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Original Article

Evaluating the impact of Viola spathulata in a rat model of brain ischemia/reperfusion by influencing expression level of caspase-3 and cyclooxygenase-2

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ABSTRACT

Introduction: Focal cerebral ischemia followed by reperfusion causes ischemia/reperfusion (I/R) brain injury. I/R injury is a complex pathophysiological process involving inflammation and apoptosis in neurons. Previous studies reported the anti-inflammatory traits of the family *Violaceae*. We aimed to evaluate the effects of *Viola spathulata* pre-treatment on infarct volume (IV), neurological deficit score (NDS) and alterations in mRNA level of cyclooxygenase 2 (COX2) and caspase 3 (CASP3) in a rat middle cerebral artery occlusion (MCAO) model.

Methods: Thirty-three male Wistar rats were randomly distributed in 4 groups: normal control, MCAO control, MCAO + 5 mg/kg *V. spathulata* and MCAO + 10 mg/kg *V. spathulata*. Two doses of *V. spathulata* extracts were injected intraperitoneally for 7 days before the onset of ischemia. Finally, IV, NDS and mRNA expression of CASP3 and COX2 genes were assessed 24h after reperfusion.

Results: IV and NDS in MCAO rats were remarkably higher compared with normal control rats and pre-treatment with *V. spathulata* extracts markedly reduced IV and NDS in the core, penumbra and subcortical regions of MCAO rats. Also, the level of COX2 and CASP3 mRNA was higher in the MCAO control group relative to normal control. Pre-treatment with *V. spathulata* extracts markedly reduced CASP3 mRNA relative to MCAO rats.

Conclusion: It was found that *V. spathulata* might reduce ischemic damage in the brain of MCAO rats partly by decreasing apoptotic effects of CASP3. Further research is recommended to investigate signaling pathways involved in apoptosis.

Keywords:
Ischemia
Reperfusion
Viola spathulata
Caspase 3 (CASP3)
Cyclooxygenase 2 (COX2)

Introduction

Ischemic stroke occurs when the blood flow is insufficient or blocked which causes injury to particular parts of the brain. Reperfusion of the blood supply enhances the damage in ischemic tissue (Kuroda and Siesjö, 1997). Stroke is the second cause of mortality and a

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seriously disabling disease worldwide which imposes substantial economic costs to the society for post-stroke care. Although mortality associated with stroke has been declined, the overall burden is still high, therefore there is a need for more appropriate and efficient procedures (Johnson et al., 2019).

Brain ischemia/reperfusion (I/R) is a complicated progressive injury including different signaling pathways such as apoptosis and inflammation (Petrovic-Djergovic et al., 2016; Sanderson et al., 2013). It has been documented that inflammation has an important role in the late phases of cerebral ischemic injury. Anti-inflammatory agents are considered to have neuroprotective effects. Studies show that approaches targeting the enzyme's activity involved in inflammation, particularly cyclooxygenase 2 (COX2), are beneficial to the ischemic brain (Iadecola and Alexander, 2001).

COX2 as a pro-inflammatory factor is among potential biomarkers in ischemic stroke and its up-regulation along with inflammatory cytokines causes both necrotic and apoptotic cell death in I/R injury (Zhu et al., 2012). However, complexity in neuroinflammatory molecules involved in different stages of stroke, makes it difficult to find a specific target to attenuate the neuroinflammation and prevent the development of I/R injury (Gu et al., 2014). Both ischemia and reperfusion contribute to an increase in caspase 3 (CASP3) activity (Haylor et al., 2011). It is reported that apoptosis might start reperfusion-induced inflammation, consequently tissue injuries (Daemen et al., 1999). So inflammation and apoptosis seem to have a reciprocal relationship in I/R injury.

There is an urgent need to identify pharmacological strategies that limit the secondary damage following a stroke. Numerous studies have examined different strategies to reduce neuronal cell death after cerebral ischemia. Early thrombolytic therapy using tissue-type plasminogen activator is the only FDA-approved approach to treat ischemic stroke (Bansal et al., 2013).

Accessibility, lower price and limited side effects of herbal antioxidants make them an interesting topic in researches focusing on ischemic and neurodegenerative cell damage. *Viola* is the largest genus of *Violaceae* consisted of around 500 species in which *Viola spathulata* is the only species endemic to the north of Iran. Previous studies showed that members of the family *Violaceae* have anti-inflammatory (Muhammad et al., 2012), antioxidant and neuroprotective activity (Moliner et al., 2019). Studies on *Viola* demonstrated this genus as a rich source of natural compounds including antioxidant flavonoids, terpenoids, phenylpropanoids, amides, sterols, cyclotides and other derivatives which its biological activities are attributed to them (Svangård et al., 2004; Vukics et al., 2008; Zhu et al., 2015).

Furthermore, high contents of melatonin have been reported in some medicinal plants including *Viola* plants (Kim et al., 2011). It has been reported that melatonin has neuroprotective and antioxidant effects and prevents I/R damages (Pasbakhsh et al., 2008). It has been shown that the hydroalcoholic solution can extract more melatonin from *Viola* plants (Ansari et al., 2010).

The present study aimed to evaluate the protective effects of *V. spathulata* extract after I/R brain injury and elucidate possible molecular mechanisms by which *Viola* extract may increase tissue regeneration and prevent further brain cell damage.

Materials and methods

The extraction of Viola

Aerial parts of *V. spathulata* were collected from Gadouk neck, Firoozkouh road, Savadkouh city (Mazandaran province) in May 2017. 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 48h. The hydroalcoholic extracts were then concentrated on a water bath and kept at -20ºC until use. Finally, the extract was dissolved in dimethyl sulfoxide (DMSO) to be used in this study. All stages were conducted at the Research Institute of Medicinal Plants of Shahid Beheshti University.

Animals

A total of 33 adult male Wistar rats (weighing 250–300g) were obtained from the Pasteur Institute (Tehran, Iran). The animals were placed at a standard condition: room temperature 22–24°C with 55% humidity under a 12h light/dark cycle, with free access to food and water.

Ethical statement

All authors hereby announce that principles of laboratory animal care (NIH publication NO. 85-23, revised 1985) were observed, as well as specific national laws

where applicable. All experiments have been tested and agreed upon by the appropriate ethics committee. All the experiments and procedures in this study were authorized by the Animal Ethical Committee of Guilan University of Medical Sciences (IR.GUMS.REC.1394.323), and all efforts were made to diminish suffering.

V. spathulata pre-treatment and model development

Intraperitoneal (IP) injection of the *V. spathulata* extracts (5 and 10mg/kg) (Letechipía-Vallejo et al., 2001; Liu et al., 2014; Robertson et al., 2013) were done for 7 days. Then 24h after the last injection, the rat middle cerebral artery occlusion (MCAO) model was developed as explained below.

The rats were weighed and anesthetized with an IP injection of chloral hydrate (400mg/kg). MCAO was done as defined by Longa et al. (1989). In brief, the neck was cut to the right, then the common carotid artery (CCA) and external carotid artery (ECA) were completely blocked and the internal carotid artery (ICA) was temporarily blocked by a clamp. A small incision was made on CCA and the blocking filament was slowly introduced up to 2cm from the start of the plug along the ICA. After 60min, the reperfusion started by removing the blocker filament. During the operation, body temperature was maintained by an anal probe at 37°C and a heating pad was used to prevent hypothermia. After surgery, the animals were allowed to recover natural breathing and were located in their cages with free access to standard pellet chow and water. Normal control rats underwent the same procedure except MCAO.

Study plan

The rats were randomly divided into four experimental groups as follows, Group 1: normal control rats (n=6) pre-treated daily with DMSO (the extract solvent); Group2: MCAO control (n=9) rats pre-treated daily with DMSO; Group3: MCAO rats (n=9) pre-treated with 5mg/kg *V. spathulata* and Group4: MCAO rats (n=9) pre-treated daily with 10mg/kg *V. spathulata*. Pre-treatment was initiated 7 days before the onset of transient MCAO.

Assessment of neurological deficit score (NDS)

Twenty-four hours after reperfusion, we employed a blinded examiner to assess the behavioral deficits. The NDS was evaluated using a six-point scale as described in our previous study (Abedinzadeh et al., 2021; Longa et al., 1989).

Assessment of infarct volume (IV)

After measuring NDS, the rats (n=5 in each group) were deeply anesthetized (100mg/kg ketamine and 10mg/kg xylazine, IP injection). The brains were quickly removed and placed at 4°C saline solution for 15min. Subsequently, the brains were placed in a brain matrix and eight 2mm diameter coronal incisions were taken. The specimens were immersed in a 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Merck, Germany) and placed in a water bath for 15min at 37°C. The samples were then photographed with a digital camera (Lumix-Panasonic camera, Japan) connected to a computer. Unstained parts of the brain were considered infarct areas. Data were analyzed using the Image J software (NIH, Bethesda, MD, USA). The IV was calculated by measuring the unstained and stained area in each hemisphere slice in three defined regions (core, penumbra and subcortex; Fig. 1; Lei et al., 2004), multiplying by slice thickness (2mm), followed by summiting all eight slices according to the method by Swanson et al. (1990): corrected IV= left hemisphere volume – (right hemisphere volume - IV).

RNA extraction and DNase I treatment

The brain samples from 4 remained rats in each group were cut into tiny segments. Core, penumbra and subcortex of the brain tissues were isolated as previously described by Lei et al. (2004) (Figure 1), then mixed with 1ml of RNA extraction Kit (BioFACT, Korea). Total RNA was extracted based on the kit instruction. To verify the purity of all RNA samples, the 260/280 nm absorbance ratio was assessed using the Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., USA). To eliminate DNA contamination, RNA extracts were treated with RNase-free DNase I in line with the manufacturer guidelines (Thermo Fisher Scientific Inc., USA). One microliter of DNase I was used per µg RNA in a 10-µl reaction mixture followed by incubating at 37ºC for 30min. Finally 1µl 50mM EDTA was added and incubated at 65ºC for 10min.

Synthesis of complementary DNA (cDNA)

Single strand cDNA was synthesized using HyperScript™ First-strand Synthesis Kit. As manufacturer

protocol: 1µg total RNA, 1µl oligo dT primer (50µM), 1µl dNTPs (10mM) was added to 0.2ml nuclease-free microtube and attained to 14µl total volume using RNase-free water. The mixture was heated at 65ºC for 5min and instantly located on ice for 1min. After a short rotation, it was transferred into the final tube that contained 2µl RTase reaction buffer (10x), 2µl of 0.1M DTT, 1µl HyperScript™ Reverse Transcriptase (200U/µl) and 1µl of ZymAll™ RNase inhibitor. Following a short rotation, the microtubes were heated for 60min at 55ºC and finally reverse transcriptase inactivated at 85ºC for 5min. In the end, the samples were briefly located on ice then stored in -20ºC.

Real-time polymerase chain reaction (RT-PCR)

The expression levels of COX2 (NCBI Ref Seq: NM-017232.3) and CASP3 (NCBI Ref Seq: NM-012922) mRNA were evaluated by RT-PCR techniques via the ABI instrument (stepOne™, USA). The mRNA expression levels of these genes were quantified in comparison with a suitable endogenous reference gene as glyceraldehyde 3-phosphate dehydrogenase.

The primers for RT-PCR were designed by Prime3web (version 4.0.0), then ordered to GenFanAvaran co, Tehran, Iran. As explained in our previous study, the specificity of primers was blasted by the Primer-BLAST program at the National Center for Biotechnology Information (Khanaki et al., 2019). The sequences and product sizes of the designed primers are mentioned in Table 2.

The reaction mixture consisted of 1µl of each primer, 10µl of SYBR Green Master Mix (Ampliqon, Denmark), 200ng of each cDNA sample and enough nuclease-free water to reach 15µl. According to the amplification protocol, reaction tubes initially heated at 95ºC for 10min, followed by 40 cycles in a two-step, 95ºC for 15s and 60ºC for 60s. The dissociation curve of each gene certified the absence of nonspecific band or primer dimers. Finally, the differences among mRNA expression of test and reference samples were assessed and the relative mRNA expression of COX2 and CASP3 were estimated by $2^{-\Delta\Delta CT}$ technique. All reactions were run in triplicate.

Statistical analysis

Data from IV and RT-PCR are presented as mean±SD. Inter-group comparisons were performed using the one-

TABLE 1: The Real-Time PCR primers sequences.

Primer name	Sequence (5' → 3')	Nucleotide count	Product size
GAPDH-F	CCACAGTCCATGCCATCACT	20	101
GAPDH-R	TGCAGGGATGATGTTCTGGG	20	
COX2-F	ATGATCTACCCTCCCCACGT	20	119
COX2-R	ACTCTGTTGTGCTCCCGAAG	20	
CASP3-F	GCTGGACTGCGGTATTGAGA	20	142
CASP3-R	CCATGACCCGTCCCTTGAAT	20	

GADPH: glyceraldehyde 3-phosphate dehydrogenase, COX2: cyclooxygenase 2, CASP3: caspase 3.

TABLE 2: Neurological deficit score (NDS) of animals in different groups 24h after middle cerebral artery occlusion (MCAO). The higher the neurological deficit score, the more severe impairment of motor motion. The neurological impairment of both groups pre-treated with *Viola spathulata (V. spathulata*) was significantly less than the MCAO group.

No.	Experimental groups	NDS in each group (N)									Median	Statistical results
		1	2	3	4	5	6	7	8	9		
1	MCAO + *V. spathulata* (5mg/kg)	3	2	2	2	1	1	1	1	0	1.44	0.003
2	MCAO + *V. spathulata* (10mg/kg)	0	0	0	1	1	1	2	2	3	1.11	0.0001
3	MCAO control	2	3	3	3	3	4	4	2	2	2.88	

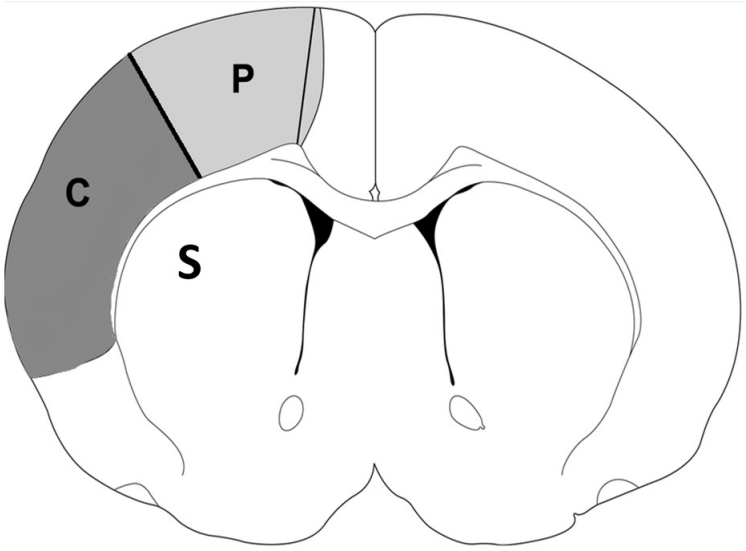

FIGURE 1. Schematic drawing showing the brain regions at a level 5mm from the frontal pole of the brain. Shaded areas indicate the ischemic areas. P: ischemic penumbra, C: ischemic core, S: subcortex.

way analysis of variance (ANOVA) followed by post hoc Tukey's test. The NDS was analyzed using Kruskal-Wallis. For all tests, $P<0.05$ was considered statistically significant. Data were analyzed using SPSS software version 16.

Results

Effects of V. spathulata on NDS in different experimental groups

NDS in the MCAO group was considerably higher than the normal control group. Pretreatment with *V. spathulata* extraction markedly improved sensorimotor function. NDS was significantly reduced in both MCAO + *V. spathulata* groups (5 and 10mg/kg) compared to the MCAO control group ($P=0.003$ and $P=0.0001$, respectively; Table 2). Comparing data from two doses of *V. spathulata* didn't show a significant difference.

Effects of V. spathulata on IV in different experimental groups

There was no infarction in the normal control group. Considerable progress of infarction was seen after blocking MCA. IP injection of *V. spathulata* in two doses of 5 and 10mg/kg for 7 days before the onset of ischemia, markedly decreased IV compared with MCAO group in brain regions including core ($P=0.037$ and $P=0.06$, respectively), subcortex ($P=0.002$ and $P=0.037$, respectively) and penumbra ($P=0.008$ and $P=0.03$, respectively). The comparison between the two doses of *V. spathulata* showed no significant difference (Figures 2 and 3).

Effects of V. spathulata on CASP3 expression in the rat brain of different experimental groups

To explore whether *V. spathulata* treatment alters the expression of pro-apoptotic markers involved in I/R, the mRNA level of CASP3 was observed. The results demonstrated that the mRNA level of CASP3 was significantly over-expressed in the core, penumbra and subcortex areas of MCAO rats compared to the normal control group ($P=0.01$, $P=0.05$ and $P=0.01$, respectively). Pretreatment with both doses of *V. spathulata* extract (5 and 10mg/kg) could markedly reduce CASP3 expression level in the core ($P=0.012$ and $P=0.02$, respectively) and penumbra ($P=0.042$ and $P=0.045$, respectively) areas compare to MCAO control but there was no remarkable change in the mRNA level of CASP3 in the subcortical area (Figure 4).

Effects of V. spathulata on COX2 expression in the rat brain of different experimental groups

To investigate the anti-neuroinflammatory effects of *V. spathulata*, we assessed the COX2 gene expression on the mRNA level. The RT-PCR results revealed that normal control rats showed low levels of COX2 in three investigated areas of the brain. After transient MCAO, the COX2 gene expression in the core and penumbra areas of MCAO groups didn't show a significant change, but a significant increase in the subcortex area of this group

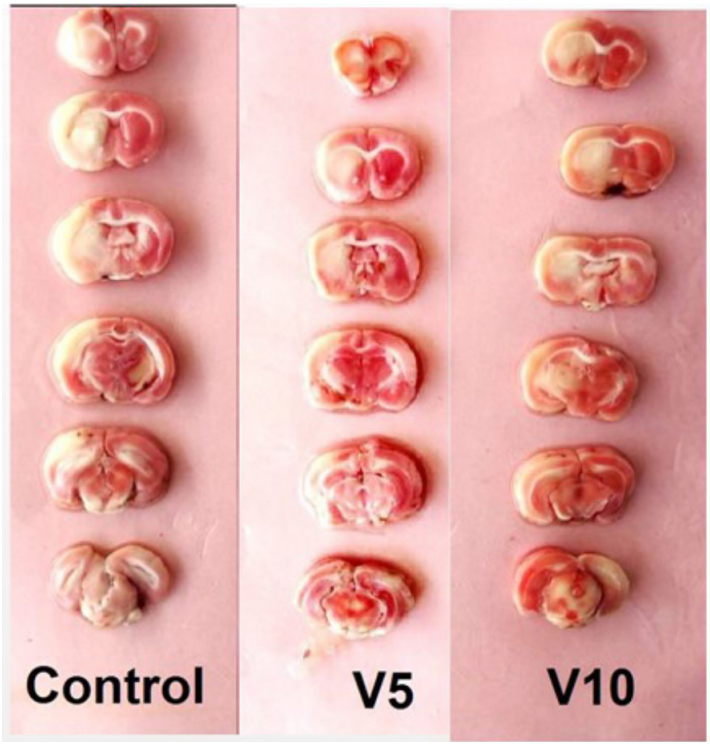

FIGURE 2. Images shown 2,3,5-triphenyltetrazolium chloride (TTC) stained sections. Red areas represent normal tissue, while the white is infarction. Control: middle cerebral artery occlusion (MCAO) control, V5: MCAO + 5 mg/kg *Viola spathulata* (*V. spathulata*), V10: MCAO + 10 mg/kg *V. spathulata*.

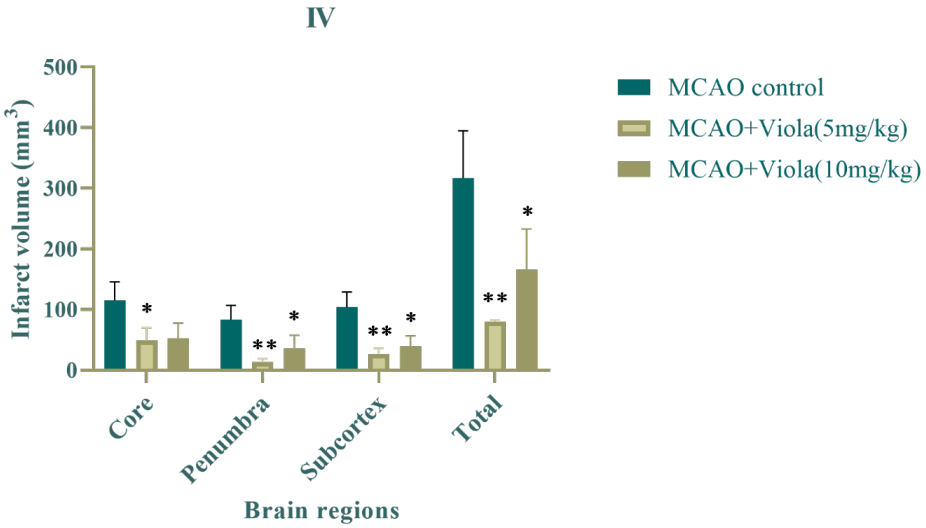

FIGURE 3. Effect of *Viola spathulata* (*V. spathulata*) on infarct volume (IV) 24h after middle cerebral artery occlusion (MCAO) in different brain areas from different experimental groups (n=5 in each group). Both *V. spathulata* groups considerably exhibit less infarct volume than the MCAO group. Data are presented as mean±SD, **P*<0.05, ***P*<0.01 compared to MCAO control.

was observed (*P*=0.038 compare to the normal control group). Pre-treatment with *V. spathulata* extracts (both doses of 5 and 10mg/kg) didn't significantly change the COX2 expression in the subcortex area (Figure 5).

Disussion

According to the promising neuroprotective potentials of viola plants (Moliner et al., 2019) and the importance to prevent stroke and ischemic brain diseases, present research assessed the impact of *V. spathulata* on NDS, IV and mRNA expression level of CASP3 and COX2 in a rat MCAO model. In this study, data showed that pre-treatment with *V. spathulata* extract remarkably reduced NDS. Also accordant to histological data, the extract could significantly decrease IV in the core, penumbra and subcortical areas of the brain in rats subjected to ischemia. These findings confirm the protective role of *V. spathulata* in I/R injury.

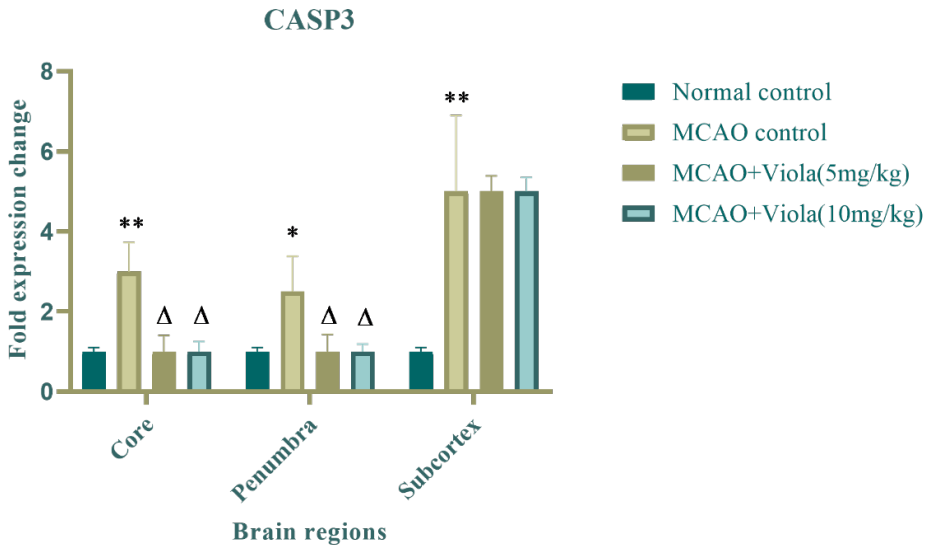

FIGURE 4. Relative mRNA expression of caspase 3 (CASP3) in three regions of the brain from different experimental groups (n=4 in each group). Values are presented as mean±SD. $^{*}P<0.05$, $^{**}P<0.01$ by comparison with the normal control group and $^{\Delta}P<0.05$ by comparison with middle cerebral artery occlusion (MCAO) control.

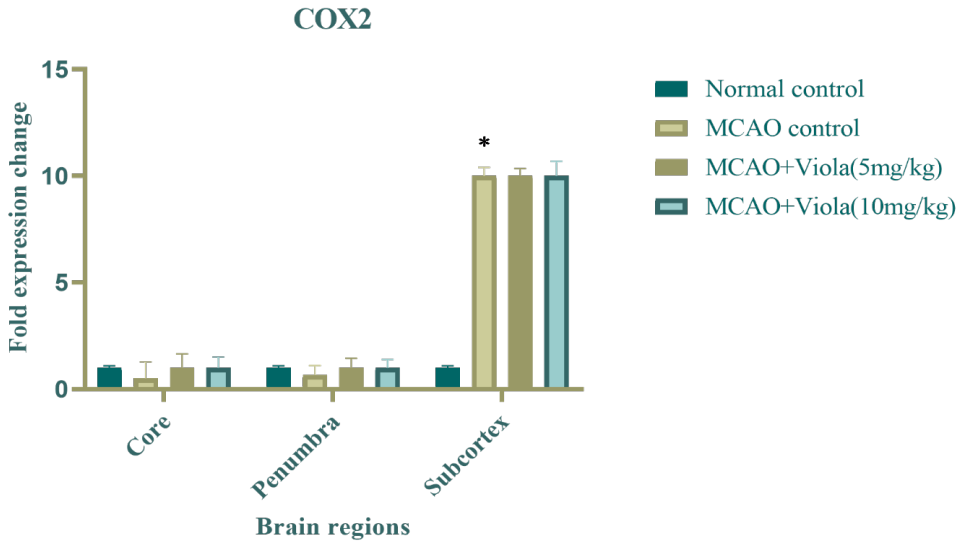

FIGURE 5. Relative mRNA expression of cyclooxygenase 2 (COX2) in three regions of the brain from different experimental groups (n=4 in each group). Values are presented as mean±SD. $^{*}P<0.05$ by comparison with the normal control group.

Viola has been reported to exert different properties which are beneficial to neuron cell survival for instance blocking the reactive oxygen species production and decreasing the pro-apoptotic/anti-apoptotic markers ratio (Tayarani-Najaran et al., 2019). Mousavi et al. (2016) investigated the potential effects of *Viola tricolor* and *Viola odorata* in an *in vitro* model of ischemia and neurodegeneration on the PC12 neuronal cell line. They demonstrated that pre-treatment with *V. tricolor* and *V. odorata* extracts raised cell vitality in a dose-dependent way and inhibited neuronal cell damage induced by serum/glucose deprivation condition. In a similar study by Rasouli Vani et al. (2019), pre-treatment of ischaemic rats with the extract of *Dorema aucheri* Bilhar (Umbelliferae) leaves could considerably attenuate IV and tissue swelling. The study suggested that the protective effect of *D. aucheri* is due to containing the flavonoids constituents. Flavonoids can exert antiapoptotic and anti-inflammatory effects in neurodegenerative diseases (Singh and Hembrom, 2019). It is reported that flavonoids can cross through the blood-brain barrier (Youdim et al., 2003), these chemicals are common major constituents of the genus *Viola* (Zhu et al., 2015).

In a study conducted by Gao et al. (2009), IP admin-

istration of puerarin (dominant essential element derivate from the traditional Chinese medicine Ge Gen) after transient MCAO in rat, decreased IV and improved functional neurological outcome. They concluded that these results, other than the attenuation of apoptosis could be in part because of the erythropoietin over-expression. The critical role of caspase-3 as a mediator of apoptosis in animal models of ischemic stroke has been reported (Asahi et al., 1997) and its inhibition has been found to have a strong neuroprotective effect in experimental stroke (Endres et al., 1998). Our findings showed that CASP3 mRNA expression level in the core, penumbra and subcortical brain areas of MCAO rats was remarkably higher than that of normal control rats. The *V. spathulata* extract pre-treatment in both doses attenuated the apoptosis occurrence in core and penumbra compared to MCAO groups, since represented as remarkable downregulation of CASP3 mRNA expression. Our data were similar to those carried out by Gu et al. (2018) in which the effects of *Ligusticum chuanxiong* and *Radix paeoniae* combination on focal cerebral ischaemic stroke was assessed. This study demonstrated that expression of apoptotic factors such as CASP3 was raised after ischaemic injury but reduced in *L. chuanxiong* and *R. paeoniae* treated groups both in mRNA and protein level. These data might exhibit the vital role of apoptosis in stroke. Previous studies had reported the association of cerebral I/R injury and apoptosis mediated by caspase family members (Erfani et al., 2018). In another research, the potential effect of extract from *Sophora flavescens* was investigated *in vitro* (human neuroblastoma SH-SY5Y cells) and *in vivo* (rat MCAO model). Two main flavonoids of *S. flavescens* are kurarinone (45.5%) and *sophora flavone* G (14.7%). The S. *flavescens* could attenuate CASP3 activity in a dose-dependent way and reduced IV. It was suggested that the main neuroprotective features of *S. flavescens* might be due to its flavonoids (Park et al., 2009).

Our findings were similar to the data achieved by Bai et al. (2016) in which triptolide (a diterpene triepoxide and the main active element of *Tripterygium wilfordii* Hook F. extract) reduced NDS and the cerebral IV and attenuated brain edema in rats with focal cerebral I/R injury. Also, this extract could down-regulate CASP3 and COX2 expression. The study suggested triptolide as a potential anti-inflammatory agent due to preventing the NF-κB pathway. Activation of NF-κB may enhance pro-inflammatory gene expression such as COX2 (Lee et al., 2004). Over-expression of pro-inflammatory agents plays a critical role in neuron loss in some central nervous system diseases (Lucas et al., 2006). Also, increased levels of COX2 are observed within ischemia and neural injury(Chan, 2001).

Lee et al. (2010) showed that IP injection of *Viola mandshurica* W. Becker ethanolic extract has anti-inflammatory and anti-asthmatic effects on airway inflammation in an ovalbumin induced asthmatic BALB/c mouse model. Our results showed that mRNA expression of COX2 was more enhanced in the subcortex region of MCAO rats in contrast with the normal control group. These findings showed that there might be a relation between cerebral I/R-induced neuroinflammation and COX2 expression, particularly in the subcortical area. In the present study, pre-treatment with *V. spathulata* extract in both doses showed no significant reduction in COX2 mRNA expression in the subcortical region comparing the MCAO control group.

In similar research by Li et al. (2016), COX2 was up-regulated in the cortical area of MCAO rats comparing the normal control group. Then after carvacrol (a monoterpene phenol naturally in various plants belonging to the family *Lamiaceae*) treatment, mRNA and protein expression of COX2 markedly decreased in ischemic cortical tissue in a dose-dependent manner. According to the study, carvacrol exerts its anti-inflammatory effect by interrupting inflammatory responses such as inhibition of the NF-kB signaling pathway resulting in decreased COX2 expression. In that study, COX2 mRNA and protein levels were examined by RT-PCR and western blotting, and three different doses of carvacrol were used. However, in our study protein assessment of COX2 was not examined and only two different doses of *V. spathulata* were used.

Ha et al. (2008) reported that oral administration of apigenin (5,7,40-trihydroxyflavone) 30min after MCAO in mice, inhibited COX2 protein expression and led to reduction of neuronal cell death and IV. These data showed the important role of COX2 in inflammation and cell damage. The study conducted by Chen et al. (2016) represented that trans-cinnamaldehyde, the fundamental oil in cinnamon powder, could remarkably attenuate the infarct region in a mouse model of cerebral ischemia in a dose-dependent manner and inhibited inflammation. Trans-cinnamaldehyde also prevented

the rise in COX2 protein levels induced by lipopolysaccharides and down-regulated mRNA level of COX2 in BV-2 microglial cell line cultured *in vitro*. The present findings might be indicative of the neuroprotection effect of *V. spathulata* against cerebral ischemia *in vivo*. Since levels of melatonin isomers in plants are high (Tan et al., 2014), there is a possibility that the neuroprotective effect of *Viola* might be due to its different melatonin isomers. Although in the current study in which *V. spathulata* was studied for the first time, evaluation of melatonin concentration in extracts was not examined.

Conclusion

Overall, it seems that V. spathulata might elevate neuronal cell survival in a MCAO ischemia model and decrease apoptotic cell death in core and penumbra regions of the brain by decreasing CASP3 expression. Since the Viola plant extracts are combined of different compounds and may influence cerebral ischemia through various procedures, much more research in this issue investigating the possible impacts of various doses of V. spathulata extracts along with its effective compounds on other inflammatory or apoptotic markers together accompanied by protein quantification of cleaved CASP3 are warranted.

Acknowledgment

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Conflict of interest

All authors declared that there is no conflict of interest in the present study.

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