAGTTA-AGAACACG3', and

Reverse primer: 5'AGGCAACAGTGT-CAGAGTCC3'

(size of PCR product: unspliced *uXBP-1*=205 bp and spliced *sXBP-1*=179 bp) as described [27], and β -actin housekeeping primers were obtained from CinnaGen, Iran.

Statistical analysis

The results of this experiment were analyzed in IBM SPSS software v. 16. For comparing the spliced or unspliced *XBP-1* mRNA gene in the studied groups, the χ^2 test was utilized. For all tests, $P < 0.05$ was consid-

ered statistically significant. The obtained data were expressed as Mean \pm SD.

Results

Three herbs were used for evaluating their effects on *XBP-1* gene splicing in a male rat stroke model:

Effects of *V. Spathulata* on the *XBP-1* gene splicing: In the healthy group (group 1), we had no *XBP-1* gene splicing. In the MCAO group (group 6), all rats had spliced the *XBP-1* gene. In the MCAO group treated with *V. spathulata* 5 (group 7), only two mice showed spliced *XBP-1* gene, and this value was significantly lower than that in the MCAO group ($P < 0.05$). In addition, in the MCAO group treated with *V. spathulata* 10 (group 8), only one rat had *XBP-1* gene splicing ($P < 0.05$). Treatment with *V. spathulata* 5 and *V. spathulata* 10 in healthy rats caused no *XBP-1* gene splicing (groups 2 and 3) (Figure 1).

Effects of *L. album* on *XBP-1* gene Splicing: In the healthy group (group 1), we found no splicing *XBP-1* gene. In the MCAO group (group 6), all rats had spliced *XBP-1* gene. In the MCAO group treated with the *L. album* (group 9), only two rats had *XBP-1* gene splicing ($P < 0.05$). Treatment with *L. album* in the healthy rats stopped *XBP-1* gene splicing (group 4) (Figure 2).

Effects of *U. dioica* on *XBP-1* gene Splicing: In the healthy group (group 1), we had no *XBP-1* gene splicing. In the MCAO group (group 6), all rats had spliced *XBP-1* gene. In the MCAO group treated with the *U. dioica* (group 10), only two rats had *XBP-1* gene splicing

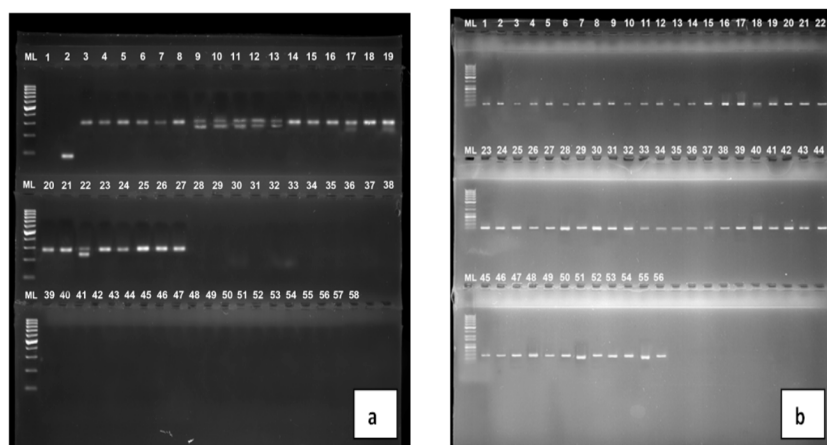


Figure 1. Effects of *V. Spathulata* on ER stress

A: Effects of *V. Spathulata* 5 and 10 mg/kg on the *XBP-1* gene splicing in different study groups; B: Internal control of GAPDH-RT-PCR results to present effects of *V. spathulata* on the *XBP-1* gene splicing in different study groups.

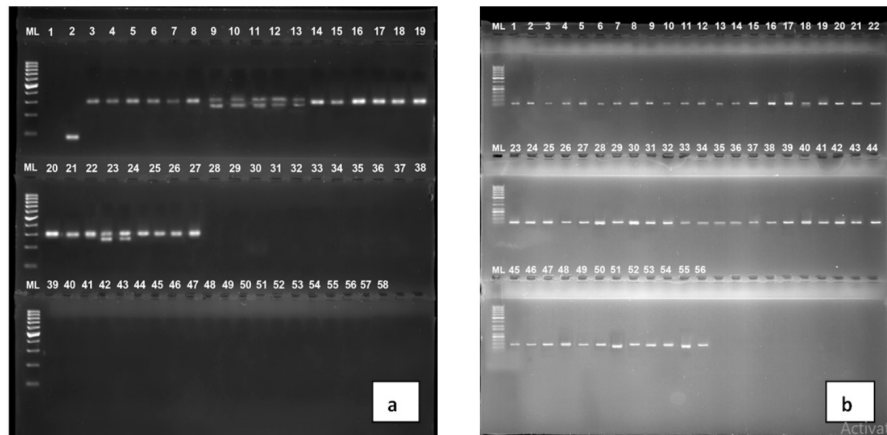


Figure 2. Effects of L.Album on ER stress

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A: Effects of L.album on the XBP1 gene splicing in different study groups.AA'GI; B: Internal control of GAPDH-RT-PCR results to present effects of L.album on the XBP-1 gene splicing in different study groups.

($P < 0.05$). Treatment with *U. dioica* in the healthy rats stopped *XBP-1* gene splicing (group 5) (Figure 3).

tracts dramatically decreased target gene splicing in the MCAO studied groups.

Discussion

In the present study, we examined the probable neuro-protective effects of *V. spathulate*, *L. album*, and *U. dioica* on splicing of ER stress mRNA gene marker (*X-box binding protein-1 [XBP-1]*) and Unfolded Protein Response (UPR) activation in the brain of the rat stroke model.

In the present study, we used a novel index to investigate and compare the effects of three herbs (*V. spathulata*, *L. album*; *U. dioica*) on the UPR response in vivo.

MCAO-induced ischemia caused a marked increase in *XBP-1* splicing in all rats of the MCAO group compared to the control groups, and pretreatment with 3 herbal ex-

Hypothetically, the ER stress in the eukaryotic endoplasmic reticulum results in the activation of the UPR in response to increased levels of miss-folded proteins. This effect will often indicate its presence, either in the form of an increase in the bioavailability of the living tissue and cells or causing the death of the cells and, ultimately, the subject [16]. To cause cell death, calcium overload of cells occurs in different ways [8, 9, 11-13, 15].

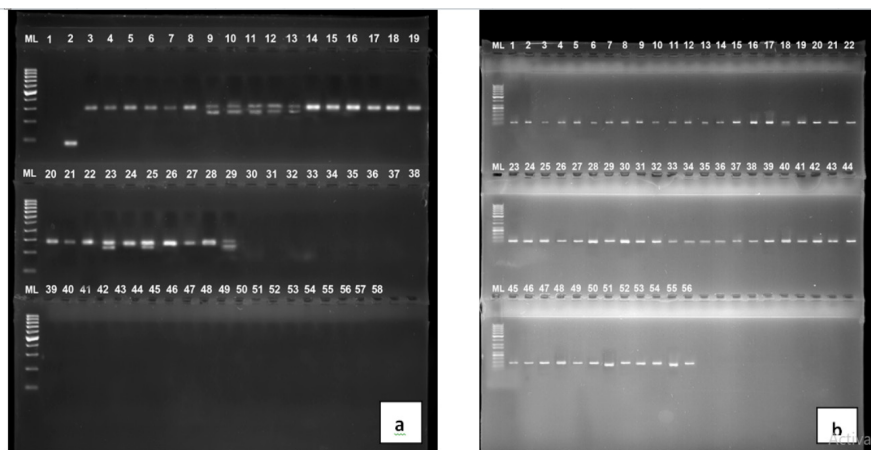


figure 3. Effects of U.dioica on ER stress

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A: effects of U.dioica on the XBP-1 gene splicing in different study groups: AA'FJ; B: Internal control of GAPDH-RT-PCR results to present effects of U.dioica on the XBP-1 gene splicing in different study groups.

We looked at the effects of three different plant extracts on ER stress and altered *XBP-1* mRNA gene transcripts splicing in the animal model of stroke. Normally, blood flow to the affected tissue is impaired, leading to hypoxia and regional tissue damage. One of the intracellular organelles involved in this event is the endoplasmic reticulum. In this study, the efficacy of three herbal extracts on strokes was investigated with changes in gene target editing. It was observed that one hour of closing the middle cerebral artery activated the splicing of the target gene, and the use of herbal extracts reduced ER stress.

Various herbal medicines have been used in previous studies to reduce the inflammatory effects and cellular stress caused by MCAO. Each method has advantages and disadvantages. Given the extent of the damage caused by MCAO, it is always necessary to find new treatment strategies.

This study showed that the use of *V. spathulata* at a dose of 5 and 10 mg/kg reduced the splicing of the target gene. This effect can be due to the presence of anti-inflammatory compounds like polyphenols and melatonin of the plant. In another study by Karimifar et al. [25], doses of 25, 50, and 75 mg/kg of *Viola odorata* extract were tested in a focal cerebral ischemic model of male rats. They observed that a 50 mg/kg dose of the extract could reduce infarct volume and neurological defects. Their reports were based on calculating Neurological Deficit Scores (NDS) and Infarct Volume (IV) in an MCAO stroke model. According to their investigation of IV in the core, penumbra, and subcortex, different doses of extract may lead to different score recordings in each anatomic area of the brain [25]. In the present study, this is the first time that the effectiveness of *V. spathulata* extract is measured by evaluating the mechanism of UPR. It is worth noting that the *V. spathulata* species is a native species of Iran, and there has not been much information about its effectiveness on the stroke.

The present study results show that *U. dioica* at a dose of 100 mg/kg reduced the target gene splicing. This effect can be due to the presence of antioxidant compounds of the *U. dioica*. The healing effect of *U. dioica* extract has already been evaluated for some disorders like experimental acute pancreatitis model in rats [28] or combination therapy of nettles with nonsteroidal anti-inflammatory drugs [29]. Although much research has been done on the role of the UPR mechanism in stroke, no study has been conducted on the effectiveness of nettle mechanisms in this way.

The use of *Lamium* at a dose of 100 mg/kg also reduced target gene splicing. This effect can be due to the presence of antioxidant compounds of the *Lamium*. The therapeutic effects of *U. dioica* and *L. album* extracts have previously been investigated in streptozotocin-induced diabetic rats. The assessment method evaluated the expression level of cyclooxygenase-2 and caspase-3 in the liver and kidney of rats [30]. In another study, the effects of combination therapy with *U. dioica* and *L. album* extracts were investigated on rat tracheal smooth muscle contraction [24]. Thus, the therapeutic efficacy of *Lamium* and its combination therapy have already been demonstrated. Nevertheless, no study has compared the therapeutic efficacy of the extract of these plants on the stroke animal model via UPR evaluation.

Examining the sensors of the UPR system and its transcription factor splicing (*XBP-1*) can be an indicator of the body's homeostatic balance or deadly inflammatory responses. The more inflammation, the more splicing will occur in the *XBP-1* gene of the UPR system. In the animal model group of strokes that received 5 mg/kg *V. spathulata*, only two rats presented with *XBP-1* splicing and, in the stroke group treated with 10 mg/kg *V. spathulata* extract, only one rat had spliced *XBP-1*. In the animal model group of strokes that received *L. album* and *U. dioica*, only two rats had *XBP-1* splicing. This study shows that the use of these plant extracts targets inflammatory mechanisms in the brain at the molecular level and can control some of the deadly cellular stress caused by strokes. Recent findings could confirm and suggest the neuroprotective effects of pretreatment with these herbal extracts to prevent stroke in sensitive or high-risk individuals. It is recommended that other tests be performed on other sensors in the UPR system to support this claim.

Conclusion

All three herbal extracts of *V. spathulata*, *L. album*, and *U. dioica* had the promising potential to use as a neuroprotective agent with diminished ER stress. Further study is needed to reveal more pathways that are mechanistic.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Guilan University of Medical Sciences (IR.GUMS.REC.1395.161). All study procedures were done in compliance with the ethical guidelines of the Declaration of Helsinki, 2013.

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Authors' contributions

Conceptualization and methodology: All authors; Project administration: Mahmood Abedinzade, Ekram Mohammadi, Behrooz Khakpour Taleghani, and Korosh Khanaki; Acquisition: Mojtaba Hedayati, Ekram Mohammadi, and Mahmood Abedinzade; Formal analysis: Mojtaba Hedayati Ch, Mahmood Abedinzade, Behrooz Khakpour Taleghani, and Korosh Khanaki; Writing, review, and editing: Mojtaba Hedayati and Mahmood Abedinzade; Resources, data curation: Korosh Khanaki, Ekram Mohammadi, Behrooz Khakpour Taleghani, and Mostafa Golshekan.

Conflict of interest

The authors declared no conflict of interest.

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