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Attenuating *NF-κB/VCAM-1* Expression in the Middle Cerebral Artery Occlusion Model by Viola Odorata: Protection Against Ischemia-Reperfusion Injury in Rats

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ABSTRACT

Introduction: The death of neurons and cerebral edema are the main consequences of stroke. However, inflammatory processes play key roles in aggravating cerebral damage following a stroke. This study aimed to investigate the effects of Viola odorata extract (VOE) on the infarct volume (IV), neurologic deficits (ND), and the expression of *NF-κB* and *VCAM-1* in the Middle Cerebral Artery Occlusion (MCAO) model.

Methods: The animals were randomly separated into 6 groups: (1) control group, (2) vehicle-treated group, (3) MCAO group, (4) VOE25 group, (5) VOE50 group, and (6) VOE75 group (n= 12). VOE (25, 50, and 75 mg/kg) and distilled water were administered daily for 30 days. Two hours after the last gavage, the rats were exposed to MCAO for 60 minutes. Twenty-four hours later, *IV, ND*, and *NF-κB/VCAM-1* expressions were evaluated.

Results: Viola odorata extract exhibited excellent neuroprotective effects by reducing IV (mainly in the core and subcortex areas), and induced downregulation of *NF-κB* and *VCAM-1* expression.

Conclusion: Viola odorata could also activate intracellular pathways, reducing the expression of *NF-κB* and *VCAM-1*. It is useful for developing a novel medical herb for treating cerebral ischemia.

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Highlights

- A stroke occurs when the blood supply to a part of the brain is interrupted or reduced.
- Viola odorata extract (VOE) reduced the infarct volumes (IV) in rats' brains 24 h after middle cerebral artery occlusion (MCAO).
- VOE may decrease *IV* in the *MCAO* model by downregulating the *NF-κB/VCAM-1* expression.

Plain Language Summary

A stroke is a medical condition in which poor blood flow to the brain results in cell death. After an interruption of blood flow, energy stores are rapidly depleted, and complex cellular cascades induce excitotoxic cell death. So far, no effective and specific treatment has been suggested for the stroke. In this study, the neuroprotective effects of the viola odorata extract (VOE) were screened. The V. odorata extract exhibited excellent neuroprotective effects by reducing infarct volume and inducing downregulation of *NF-κB/VCAM-1* expression. This finding suggests that *V. odorata* may be useful for developing a novel medical herb for treating cerebral ischemia.

1. Introduction

Stroke is an acute and progressive neurodegenerative disorder resulting in Central Nervous System (CNS) injuries of vascular origin that interrupts blood flow of the brain and is the second most common cause of death throughout the world (Heo & Kim, 2013; Neuhaus, Couch, Hadley, & Buchan, 2017; Taştepe, Gül, Özmen, & Demirci, 2017). The death of neurons and cerebral edema are the main consequences of stroke. Inflammatory processes are key in exacerbating stroke-related brain damage (Panickar, 2015). A variety of mechanisms are responsible for ischemic brain injury. After the interruption of blood flow, energy stores become rapidly depleted, and excitotoxic cell death is induced by complex cellular cascades such as cellular depolarization and Ca^{2+} influx (Lee, Grabb, Zipfel, & Choi, 2000). In the pathogenesis of cerebral ischemia, evidence indicates that a Tumor Necrosis Factor (TNF) like cytokine has both cytotoxic and cytoprotective activities (Botchkina, Geimonen, Bilof, Villarreal, & Tracey, 1999). The development of TNF cytotoxic activity depends on the balance between the activities of intracellular signaling pathways mediating either apoptotic or antiapoptotic effects (Botchkina et al., 1999). Tumor necrosis factor is a pro-inflammatory cytokine that targets endothelial cell by binding to its surface receptor (Tumor Necrosis Factor Receptor 1 (TNF-R1)), and stimulates intracellular cascades (Wojdasiewicz, Poniatowski, & Szukiewicz, 2014). The activated signaling pathway increases the expression of *NF-kB* and cell adhesion molecules (*VCAM-1* and *ICAM-1*) (Lin, Pan, Wang, Liu, Hsiao, & Yang, 2015). Finally, cell adhesion molecules stimulate leukocytes' migration from the peripheral circulation to brain tissue and reinforce inflammatory signaling cascades (Wang, Tang, & Yenari, 2007). Inflammatory neuron changes could result in blood-brain barrier breakdown and edema and induce cell death in the advanced stage. Therefore, neural inflammatory pathways can be the targets for cerebral ischemia (Kim, Kawabori, & Yenari, 2014).

Medicinal plants have historically proven their value as a source of new therapeutic agents for all sorts of human ailments (Atanasov et al., 2015; Gaire, 2018). This is mainly for two reasons, which may suggest the promising future of natural medicine for stroke treatment: 1) stroke is a complication of primary pathologic conditions, and 2) the multifactorial effect of herbal medicine (Gaire, 2018). *Viola odorata* is a member of the Violaceae family and is grown as a medical herb (Alipanah, Bigdeli, & Esmaeili, 2018). Traditionally, V. odorata has been used to treat kidney and liver disorders, anxiety, blood pressure, insomnia, hypertension, and relieving cancer pain. Previous studies verified some medical features of *V. odorata*, including its antioxidant, anti-inflammatory, and anticancer activities (Barekat, Otroshy, Samsam-Zadeh, Sadrarhami, & Mokhtari, 2013; Hartwell, 1967; Kapoor, 2000; Mittal, Gupta, Goswami, Thakur, & Bansal, 2015).

So far, no effective and specific treatment for stroke has been suggested. In developing countries, the rate of cerebral ischemia is increasing significantly (Hackett, Duncan, Anderson, Broad, & Bonita, 2000). Several studies

have shown the anti-inflammatory effects of different species of Viola (Drozdova & Bubenchikov, 2005; Muhammad, Saeed, & Khan, 2012). Mousavi, Naghizade, Pourgonabadi, and Ghorbani (2016) demonstrated that V. tricolor and V. odorata protect neuronal cells against serum/glucose deprivation-induced cell death partially by their antioxidant activities (Mousavi, et al., 2016). Our previous study showed the antioxidant activity of V. odorata hydroalcoholic extract and the presence of several flavonoids. Flavonoids have been investigated for their ability to prevent oxidant injury caused by reperfusion of ischemic tissue, a scenario similar to clinical conditions such as stroke and myocardial infarction. *Viola odorata* extract may change the expression of *NF-kB*, *VCAM-1* and *TNF-R1* in breast cancer model (Alipanah et al., 2018; Alipanah, Bigdeli, Esmaeili, & Akbari, 2017). Hence, we selected V. odorata as an independent variable in our investigation. Therefore, this study aimed to determine whether V. odorata extract would effectively reduce Infarct Volume (IV) and neurologic deficit in the Middle Cerebral Artery Occlusion (MCAO) model and whether there would be changes in expression of NF-kB and VCAM-1 determined by western blot technique in rats treated with *V. odorata* hydroalcoholic extract (VOE).

2. Materials and Methods

Study animals

Male Wistar rats (200-300g, 10-12 weeks old) were housed in a standard 12:12 light-dark cycle and controlled temperature (lights on at 9 AM), and food and water were available ad libitum. All animal experiments were conducted following a protocol approved by Institutional Animal Care and Use Committee at Shahid Beheshti University (Iran).

Viola odorata extract

Aerial parts of V. odorata were collected in summer from Rostamabad, Gilan, Iran, and authenticated by Dr Mohammad Reza Kanani (Shahid Beheshti University). The plants were kept in the herbarium (Index Herbarium code: MPH-615) for future reference. Department of Biology, Medicinal Plants and Drugs Research Institute (Shahid Beheshti University, Iran) prepared hydroalcoholic (50/50) extract.

Experimental protocol

Animals were randomly divided into MCAO groups (4 subgroups, n=6) and Western groups (5 subgroups, n=6) and received different doses (25, 50, and 75 mg/kg bw) of *Viola odorata* hydroalcoholic extract. The sham-operated animals underwent the MCAO surgical procedure. Animals in the control-treated group were almost the same as the sham group, except they received distilled water by gastric gavage. In VOE-treated groups, animals underwent the MCAO surgical procedure, and VOE was administrated by gastric gavage at doses of 25, 50, and 75 mg/kg, respectively. VOE or distilled water was administered daily for 30 days. Six rats from each group were sacrificed to determine *NF-κB* and *VCAM-1* expression rates by western blotting technique. The IV and ND in MCAO groups were assessed. Two hours after the last gavage, the rats were exposed to MCAO surgery for 60 min. Twenty-four hours later, the IV and ND were evaluated.

Protocol of middle cerebral artery occlusion (MCAO)

Animals are anesthetized with chloral hydrate (400 mg/kg) intraperitoneally. After turning the animal to the supine position, an incision was created along the midline in the neck, with a length of 2 cm, then the Common Carotid Artery (CCA) was isolated from the vagus nerve carefully. Two permanent knots were placed at the distal part of the External Carotid Artery (ECA) and CCA. The Internal Cerebral Artery (ICA) blood flow was blocked by a microvascular clip, and CCA was incised using micro scissors to insert the monofilament. Monofilament was carefully advanced into the Middle Cerebral Artery (MCA) at the CCA junction. Once mild resistance was felt, the microvascular clip was removed permanently from the CCA (Figure 1A) (Hori, M., et al., 2014). After 60 min of reperfusion, the incision of the midline neck was sewed using a surgical suture (Longa, Weinstein, Carlson, & Cummins, 1989). The animal's body temperature was maintained at 37°C±2°C during the occlusion.

Neurobehavioral evaluation: Neurological Deficit Score (NDS)

Neurological scores were assessed 24 h after the reperfusion using a modification of scoring reported by Long, Cai, Li, Zhang, Yang, & Wang, (2013) (minimum score=0, maximum score=18).

A: Raise the tail about 1 m from the earth (normal=0, maximum=3)

Four limbs extended, head deviated from the middle line less than 10 (0 score)

Forelimb crooked (1 score)

Hind limb crooked (1 score)

Head deviated from middle line less than 10º within 30 seconds (1 score)

B: Motor function (normal=0, maximum=3)

Normal working (0 score)

Did not walk along a straight line (1 score)

Rotated to the hemiplegic side (2 score)

Fell down to the hemiplegic side and could not walk (3 score)

C: Sensory function

Algesia and heat sensory (1 score)

Proprioception (2 scores)

D: Beam test (normal=0, maximum=6)

Stand stably on the beam (0 score)

Hold on to one side of the beam (1 score)

One limb fell down from the beam (2 score)

Two limbs fell down from the beam or rotated on the beam, lasting more than 60 seconds (3 scores)

Sustain on the beam for 40 seconds and fall down (4 scores)

Sustain on the beam for 20 seconds and fall down (5 scores)

Sustain on the beam for 10 seconds and fall down (6 scores)

E: Reflex activity

Corneal reflex (1 score)

Auricle reflex (1 score)

Startle reflex (1 score)

Dysmyotonia (1 score)

Infarct volume

The rats (n=5 from each main group) were sacrificed by decapitation under deep anesthesia (800 mg/kg chloral hydrate) 24 hours after the cerebral ischemia. Brain tissues were quickly removed and washed with cold saline (4°C) for 5 min. Then they were placed in the brain matrix, and the coronal sections of the frontal lobe were cut (2 mm thick). The brain sections were immediately immersed in a 2% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) (Merck, Germany) and incubated in a water bath (37°C) for 15 min. The infarct area was white, while viable tissue was red or pink. Afterward, the sections were placed on graph paper and photographed with a digital camera (Nikon, D40x digital). Calculating the infarct volume was measured by ImageJ software (version 1.46r) and multiplied by slice thickness (2 mm). Eight slices of stained brain tissue were calculated with the method described by Swanson et al (Equation 1).

1. CIV=LHV-(RHV-IAV)

, where CIV is the corrected infarct volume, LHV the left hemisphere volume, RHV the right hemisphere volume and IAV is the ischemic area volume.

Western blot analysis

Animals were deeply anesthetized with chloral hydrate (800 mg/kg, IP) and transcranial magnetic stimulation (TMS) perfused with 0.9% normal saline. In each hemisphere, the penumbra, subcortex, and core of the brain tissue were removed according to the previous methods (Lei, Popp, Capuano-Waters, Cottrell, & Kass, 2004). Tissues were placed in round-bottom microcentrifuge tubes, and ice-cold lysis buffer (0.25 g sodium deoxycholate, 0.08 g NaCl, 0.01 g SDS, 0.003 g EDTA, 10 µL NP40 [0.1%], 1 tablet protease inhibitor cocktail [Roche] and 500 µL Tris-HCl with pH 8.0) was added rapidly. Tissues were homogenized by an ultrasound homogenizer (4000 rpm). The homogenate was centrifuged in a microcentrifuge for 20 min at 12000 rpm at 4°C. The supernatant was aspirated and placed in a fresh tube kept on the ice, and the samples were stored at -80°C for later use. The protein concentration for each sample was determined by a protein quantification assay. Samples were boiled in sample buffer at 100°C for 5 min. Equal amounts of proteins with a protein ladder (Thermo Scientific) were loaded into the wells of the SDS-PAGE gel. Proteins were transferred from the gel to the PVDF (polyvinylidene fluoride) membrane (Millipore). The membrane was blocked at room temperature for 1 h using blocking buffer and subsequently incubated with specific

primary polyclonal rabbit antibodies to NF-κB (1:500 dilution; Santa Cruz), goat anti-VCAM-1 polyclonal antibody (1:500 dilution; Santa Cruz), and goat anti-GAPDH (1:1000 dilution; Santa Cruz). The membrane was washed in three washes of TBST, 5 min each. Then, the membrane was incubated with secondary antibodies, including goat anti-rabbit and rabbit anti-goat (1:500 dilution; Santa Cruz Biotechnology, CA, USA). For the second time, each membrane was washed in three washes of TBST for 5 min. The darkroom technique for chemiluminescence was used to detect *NF-κB* and *VCAM-1* immunoreactive proteins. Signal bands were quantified by ImageJ densitometry software and expressed relative to the GAPDH signal (loading control).

Statistical analysis

The Kruskal-Wallis test was used to analyze the neurological deficits score. Infarct volume and protein expression in experimental groups were compared with the control group using the 1-way ANOVA SPSS software v. 22, and the post hoc LSD test was used. Data were expressed as Mean±SD. P<0.05 was considered significant.

3. Results

Effect of *viola odorata* on neurological deficits scores

As shown in Table 1, pre-feeding of V. odorata (VOE50) significantly reduced neurological deficit (P<0.01) in comparison with the control group.

Effect of *viola odorata* on the volume of tissue damage (infarct volume)

The present results indicated that total IV was significantly decreased (P<0.001) in the brain hemisphere of rats that received 50 mg/kg/d of VOE (Figure 2A). The effect of VOE75 and VOE25 on the reduction of total IV was not significant compared with the control group (Figure 2B). The effective dose of the extract was 50 mg/kg.

The effect of hydroalcoholic extract of *Viola odorata* on the IV in three areas of penumbra (P), core (C), and subcortex (SC) was assessed (Figure 3). Infarct volume was reduced in the P area (P<0.01) at a dose of 50 mg/kg of VOE compared to the control group. VOE50 (P<0.001) and VOE25 (P<0.05) reduced the IV in the C area significantly. Infarct volume of SC was significantly decreased (P<0.001) in rats treated with VOE25, VOE50, and VOE75 compared to the control group (Figure 3).

Effects of VOE on *NF-κB* expression in penumbra area

The assessment of *NF-κB* protein by western blotting technique demonstrated that VOE in doses of 50 and 75 mg/kg decreased *NF-κB* expression in the P area compared to the control group (Figure 4A-B). VOE25 did not affect the *NF-κB* expression significantly in the P area. Besides, there is no significant difference between the sham and the control groups (Figure 4).

Effects of VOE on *NF-κB* expression in core area

The western blotting technique demonstrated no significant difference in the *NF-κB* expression between VOE in doses of 25 and 75 mg/kg with the control group. Unlike VOE25 and VOE75, treatment with VOE50 significantly decreased the *NF-κB* expression in the C area compared to the control group (P<0.001) (Figure 5). In addition, there was no significant difference between the sham and the control groups.

Effects of VOE on *NF-κB* expression in sub-cortex area

Analysis of the western blotting technique demonstrated a significant decrease in the NF-κB expression in doses of 50 and 75 mg/kg of VOE compared to the control group in the SC area (P<0.05), while there was no significant difference between VOE25 and the control group (Figure 6). Also, there was no significant difference between the sham and the control groups.

Effects of VOE on VCAM-1 expression in penumbra area

As shown in Figure 7, VCAM-1 expression was reduced in doses of 50 mg/kg of VOE compared to the control group in the P area (P<0.05), while there was no significant difference between VOE25 and VOE75 with the control group. Also, there was no significant difference between the sham and control groups.

Effects of VOE on VCAM-1 expression in core area

There was no statistically significant difference in the VCAM-1 expression between the VOE25 and control group, while VOE75 and VOE50 significantly decreased VCAM-1 expression in the C area compared to the control group (Figure 8). In addition, there was no significant difference between the sham and control groups.

Table 1. Neurological deficits scores

Groups	Rat	Neurological Deficits Score					Sum	Mean	Median
		Raise the Tail	Motor Function	Sensory Function	Beam Test	Reflex Activity			
Control	1	3	3	2	6	2	16	15.83	16
	2	3	3	2	6	4	18		
	3	3	2	2	4	1	12		
	4	3	3	2	6	4	18		
	5	3	3	2	6	2	16		
	6	3	2	2	6	2	15		
VOE25	1	3	3	2	6	4	18	15.66	16.5
	2	3	2	1	4	2	12		
	3	3	3	2	6	4	18		
	4	2	2	2	5	2	13		
	5	3	2	2	5	1	15		
	6	3	3	2	6	4	18		
VOE50	1	2	2	2	1	0	7	7.1**	7
	2	3	2	0	5	0	10		
	3	2	0	1	1	0	4		
	4	2	2	1	3	1	9		
	5	1	2	1	2	1	6		
	6	2	1	2	2	0	7		
VOE75	1	3	2	2	4	2	13	14.83	14.5
	2	3	2	2	6	3	16		
	3	2	3	2	5	2	14		
	4	3	3	2	6	4	18		
	5	3	2	1	5	2	13		
	6	3	3	2	5	2	15		

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Comparison of neurological deficits in experimental groups treated with different doses of Viola odorata extract (VOE). Pre-feeding of Viola odorata (VOE50) significantly reduced neurological deficit **(P<0.01) in comparison with the control group.

VOE25, 50, 75, Viola odorata extract in different concentration (25, 50 and 75 mg/kg bw); Ctrl, control group, (n= 6).

** (P<0.01).

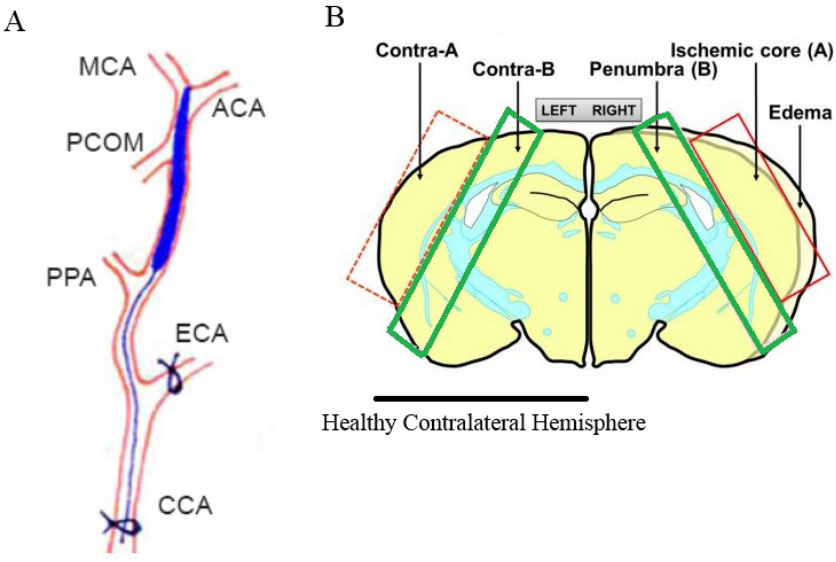

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Figure 1. A) Experimental design using Middle Cerebral Artery Occlusion (MCAO) model; B) Ischemic areas of the ipsilateral hemisphere (Right) along with the corresponding regions in the healthy contralateral hemisphere (left)

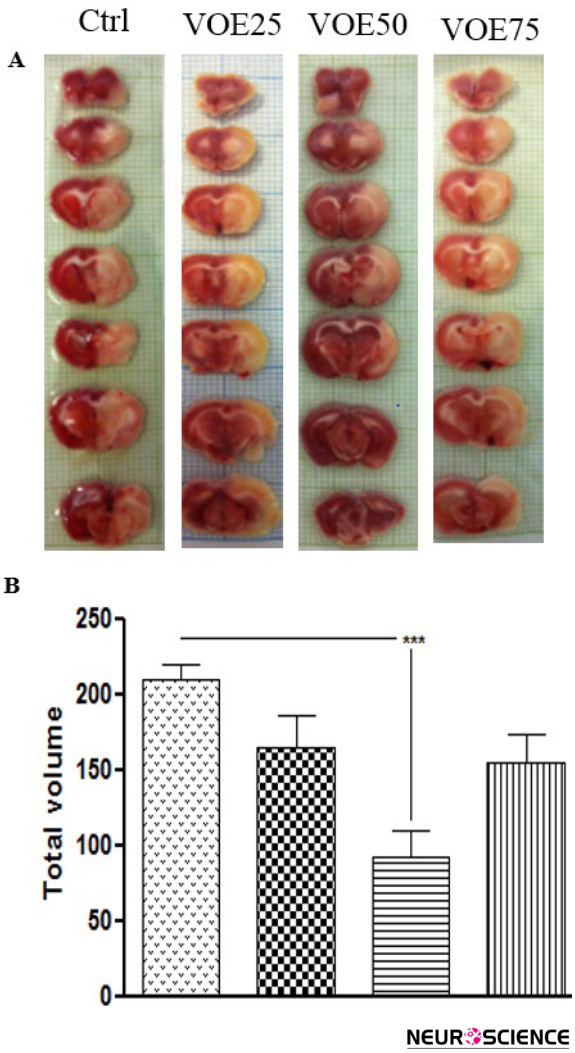

Figure 2. Effect of hydro-alcoholic extract of viola odorata on total Infarct Volume (IV)

Representative photographs of TTC-stained brain slices (2mm) showing the infarct area; B) Quantitative analysis of the infarct area in each slice from rats treated with VOE or vehicle.

VOE25, 50, 75: Viola odorata extract in different concentration (25, 50, and 75 mg/kg bw); Ctrl: control group; (n= 6).

*** P<0.001.

Note: White indicates ischemic tissue, and red or pink areas are healthy sites.

Effects of VOE on VCAM-1 expression in subcortex area

As shown in Figure 9, VCAM-1 expression was decreased in doses of 50 mg/kg of VOE compared to the control group in the SC area, whereas there was no significant difference between VOE25 and VOE75 in the control group. Also, there was no significant difference between the sham and control groups.

4. Discussion

In the present study, screening of the neuroprotective effects of the hydroalcoholic extract of the V. odorata was carried out. The VOE exhibited excellent neuroprotective effects by reducing IV (mainly in core and subcortex areas) (Figure 3), and induced downregulation of NF-κB and VCAM-1 expression.

Increasing attention has been drawn to the role of inflammation in ischemic stroke. The intensity of the inflammatory reaction is correlated with the extent of the ischemic lesion so that small infarcts produce less inflammation than large infarcts (Akopov, Simonian, & Grigorian, 1996). Our results showed that VOE pretreatment reduces the IV in mice brains 24 h after MCAO (Figures 2 & 3) and improves the neurological and behavioral deficits significantly (Table 1). Recent studies have shown that Viola species extracts have neuroprotective, anti-inflammatory, and antioxidant properties. The polysaccharide complex of V. odorata (Sweet violet) has an anti-inflammatory activity that prevents inflammation progression and changes capillary permeability (Drozdova & Bubenchikov, 2005). Several studies have demonstrated that V. odorata extract contains antioxidant activity like phenolic compounds (Ebrahimzadeh, Nabavi, Nabavi, Bahramian, & Bekhradnia, 2010; Stojković, Glamočlija, Ćirić, Šiljegović, Nikolić, M., & Soković, 2011). Phenolic compounds could be the main cause of the antioxidant activity of Viola species (Muhammad & Saeed, 2011). One of the examples of Viola species antioxidant activity is presented in Vukics, Kery, Bonn, & Guttman (2008). Their study shows the antioxidant activity of different flavonoid fractions of V. tricolor. Jeon, Yoon, Park, Lee, & Park (2009) studied the neuroprotective effects of acetone extracts from V. mandshurica and showed that the V. mandshurica extract (VME) (100 and 250 µg/mL) was a dose-dependent inhibitor of H_2O_2 inducing DNA damage. On the other hand, their researchers have concluded that VME significantly suppresses apoptosis in the H_2O_2-stressed PC12 cells (Jeon et al., 2009). Therefore, the anti-inflammatory effect of VOE could be a consequence of the reduced infarct size. As a result, VOE could be a new candidate against neuronal diseases.

Our data also showed that VOE significantly decreased *NF-κB* and VCAM-1 expression in the penumbra, core, and subcortex areas (Figures 4-9). It has been widely acknowledged that *NF-κB* regulates the expression of several genes involved in pro-inflammatory (cytokines and chemokines), innate and adaptive immune functions, apoptosis, cell survival, and proliferation (Liu, et al., 2017). These genes are activated during reperfusion injury (Nichols, 2004). Modulation of *NF-κB* signal-

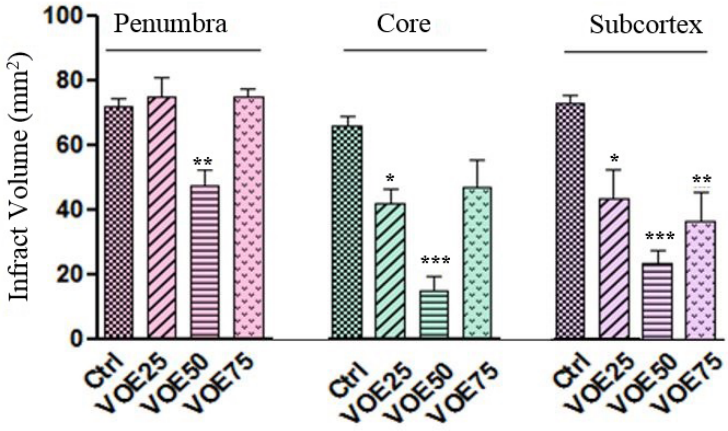

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Figure 3. Quantitative analysis of the infarct area in the penumbra, core, and subcortex areas from rats treated with VOE or vehicle

VOE25, 50, 75: Viola odorata extract in different concentration (25, 50 and 75 mg/kg bw); Ctrl: control group, (n= 6).

*P<0.05; **P<0.01; ***P<0.001.

ing has the potential to interrupt multiple inflammatory and apoptotic mechanisms by one specific molecular target (Ridder & Schwaninger, 2009). As reported by Schneider, Martin-Villalba, A., Weih, Vogel, Wirth, & Schwaninger (1999), *NF-κB* is activated and promotes cell death in focal cerebral ischemia, and DNA binding of NF-κB subunits RelA and p50 enhances in the ischemic hemisphere. On the other hand, they also showed that in p50 knockout mice, ischemic damage was significantly reduced(Schneider et al., 1999). Furthermore, the results offered by Stephenson et al. (2000) suggest that transient focal cerebral ischemia activates *NF-κB* in neurons. Clemens et al. (1997) also indicated that *NF-κB* has a role in programmed cell death in hippocampal CA1 neurons. Inhibition of *NF-κB* has also been reported to suppress inflammation following MCAO (Kunz et al., 2008). Zhang et al. (2005) also found that *NF-κB* activity is increased in neurons and astrocytes during cerebral ischemia and

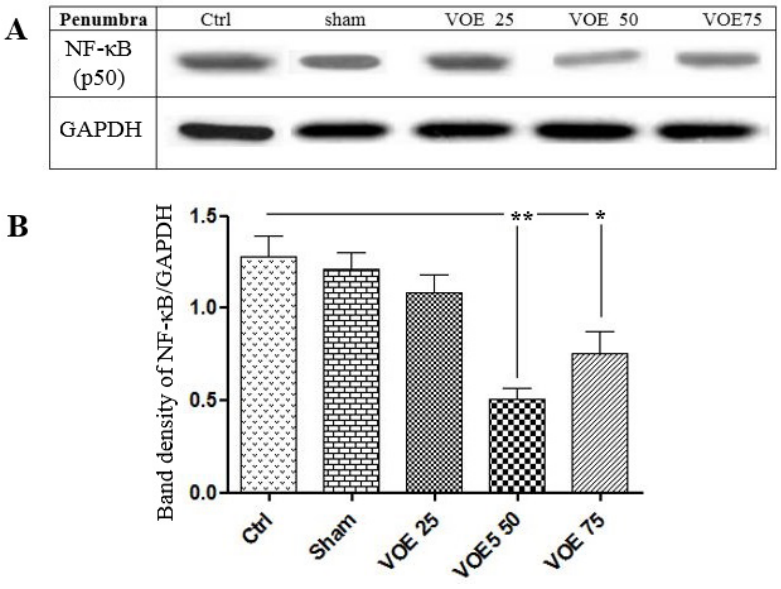

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Figure 4. Effects of Viola Odorata Extract (VOE) on *NF-κB* expression in penumbra area

Representative Western blot showing *NF-κB* and GAPDH in penumbra area; B) Quantitative analysis of the NF-κB level in rats treated with VOE or vehicle.

VOE25, 50, 75: Viola odorata extract in different concentration (25, 50 and 75 mg/kg bw); Ctrl: control group, (n= 6).

*P<0.05 and **P<0.01 compared to the control group.

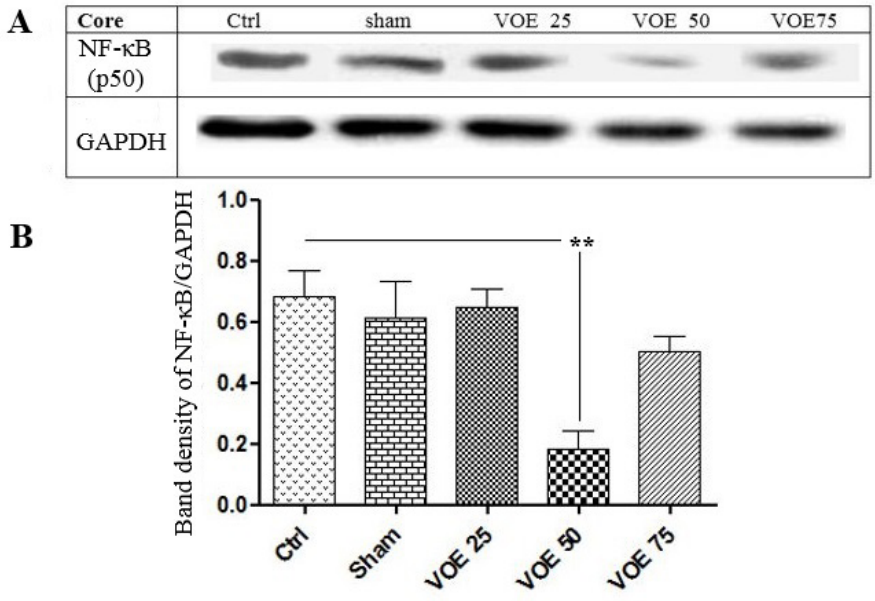

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Figure 5. Effects of Viola Odorata Extract (VOE) on NF-κB Expression in Core Area

Representative Western blot showing *NF-κB* and GAPDH in the core area; B) Quantitative analysis of the *NF-κB* level in rats treated with VOE or vehicle.

VOE25, 50, 75: Viola odorata extract in different concentration (25, 50 and 75 mg/kg bw); Ctrl: control group, (n= 6).

**P<0.01 compared to the control group.

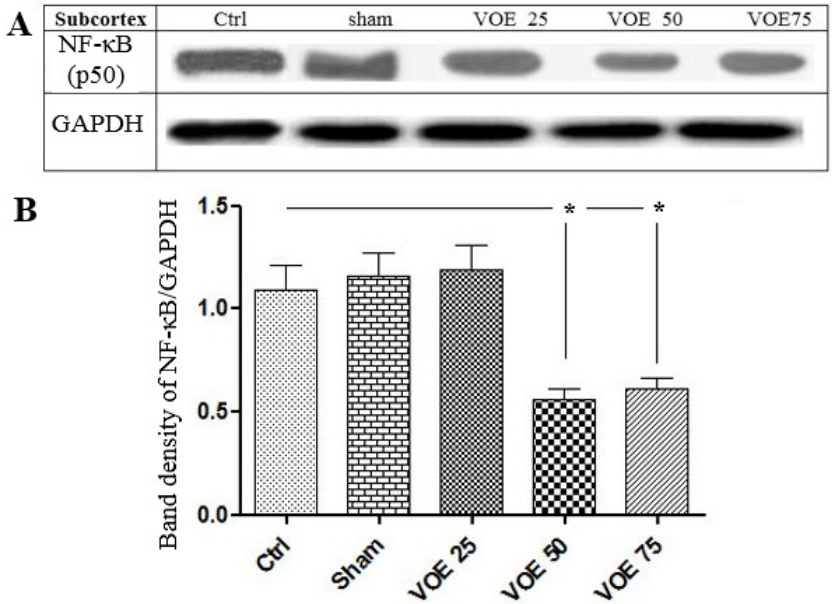

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Figure 6. Effects of Viola Odorata Extract (VOE) on *NF-κB* Expression in Sub-Cortex Area

Representative Western blot showing NF-κB and GAPDH in sub-cortex; B) Quantitative analysis of the NF-κB level in rats treated with VOE or vehicle. Rats treated with VOE or vehicle.

VOE25, 50, 75: Viola odorata extract in different concentration (25, 50 and 75 mg/kg bw); Ctrl: control group, (n=6).

*P<0.05 compared to the control group.

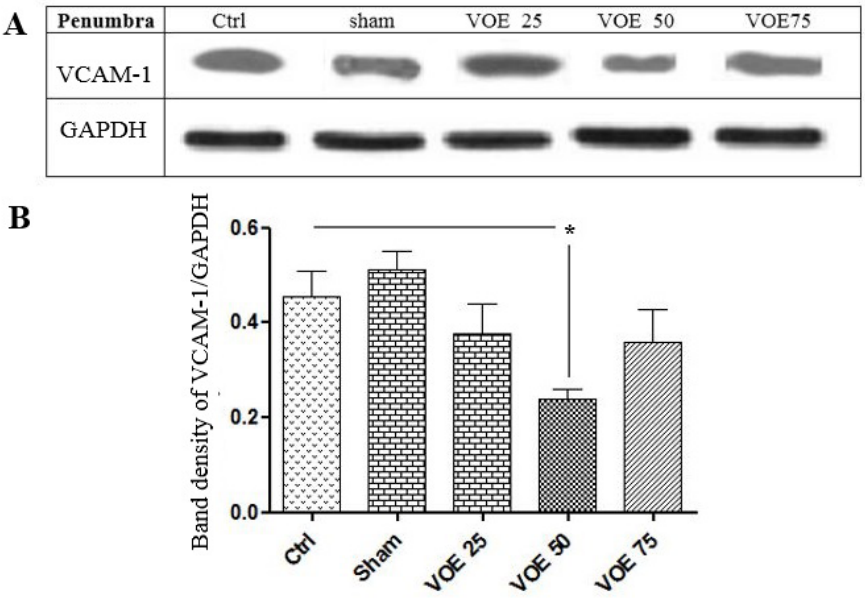

Figure 7. Effects of viola odorata extract (VOE) on VCAM-1 Expression in Penumbra Area

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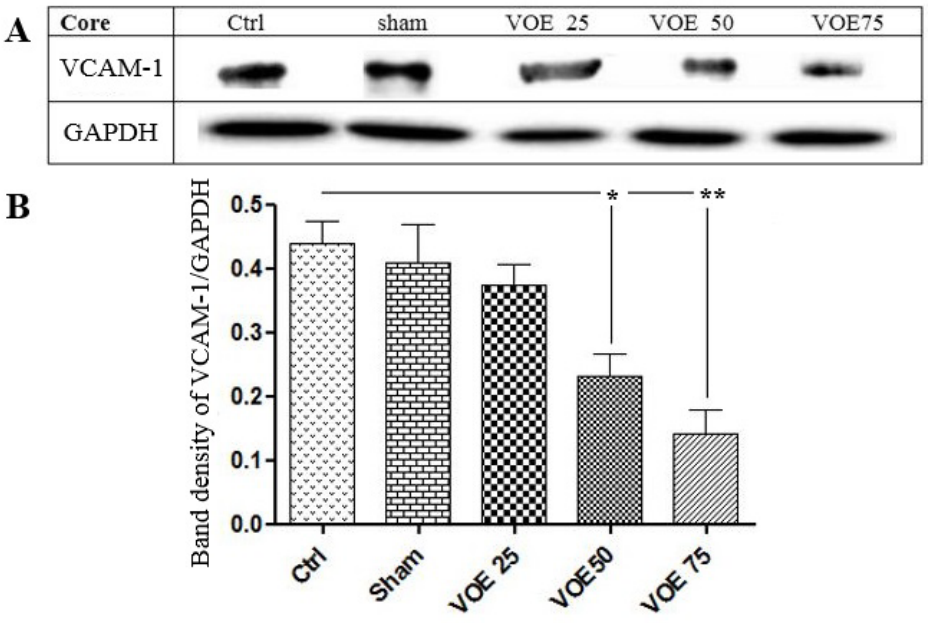
Representative Western blot showing VCAM-1 and GAPDH in penumbra; B) Quantitative analysis of the VCAM-1 level in rats treated with VOE or vehicle. Rats treated with VOE or vehicle.

VOE25, 50, 75: Viola odorata extract in different concentration (25, 50 and 75 mg/kg bw); Ctrl: control group, (n=6).

*P<0.05 compared to the control group.



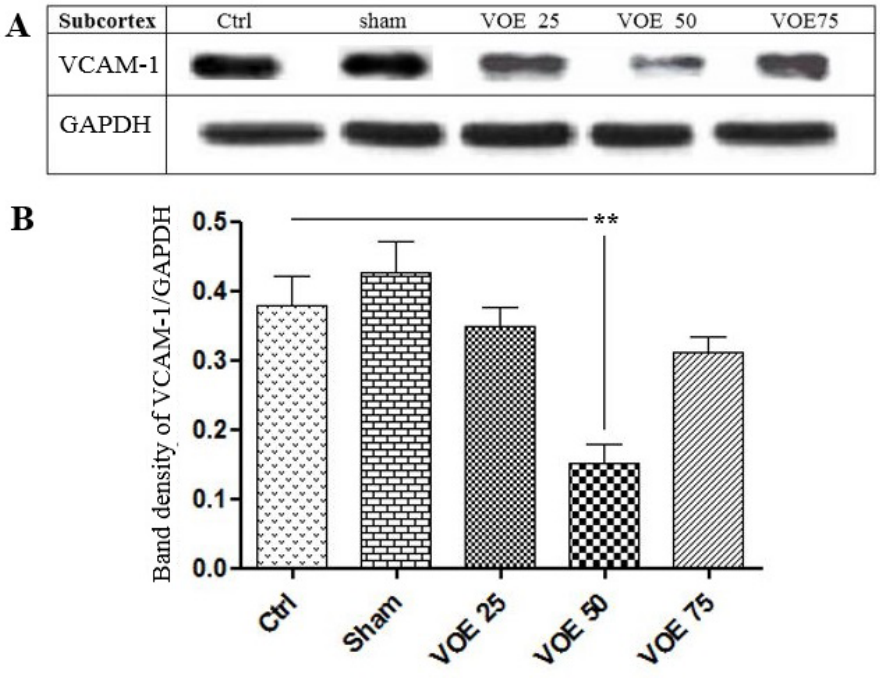
NEUROSCIENCE

Figure 8. Effects of viola odorata extract (VOE) on VCAM-1 Expression in Core Area

Representative Western blot showing VCAM-1 and GAPDH in sub-cortex; B) Quantitative analysis of the VCAM-1 level in rats treated with VOE or vehicle.

VOE25, 50, 75: Viola odorata extract in different concentration (25, 50 and 75 mg/kg bw); Ctrl: control group, (n=6).

*P<0.05; **P<0.01 compared to control group.



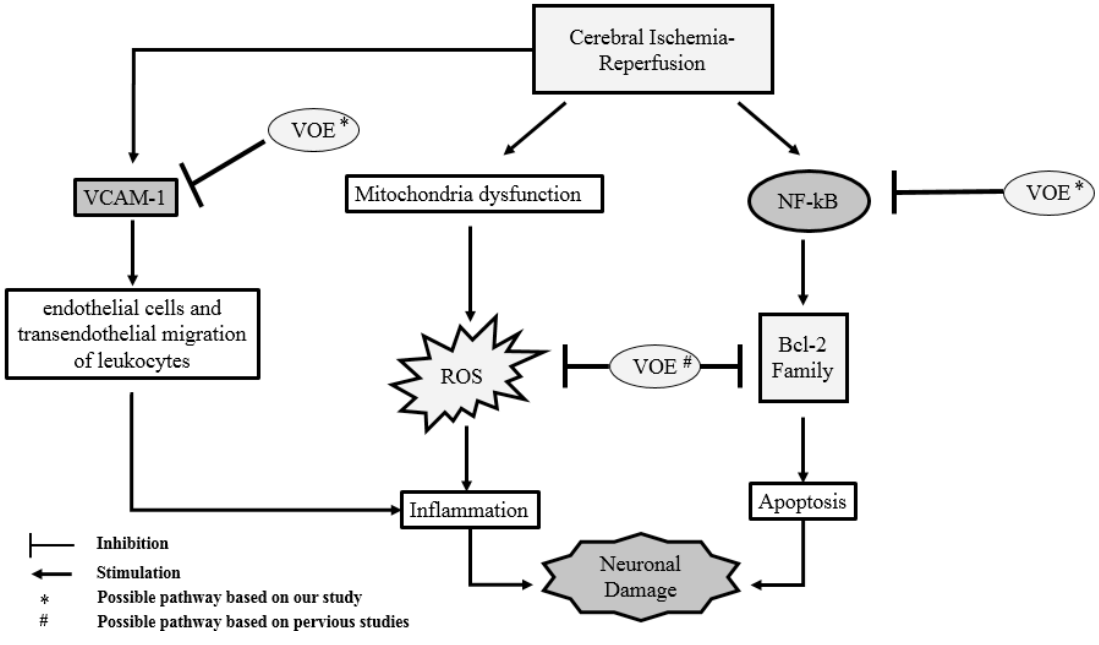
NEUROSCIENCE

Figure 9. Effects of viola odorata extract (VOE) on VCAM-1 Expression in Subcortex Area

Representative Western blot showing VCAM-1 and GAPDH in the core; B) Quantitative analysis of the VCAM-1 level in rats treated with VOE or vehicle. Rats treated with VOE or vehicle.

VOE25, 50, 75: *Viola odorata* extract in different concentration (25, 50 and 75 mg/kg bw); Ctrl: control group, (n= 6).

** P<0.01 compared to the control group.



NEUROSCIENCE

Figure 10. Possible Mechanism of VOE on Cerebral Ischemia

Based on a previous study (#), VOE could reduce neural damage by inhibiting ROS and Bcl-2 family. In addition, brain damage may be reduced by down-regulation of NF-κB and VCAM-1 expression (*).

VOE: *Viola odorata* extract; ROS: reactive oxygen species.

contributes to ischemic neuronal damage. Previous studies have shown that the expression of adhesion molecules such as ICAM-1 and VCAM-1 activate endothelial cells, inflammation, and progression of ischemic injury following stroke CNS migration of leukocytes. In the CNS, leukocytes facilitate cell death and increase the IV by producing several cytotoxic molecules (Danton & Dietrich, 2003). Frijns and Kapelle (2002) reported an increase in VCAM-1 after acute stroke (Frijns & Kappelle, 2002). The VCAM-1 and ICAM-1 expressions were evaluated in patients with acute ischemic stroke and the control group (Supanc, Biloglav, Kes, & Demarin, 2011). No significant difference was observed between VCAM-1 and ICAM-1 in the control group, but the level of VCAM-1 in thromboembolic stroke is significantly higher than in the control group (Supanc, etal., 2011). Zhou, Chen, & Wang (1997) also reported that VCAM expression is much higher in patients with large cerebral infarctions than in patients with medium and small cerebral infarctions. However, in knockout animals, the absence of CAMs reduces infarct size (Frijns & Kappelle, 2002). Both the sizes of the infarct and cell death decrease 48 hours after permanent MCAO by neuronal expression of the *NF-κB* inhibitor (Zhang et al., 2005).

The Reactive Oxygen Species (ROS) are involved in ischemia-induced neuron damage as well as the pathogenesis of many general neurodegenerative. Therefore, the use of antioxidants that suppress the effects of ROS is a promising approach to neuroprotection. Recently, there has been an increasing interest in the use of herbal antioxidants in preventing and treating ischemic and neurodegenerative cell damage. Oxidative stress is a key deleterious factor in neuronal cell damage during ischemia-reperfusion injury. In acute ischemia, the increased level of ROS can cause oxidative damage to cellular macromolecules, including lipids, proteins, and nucleic acids. Therefore, novel antioxidant agents might be a good therapeutic approach against neuronal damage during brain ischemia. In our previous study, the antioxidant capacity of hydroalcoholic extract of *V. odorata* (VOE) was confirmed by DPPH assay. Our results determined that the hydro alcoholic extract of V. odorata has more appropriate antioxidant activity than its water (IC50:140.7 µg/mL or 163.6 µg/mL) and methanol extract (IC50: 245.1 µg/mL) (Alipanah et al., 2018). In addition, the most oxidant and ROS in the biological system affect the *NF-κB* signaling pathway (Morgan & Liu, 2011). Activating *NF-κB* signaling is mediated by the upstream kinase inhibitor of kappa B kinase and triggered by hypoxia, ROS, and several inflammatory mediators (Ridder & Schwaninger, 2009). The importance of ROS on *NF-κB* activation is further supported by various investigations that demonstrated the inhibition of NF-κB activity by antioxidants, such as polyphenols and vitamin E (Ahmed, Donovan, Yujiao, & Zhang, 2015; Ridder & Schwaninger, 2009). Based on the previous studies, our researchers have concluded that VOE may be decreased the IV in the MCAO animal model by downregulating the *NF-κB* pathway. It also attenuates the inflammatory responses, as evidenced by a reduction in *NF-κB* and VCAM-1 expressions (Figure 10). It should be noted that its mechanism needs to be further investigated.

In general, in this study, the neuroprotective effects of VOE showed the highest effects in the intermediate dose (50 mg/Kg) (Figure 2). Moreover, in some cases, such as the effects on the infarct volume, no significant effects at the highest dose (75 mg/kg) were observed. VOE has an enhanced catalase activity, leading to cell protection against oxidative injuries. In our previous study, we showed that the maximum effect of VOE on decreasing superoxide dismutase activity was observed when the intermediate dose was used (50 mg/kg)(Alipanah et al., 2018). Probably, the effect of VOE on reducing the infarction area size is partly related to its antioxidant effect. Besides, different neuroprotective antioxidants with various structures and effects show a biphasic dose-response relationship; that is, they produce a lower level of neuroprotection with a higher dose compared to the lower dose (Castagné & Clarke, 2000; Farinelli, Greene, & Friedman, 1998; Green & Ashwood, 2005; Levin, Clark, & Johns, 1996). Hormesis is a term used to refer to a process by which an intermediate dose can generate the opposite effect of a higher or lower dose (Alabrese & Blain, 2005). Perhaps, VOE dose-dependence on neuroprotective effect follows this rule. High doses of antioxidant supplementation have no benefit and even can be potentially detrimental (Virtamo et al., 2003). Free radicals are normal components of second messenger signaling pathways and required for the normal function of the cell, although these and ROS are potentially harmful at levels that overwhelm antioxidant defenses (Valko et al., 2007). Consequently, the efficacy of free-radical antioxidants may be limited to their ability to restore the level of free radicals and oxidative stress within narrow homeostatic limits (Castagne, Lefevre, Natero, Becker, & Clarke, 1999).

5. Conclusion

In this study, we used in vivo techniques to investigate the protective effects of V. odorata alcoholic extract on the MCAO model. Based on this research, V. odorata alcoholic extract can reduce IV, ND, *NF-κB*, and VCAM-1 expressions in the MCAO model. Therefore,

V. odorata could be useful for developing a novel medical herb for treating cerebral ischemia. A better understanding of the role of VOE in the MCAO model will open new perspectives for discovering new therapeutics for cerebral ischemia.

Ethical Considerations

Compliance with ethical guidelines

All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Shahid Beheshti University, Iran.

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Authors' contributions

Conceptualization and Supervision: Mohammad Reza Bigdeli; Methodology: Kiana Karimifar and Hiva Alipanah; Investigation, Writing-original draft, and Writing -review: Mohammad Reza Bigdeli, Kiana Karimifar and Hiva Alipanah, Editing: Hiva Alipanah and Ava Soltani Hekmat; Data collection: Kiana Karimifar; Data analysis: Kiana Karimaifar, Hiva Alipanah ; Funding acquisition and Resources: Mohammad Reza Bigdeli; Approving the final version for publication: All authors

Conflict of interest

The authors declared no conflict of interest.

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