Title: Attenuating of NF-Kb/VCAM-1 Expression in Middle Cerebral Artery Occlusion (MCAO) Model by Viola Odorata: Protection Against Injury Ischemia- Reperfusion Injury in Rats

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To appear in: Basic and Clinical Neuroscience

Received date: 2019/09/23
Revised date: 2020/05/26
Accepted date: 2020/08/25

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Abstract

Background: The death of neurons and cerebral edema are the main consequences of stroke. However, inflammatory processes play a key role in aggravating cerebral damage following stroke. The aim of this study was to investigate the effects of Viola odorant extract (VOE) on infarct volume (IV), neurologic deficits (ND), and expression of NF-κB and VCAM-1 in the MCAO model.

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

Results: V. odorata extract (VOE) exhibited excellent neuroprotective effects by reducing infarct volume (mainly in the core and sub-cortex areas), and induced down-regulation of NF-κB and VCAM-1 expression.

Conclusion: This finding suggests that V. odorata could also activate intracellular pathways, which ultimately reduces the expression of NF-κB and VCAM-1 and be useful for developing a novel medical herb for treating cerebral ischemia.

Keywords: Cerebral Infarction, Inflammation, NF-kappa B, VCAM-1, Viola
Highlights

- A stroke occurs when the blood supply to part of your brain is interrupted or reduced.
- VOE reduced the infarct volumes (IV) in mice brains 24 h after MCAO.
- VOE may decrease IV in MCAO model by down-regulation on 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 interruption of blood flow, energy stores become rapidly depleted and excitotoxicity cell death induced by cellular complex cascades. Unfortunately, despite the efforts made, there is still no effective and specific treatment for the stroke. In this study, screening of the neuroprotective effects of the VOE was carried out. The V. odorata extract exhibited excellent neuroprotective effects by reducing of infarct volume and induced downregulation of NF-kB and VCAM-1 expression. This finding suggests that V. odorata may be useful for developing a novel medical herb for treatment cerebral ischemia.
1. Introduction

Stroke is an acute and progressive neurodegenerative disorder, and central nervous system (CNS) injuries of vascular origin that interrupts blood flow of 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 play a key role in exacerbating stroke-related brain damage (Panickar, 2015). A variety of mechanisms are responsible for ischemic brain damage. After interruption of blood flow, energy stores become rapidly depleted and excitotoxicity cell death 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 indicated that tumor necrosis factor (TNF) as cytokine has both cytotoxic and cytoprotective activities (Botchkina, Geimonen, Bilof, Villarreal, & Tracey, 1999). The development of TNF cytotoxicity activity is dependent on the balance between the activities of intracellular signaling pathways that mediate either apoptotic or anti-apoptotic effects (Botchkina et al., 1999). TNF as a pro-inflammatory cytokine, targets endothelial cell by binding to its surface receptor (TNF-R1) and stimulates intracellular cascades (Wojdasiewicz, Poniatowski, & Szukiewicz, 2014). Activated signaling pathway increases expression of NF-κB and cell adhesion molecules (VCAM-1 and ICAM-1) (Lin et al., 2015). Finally cell adhesion molecules stimulate leukocytes migration from peripheral circulation to brain tissue and reinforce inflammatory signaling cascade (Wang, Tang, & Yenari, 2007). Inflammatory changes in neurons could result in blood-brain barrier breakdown and edema, and in advance stage induce cell death. Therefore, neural inflammatory pathways can be targets for the 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 the complication of major pathologic conditions 2) multifactorial effect of herbal medicine (Gaire, 2018). *Viola odorata* is a member of Violaceae family and 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 have shown some medical features of *V. odorata* including antioxidant, anti-inflammatory and anti-cancer activates (Barekat, Otroshy, Samsam-Zadeh, Sadrahami, & Mokhtari, 2013; Hartwell, 1967; Kapoor, 2000; Mittal, Gupta, Goswami, Thakur, & Bansal).

Unfortunately, despite the efforts made, there is still no effective and specific treatment for the stroke, and in developing societies, the rate of cerebral ischemia is increasing significantly (Hackett, Duncan, Anderson, Broad, & Bonita, 2000). Several studies have shown that the anti-inflammatory effects of different species of Viola (Drozdova & Bubenchikov, 2005; Muhammad, Saeed, & Khan, 2012). Mousavi et al. 2016 showed that *V. tricolor* and *V. odorata* protect neuronal cells against serum and glucose deprivation-induced cell death, at least in part, by their antioxidant activities (Mousavi, Naghizade, Pourgonabadi, & Ghorbani, 2016). Our pervious study showed antioxidant activity of *V. odorata* hydro-alcoholic extract and presence 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. In other hands we have shown that VOE maybe change the expression of NF-kB, VCAM-1 and TNF-R1 in breast cancer model (Alipanah et al., 2018; Alipanah, Bigdeli, Esmaeili, & Akbari, 2017), on this basis, we selected *V. odorata* as independent variable in our
investigation. Therefore, the aims of this study were to determine: 1) whether *V. odorata* extract would effectively reduce infarct volume (IV) and neurologic deficit in MCAO model, 2) whether there would be changes in expression of NF-kB and VCAM-1 determined by western blot technique in Rat treated with *V. odorata* hydro-alcoholic extract (VOE).

2. Material and Methods

2.1. Animal

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 9:00 a.m.) and food and water were available *ad libitum*. All animal experiments were performed in accordance with a protocol approved by institutional animal care and use committee in Shahid Beheshti University (Iran).

2.2. *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 hydro-alcoholic (50:50) extract.

2.3. Experimental protocol

Animals were randomly divided into MCAO groups (4 subgroup, n=6) and Western groups (5 subgroup, n=6) and received different doses (25, 50 and 75 mg/kg) of *Viola odorata* hydro-
alcoholic extract. The control group (sham operated animals) underwent the MCAO surgical procedure, except the silicone coated nylon was not inserted into the common carotid artery. The animals in the vehicle-treated group were almost same with the control group except received distilled water by gastric gavage. VOE treated groups, animals went through the MCAO surgical procedure, and VOE were administrated by gastric gavage at a dose of 25, 50 and 75 mg/kg, respectively. VOE or distilled water were administered daily for 30 days. Six rats from each group were sacrificed for determination of NF-kB and VCAM-1 expression rate by western blotting technique. The infarction volume (IV) and neurological deficits scores (NDS) in MCAO groups were assessed. Two hours after the last gavage, rats were exposed to 60 min MCAO surgery. Twenty-four hours later, the IV and NDS were evaluated.

2.4. 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, incision was created along the mid-line in the neck, with a length of 2 cm, then common carotid artery (CCA) was isolated from the vagus nerve, carefully. Two spaced permanent knots were placed at the distal part of external carotid artery (ECA) and CCA. The internal cerebral artery (ICA) blood flow was blocked by a microvascular clip and the 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). after 60 minutes of reperfusion, the incision of the midline neck was sewed using a surgical suture(Longa, Weinstein, Carlson, & Cummins, 1989). The body temperature of the animal was maintained at 37 ± 2°C during the occlusion.
2.5. Neurobehavioral evaluation: Neurological deficit score (NDS)

Neurological scores were assessed 24h after the reperfusion using a modification of scoring reported by Long et al., 2013 (minimum score=0, maximum score=18)(Long et al., 2013).

A: Raise the tail, about 1m 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 10° 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)
   - Fall down to the hemiplegic side and could not walk (3 score)

C: Sensory function
   - Algesia and heat sensory (1 score)
   - Propnoception (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 falling down from the beam (2 score)
   - Two limbs falling 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)
2.6. 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 quickly were removed and washed with cold saline (4 °C) for 5 minutes. Then they were placed in the brain matrix and the coronal sections of frontal lobe was cut (2mm thick). The brain sections were immediately immersed in 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Merck, Germany) and incubated in water bath (37 °C) for 15 min. The infarct area was white while viable tissue was red or pink. Then the sections were placed on graph paper and photographed with a digital camera (Nikon, D40x digital). Calculation of the infarct volume was measured by Image J software (version 1.46r) then multiplied by slice thickness (2mm). Eight slices of stained brain tissue were calculated with the method described by Swanson et al.

\[
CIV = LHV - (RHV - IAV)
\]


2.7. Western blot analysis

Animals were deeply anesthetized with chloral hydrate (800 mg/kg, i.p) and transcranial perfused with 0.9% normal saline. In each hemisphere the penumbra, subcortex, and core of the brain tissue were removed according to previous methods (Lei, Popp, Capuano-Waters, Cottrell, & Kass, 2004). Tissues were placed in round-bottom microcentrifuge tubes and ice-cold lysis buffer (0.25gr sodium deoxycholate, 0.08gr NaCl, 0.01gr SDS, 0.003gr EDTA, 10μL 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 ultrasound homogenizer (4000 r.p.m). The homogenate was centrifuged in a microcentrifuge for 20 min at 12,000 rpm at 4°C. 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 protein quantification assay. Samples were boiled in sample buffer at 100°C for 5 min and 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). Membrane was blocked at room temperature for 1 h using blocking buffer and subsequently incubated with specific primary polyclonal rabbit antibody to NF-κB (1:500 dilution; Santa Cruz), goat anti-VCAM-1 polyclonal antibodies (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. The membrane was then incubated with secondary antibodies including goat anti-rabbit and rabbit anti-goat (1:500 dilution; Santa Cruz) separately. For the second time the membrane was washed in three washes of TBST, 5 min each. Darkroom development technique for chemiluminescence was used to detect NF-κB and VCAM-1 immuno-reactive proteins. Signal bands were quantified using ImageJ densitometry software, and expressed relative to GAPDH signal (loading control).

2.8. Statistical analysis

Non-parametric Kruskal-Wallis test was used to analyze the neurological deficits score. Infarct volume and proteins expression in experimental groups were compared with control group using one-way ANOVA analysis of variance (SPSS 22.0 post hoc LSD). Data were expressed as mean ± sd. P<0.05 was considered significant.
3. Results

3.1. The effect of Viola odorata on neurological deficits scores

As shown in table 1, pre-feeding of Viola odorata (VOE50) significantly reduced neurological deficit ** (p < 0.01) in comparison with the control group.

3.2. Effect of Viola odorata on the volume of tissue damage (infarct volume)

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

Location Figure 2

The effect of hydro-alcoholic extract of Viola odorata on the infarct volume (IV) in three areas of Penumbra (P), Core (C) and sub-cortex (SC) was assessed (Figure 3). Infarct volume was reduced in P area ** (P<0.01) in dose of 50 mg/kg of VOE compared with the control group. VOE50 *** (P<0.001) and VOE25 * (P<0.05) reduced infarct volume in C area, significantly. Infarct volume of sub-cortex was significantly decreased (P<0.001) in rats that were treated with VOE25, VOE50 and VOE75 compared with the control group (Figure 3).

Location Figure 3

3.3. Effects of VOE on NF-kB expression in penumbra area
The assessment of NF-kB protein by western blotting technique demonstrated that VOE in doses of 50 and 75 mg/kg decreased NF-kB expression in P area compared with the control group (Figure 4A-B). VOE25 did not a significant effect on NF-kB expression in P area. Besides, there is no significant difference between the sham and control groups (Figure 4).

Location Figure 4

3.4. Effects of VOE on NF-kB expression in core area

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

Location Figure 5

3.5. Effects of VOE on NF-kB expression in sub-cortex area

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

Location Figure 6
3.6. 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 with the control group in P area *(P<0.05)*, while there was no significant difference between VOE25 and VOE75 with the control group. There was also no significant difference between the sham and control groups.

Location Figure 7

3.7. Effects of VOE on VCAM-1 expression in core area

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

Location Figure 8

3.8. Effects of VOE on VCAM-1 expression in sub-cortex area

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

Location Figure 9
4. Discussion

In the present study, screening of the neuroprotective effects of the hydro-alcoholic extract of the *V. odorata* was carried out. The *V. odorata* extract exhibited excellent neuroprotective effects by reducing of infarct volume (mainly in core and sub-cortex areas) (Figure 3), and induced downregulation of NF-kB and VCAM-1 expression.

Increasing attention has been drawn to inflammatory role in ischemic stroke. The intensity of the inflammatory reaction is correlated with the extent of the ischemic lesion, so that small infarcts have less inflammation than large infarcts (Akopov, Simonian, & Grigorian, 1996). Our results showed that VOE pretreatment was found to reduce the infarct volumes in mice brains 24 h after MCAO (Figures 2 & 3) and significantly improve the neurological behavioral deficits (Table, 1). Recent studies have shown that Viola species extracts have neuroprotective, anti-inflammatory and anti-oxidant properties. As reported by Drozdova *et al*. 2005, polysaccharide complex of sweet violet has an anti-inflammatory activity, and prevents the inflammation progression and changes capillary permeability (Drozdova & Bubenchikov, 2005). Several studies have experimentally been demonstrated that Viola odorata extract contains the antioxidant activity such as phenolic compounds (Ebrahimzadeh, Nabavi, Nabavi, Bahramian, & Bekhradnia, 2010; Stojković et al., 2011). In these studies and related references, it was observed that phenolic compounds could be the main cause of antioxidant activity of viola species (Muhammad & Saeed, 2011). One of the examples of Viola species antioxidant activity is presented in Vukics *et al.*, 2008, their study shows the antioxidant activity of different flavonoid fractions of *Viola tricolor* (Vukics, Kery, Bonn, & Guttmann, 2008). Joen *et al*. 2009, studied the neuroprotective effects of acetone extracts from *Viola mandshurica* (VME) and showed that VME (100 and 250 μg/mL) was a dose-dependent inhibitor of H2O2 induced DNA damage (Jeon, Yoon, Park, Lee, & Park, 2009). On the other
hand, their researchers have arrived at the conclusion that VME had greatly suppresses apoptosis in the H2O2-stressed PC12 cells (Jeon et al., 2009). Therefore, the anti-inflammatory effect of VOE could be a consequence of the reduced infarct size. The results suggest that VOE could be a new candidate against neuronal diseases.

Our date also showed that VOE significantly decreased NF-κB and VCAM-1 expression in penumbra, core and sub-cortex 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, Zhang, Joo, & Sun, 2017). Many of these same genes are activated during reperfusion injury (Nichols, 2004). Modulation of NF-κB signaling has the potential to interrupt multiple inflammatory and apoptotic mechanisms by one specific molecular target (Ridder & Schwaninger, 2009). As reported by Schneider et al, 1999, NF-κB is activated and promotes cell death in focal cerebral ischemia and DNA binding of NF-κB subunits RelA and p50 enhance in the ischemic hemisphere (Schneider et al., 1999). On the other hand, Schneider et al. 1999, also showed that in p50 knockout mice, ischemic damage was significantly reduced (Schneider et al., 1999). Furthermore, the results offered by Stephenson et al, in 2000 suggest that transient focal cerebral ischemia results in activation of NF-κB in neurons (Stephenson et al., 2000). Clemens et al., 1997 also indicated that NF-κB has a role in programmed cell death in hippocampal CA1 neurons (Clemens et al., 1997). Inhibition of NF-kB has also been reported to suppress inflammation following MCAO (Kunz et al., 2008). Zhang et al 2005, has also found that NF-kB activity is increased in neurons and astrocytes during cerebral ischemia and contributes to the neuronal ischemic damage (Zhang et al., 2005). Previous studies have also shown that the expression of adhesion molecules such as ICAM-1 and VCAM-1 activate endothelial cells,
inflammation, progression of ischemic injury following stroke CNS migration of leukocytes. In the CNS, leukocytes facilitate cell death and increase the infarct volume by producing a number of cytotoxic molecules (Danton & Dietrich, 2003). Frijns and Kapelle, 2002 reported an increase in VCAM-1 after acute stroke (Frijns & Kappelle, 2002). The evaluation of VCAM-1 and ICAM-1 expression in patients with acute ischemic stroke and control group was also studied by Supanc et al, 2011. They have shown that there is no significant difference between VCAM-1 and ICAM-1 in the control group, but the level of VCAM-1 in thromboembolic stroke is significantly higher than control group (Supanc, Biloglav, Kes, & Demarin, 2011). Zhou et al, 1997 also showed that VCAM expression is much higher in patients with large cerebral infarctions than in patients with medium and small cerebral infarctions (Zhou, Chen, & Wang, 1997). On the other hand, it has been shown that in knockout animals absence of CAMs reduced infarct size (Frijns & Kappelle, 2002). As reported by Zhang et al 2005, both the size of the infarct and cell death decrease 48 hours after permanent MCAO by neuronal expression of the NF-kB inhibitor (Zhang et al., 2005).

It has been well known that reactive oxygen species (ROS) are involved in ischemia-induced neuronal cell damage as well as pathomechanism of many general neurodegenerative. Therefore the use of antioxidants, which suppress the effects of ROS, is a promising approach to neuroprotection. Recently, there has been an increasing interest toward the use of herbal antioxidants in the prevention and treatment of 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, utilization of novel antioxidant agents might be a good therapeutic approach against neuronal damage during brain ischemia. In our previuos 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/mLl)(Alipanah et al., 2018). In addition, the most oxidant and reactive oxygen species (ROS) in the biological system have an effect on the NF-kB signaling pathway (Morgan & Liu, 2011). Activation of NF-kappaB signaling is mediated by the upstream kinase inhibitor of kappaB kinase and is triggered by hypoxia, reactive oxygen species, and several inflammatory mediators(Ridder & Schwaninger, 2009). The importance of ROS on NF-κB activation is further supported by various investigations that have demonstrated 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 arrived at the conclusion that VOE may be decreased infarct volume in MCAO animal model by down-regulation on the NF-κB pathway and also attenuates inflammatory responses as evidenced by a reduction in NF-κB and VCAM-1 expression (Figure 10). It should be noted that its mechanism need to be further investigated.

Location Figure 10

In general, in this study the neuroprotective effects of VOE showed the highest effects in the intermediate dose (50mg/Kg) (Figure 2). Moreover, in some cases, such as effects on the infarct volume no significant effects at the highest dose (75 mg/kg) were observed. Apparently, VOE has an enhanced effect on the catalase (CAT) activity, which leads to cell protection against oxidative injuries. In our previous study we showed that the maximum effect of VOE on decreasing superoxide dismutase (SOD) activity observed when the intermediate dose was used (50mg/kg)(Alipanah et al., 2018). It is possible that the effect of VOE on reduction of the infarction area size partly related to its antioxidant effect. Besides, different neuroprotective antioxidants
with various structures and effects show biphasic dose-response relationship i.e. produce a lower level of neuroprotection with higher dose comparing 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 (Calabrese & Blain, 2005). Perhaps VOE dose depending neuroprotective effect follows this rule. High doses of antioxidant supplementation associated with 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 normal function of cell. Although these and reactive oxygen species 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).

**Conclusion**

In this study, we used *in vivo* techniques to investigate protective effects of *V. odorata* alcoholic extract on MCAO model. From the research that has been carried out, it is possible to conclude that *V. odorata* alcoholic extract can reduce infarct volume, neurological defects, NF-κB and VCAM-1 expression in MCAO model. This finding suggests that *V. odorata* could be useful for developing a novel medical herb for treatment cerebral ischemia. Better understanding of the role of VOE in MCAO model will open new windows for the discovery of new therapeutics for cerebral ischemia.
Authors' contributions

Study concept, design, student mentorship: Mohammad Reza Bigdeli; Acquisition of animal data: Kiana Karimifar and Hiva Alipanah; Data analysis, interpretation of the findings: Kiana Karimifar and Ava Soltani Hekmat; and Reviewing the manuscript and approving the final version for publication: All authors.

Ethical Considerations

All animal experiments were performed in accordance with a protocol approved by the institutional animal care and use committee in Shahid Beheshti University (Iran).

Declaration of interest

The authors report no conflicts of interest.

Acknowledgment

This study was supported by a grant from Shahid Beheshti University. The authors would like to express theirs thanks to Dr Sepideh Khaksar at the Nerves and Heart Physiology Lab of the Shahid Beheshti University.
References


Table 1:

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** (p < 0.01)
Figure 1

A

MCA
PCOM
PPA
ECA
CCA

B

Contra-A
Contra-B
Penumbra (B)
Ischemic core (A)
Edema

Healthy Contralateral Hemisphere
Figure 4

<table>
<thead>
<tr>
<th>Penumbra</th>
<th>Ctrl</th>
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B

Band density of NF-κB/GAPDH

- Ctrl
- Sham
- VOE 25
- VOE 50
- VOE 75

** p < 0.01
* p < 0.05
Figure 5

A

<table>
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B

Band density of NF-κB/GAPDH

Ctrl | Sham | VOE 25 | VOE 50 | VOE 75

**
Figure 6

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<tr>
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<th>Ctrl</th>
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**Band density of NF-κB/GAPDH**

- Ctrl
- Sham
- VOE 25
- VOE 50
- VOE75

*Significant difference.*
Figure 7

A

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B

Band density of VCAM-1/GAPDH

- Ctrl
- Sham
- VOE 25
- VOE 50
- VOE 75
Figure 8

A

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B

Band density of VCAM-1/GAPDH

Ctrl  Sham  VOE 25  VOE 50  VOE 75

*  **
Figure 9

(A) A table showing the expression levels of VCAM-1 and GAPDH in different subcortical conditions. The table includes columns for Ctrl, sham, VOE 25, VOE 50, and VOE 75.

(B) A bar graph comparing the band density of VCAM-1/GAPDH in Ctrl, Sham, VOE 25, VOE 50, and VOE 75 conditions. The graph includes error bars to indicate variability.
Figure 10
Table 1: neurological deficits scores. comparison of neurological deficits in experimental groups treated with different doses of viola odorata extract (VOE). As shown in table 1, 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 b.w), Ctrl: control group, (n=6).

Figure 1: A) Experimental design using the 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: The effect of hydro-alcoholic extract of Viola odorata on total infarct volume (IV). A) Representative photographs of TTC-stained brain slices (2 mm) 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 b.w), Ctrl: control group, (n=6). *** (P<0.001). White color indicates ischemic tissue and red or pink areas are healthy sites.

Figure 3: Quantitative analysis of the infarct area in 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 b.w), Ctrl: control group, (n=6). * p<0.05 and ** P<0.01 compare to control group.

Figure 4: Effects of VOE on NF-kB expression in penumbra area. A) Representative Western blot showing NF-kB and GAPDH in penumbra area. B) Quantitative analysis of the NF-kB level in rats treated with VOE or vehicle. VOE25, 50, 75: viola odorata extract in different concentration (25, 50 and 75 mg/kg b.w), Ctrl: control group, (n=6). * p<0.05 and ** P<0.01 compare to control group.

Figure 5: Effects of VOE on NF-kB expression in core area. A) Representative Western blot showing NF-kB and GAPDH in core area. B) Quantitative analysis of the NF-kB level in rats treated with VOE or vehicle. VOE25, 50, 75: viola odorata extract in different concentration (25, 50 and 75 mg/kg b.w), Ctrl: control group, (n=6). ** P<0.01 compare to control group.

Figure 6: Effects of VOE on NF-kB expression in sub-cortex area. A) Representative Western blot showing NF-kB and GAPDH in sub-cortex. B) Quantitative analysis of the NF-kB 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 b.w), Ctrl: control group, (n=6). * p<0.05 compare to control group.

Figure 7: Effects of VOE on VCAM-1 expression in penumbra area. A) 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. VOE25, 50, 75: Viola odorata extract in different concentration (25, 50 and 75 mg/kg b.w), Ctrl: control group, (n=6). * p<0.05 compare to control group.

Figure 8: Effects of VOE on VCAM-1 expression in core area. A) 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 b.w), Ctrl: control group, (n=6). * p<0.05, ** P<0.01 compare to control group.

Figure 9: Effects of VOE on VCAM-1 expression in subcortex area. A) Representative Western blot showing VCAM-1 and GAPDH in core. 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 b.w), Ctrl: control group, (n=6). ** P<0.01 compare to control group.

Figure 10: Possible mechanism of VOE on cerebral ischemia. Based on pervious study (#), VOE could reduce neural damage by inhibition of ROS and Bcl-2 family. In addition, brain damage may be reduced by down-regulation of NF-kB and VCAM-1 expression (*). VOE: Viola odorata extract, ROS: Reactive oxygen species.