



Effect of *Elettaria cardamomum* Essential Oil and Its Main Component (1, 8-cineole) on Brain Damage Caused by Cerebral Hypoperfusion by Suppressing Apoptosis and Inflammation in Rats

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Abstract

Background: Stroke is one of the most common causes of death in industrialized countries and a leading cause of permanent disability in adults worldwide. *Elettaria cardamomum* is a nutraceuticals that is used widely and characterized by numerous properties, such as remarkable effects on anxiety-like behavior features.

Objectives: This study aimed at determining the effect of *Elettaria cardamomum* essential oil and its major constituent (i.e., 1,8-cineole) on the brain injury caused by cerebral hypoperfusion among rats.

Methods: Cerebral hypoperfusion was induced in Wistar rats using the occlusion of the bilateral carotid artery for 30 min. Subsequently, *Elettaria cardamomum* essential oil (25, 50, and 75 mg/kg i.p.) and its major constituent (1,8-cineole) (5, 10, and 20 mg/kg i.p.) was injected to rats for 14 consecutive days. After the final treatment, blood samples were taken, and the brain tissues were separated and used for biochemical analysis by RT-PCR to detect the levels of proinflammatory cytokines.

Results: Administration of *Elettaria cardamomum* essential oil and 1,8-cineole significantly decreased the malondialdehyde and nitric oxide levels in the blood and brain cortex of the rat. Additionally, *Elettaria cardamomum* essential oil and 1,8-cineole significantly suppressed the expression level of Caspase 3, IL-1 β , TNF- α , and iNOS in the cerebral cortex.

Conclusion: The findings suggest that *Elettaria cardamomum* essential oil includes remarkable neuroprotective impacts that may be beneficial to cerebral hypoperfusion treatment. The pharmacological property of *Elettaria cardamomum* partly requires the modulation of inflammatory mediators and oxidative stress.

Keywords: 1,8-cineole, Apoptosis, Cerebral hypoperfusion, *Elettaria cardamomum*

1. Background

The global rate of ischemic stroke is approximately 15 million cases each year. Few clinical benefits have been obtained from the clinical and preclinical studies conducted on the prevention of neuron damage during a stroke (1,2). Production of reactive oxygen species (ROS) and high oxygen consumption leads to the low levels of endogenous antioxidant contents in non-replicating neurons. Free radicals activate lipid peroxidation of polyunsaturated fatty acids that results in the membrane impairment of functional activity and its damage. Therefore, cerebral ischemic injury in the patient is the result of inflammation, oxidative stress, necrosis, and apoptosis (3-5).

The cardamom (*Elettaria cardamomum* [EC] L. [Maton] from Zingiberaceae family) is among the most widely cultivated crops in tropical regions mainly in India. Different parts of this plant have been traditionally used as a stomachic, retentive, digestive, antiemetic, and carminative herb in the treatment of gastrointestinal complications. Moreover, it can be utilized for its antiputrefactive effects during embalmment (6). In addition, pharmacological

applications of the EC have been recently attributed to modern medicine due to its anti-inflammatory, antimicrobial, analgesic, anticonvulsant, and anti-depression properties (7).

The EC essential oil analysis using gas chromatography-mass spectroscopy (GC-MS) technique has revealed 16 major compounds that constitute 93.62% of the total components of this plant. The detected compounds included oxygenated monoterpenes (63%), α -terpinyl acetate (56.87%), monoterpenes (27.37%), 1,8-cineole (23.74%), α -terpineol (5.25%), limonene (4.05%), sesquiterpenes (1.43%), fatty acids esters (1.17%), and acetates (0.63%) (8).

Moreover, 1,8-cineole, which is also known as eucalyptol or cajepitol, is an oxidized terpene that has been found in eucalyptus (75%), rosemary (40%), psidium (40-60%), and many other plant essential oils (9).

2. Objectives

Despite the reported pharmacological properties of the EC, there is a limited number of studies

investigating its neuroprotective effects on the injury of cerebral ischemic-reperfusion. Therefore, the present study aimed at investigating the effect of EC essential oil and its major component (i.e., 1,8-cineole) on the bilateral common carotid artery (BCCA) occlusion that induce the injury of cerebral ischemia-reperfusion in the Wistar rat model.

3. Methods

3.1. Medicinal plant and the method for essential oil extraction

Dried EC whole-plant was purchased from a local market in Izeh, Iran, and identified by a herbalist in Izeh Branch, Islamic Azad University, Izeh, Iran, by comparing it to a reference sample (specimen No., 78901). Initially, the plant was ground, and the essential oil was extracted in a Clevenger apparatus via water distillation. For this purpose, 50 g of the dried plant was weighed and transferred into a 500-ml flask. Afterward, the plant was mixed with 200 ml of distilled water and heated at 40°C to reach the distillation rate of 2-3 ml per min. After four h, the essential oil was collected and stored in a dark place at -20°C (10).

3.2. Detection of essential oil components by gas chromatography-mass spectroscopy

An Agilent 5975 GC-MS system equipped with the HP-5MS fused silica column (30 m×0.25 mm I.D) was used to perform the GC-MS analysis. Oven and the injector temperature values were set at 50-240°C with a rate of 7°C/min and 280°C, respectively. Following that, helium gas flowed with a rate of 0.8 mL/min, and ionization energy was tuned to 70 eV for scanning time of 1 sec and mass range of 40-300 amu. Eventually, the compounds obtained from the essential oil were determined after making a comparison of the mass and library spectra, authentic compounds, and published literature (10).

3.3. Experimental animal model

Adult male Wistar rats (weight range: 250-300 g) were obtained from Pasteur Institute, Tehran, Iran. Subsequently, all animals were kept under a normal 12 h light-12 h dark cycle and laboratory conditions at 22±2°C and 55% relative humidity. All study procedures were approved by the Institutional Animal Care and Committee of Sanandaj Branch, Islamic Azad University, Sanandaj, Iran.

3.4. Animal grouping

The rats were assigned randomly into eight groups (n=7) of control (1), ischemia (Isc) (2), Isc+25EC (3), Isc+50EC (4), Isc+75EC (5), Isc+Cineole5 (6), Isc+Cineole 10 (7), and Isc+Cineole 20 (8). On the other hand, the control group received normal saline without the induction of cerebral Isc. The Isc rats in groups 3-5 were administered with 25,

50, and 75 mg/kg of the EC essential oil for 14 days, respectively. Furthermore, the Isc rats in groups 6-8 were respectively administered with 5, 10, and 20 mg/kg of 1,8-cineole for 14 days and afterward subjected to reperfusion for 24 h. It is worth mentioning that the rats were sacrificed 24 h following reperfusion.

3.5. Induction of ischemia

The rats underwent bilateral common carotid artery occlusion under ketamine anesthesia (50 mg/kg) on day 14. Afterward, the animals were placed on their back, and atraumatic clamps for 30 min and 24 h reperfusion by removing the clamps were utilized to expose and occlude both carotid arteries. The temperature was maintained at 37±0.5°C during the surgical procedure.

3.6. Measurement of biochemical parameters in animals

The animals were sacrificed 14 days after the treatment that followed cerebral (Isc/reperfusion) by cervical decapitation. Subsequently, the blood samples were taken, and the brains were removed and washed with cooled 0.9% normal saline. They were then kept on ice and subsequently blotted on filter paper, weighed, and homogenized in cold phosphate buffer (0.05 M, pH=7.4).

3.7. Detection of malondialdehyde levels

Thiobarbituric acid (TBA) reactive substances in the serum, and brain tissue homogenates were determined using the incubation of 0.5 ml of each serum or 10% of each homogenate with 15% trichloroacetic acid (TCA) plus 0.375% TBA and 5 N hydrochloric acids at 95°C for 15 min.

The mixture was then cooled and centrifuged, followed by the measurement of supernatant absorbance at 512 nm against an appropriate blank.

3.8. Antioxidant capacity detection

The ferric reducing antioxidant power method was employed to measure the antioxidant capacity of the serum and brain homogenate tissue. This technique can reduce Fe³⁺ ions to Fe²⁺ in the presence of TPTZ2 reagent that leads to the TPTZ-Fe²⁺ blue complex accompanied by a maximum absorbance value of 593 nm.

3.9. Detection of nitric oxide levels

In this procedure, 100 µl of each serum or tissue homogenate (in triplicate) were added into the wells of a 96-well plate. Afterward, 100 µl of sulfanilamide solution (1 g sulfanilamide in 100 ml phosphoric acid 5%) was added to each well. The plate was then incubated in a dark place at room temperature for 5-10 min. Subsequently, 50µl of NEDD solution was added to each well and incubated at room temperature for 30 min. Eventually, the light

Table 1. Forward and reverse primer sequences for the amplification of desired genes

Coding gene	Primer sequence
casp3-F*	AAGCCGAAACTCTTCATCATTCA
casp3-R**	GCCATATCATCGTCAGTTCAC
Tnf α -F	CTGAACTTCGGGTGATCGG
Tnf α -R	GGCTTGTCACCTCGAATTTTGAGA
IL-1 β -F	GAAATGCCACCTTTTGACAGTG
IL-1 β -R	TGGATGCTCTCATCAGGACAG
Bax F	GAGGATGATTGCTGATGTGGATA
Bax-R	CAGTTGAAGTTGCCGTCTG
Bcl2-F	GGAGCGTCAACAGGGAGATG
Bcl2-R	ACAGCCAGGAGAAATCAAACAGA
iNOS-F	TCAGCCAAGCCCTCACCTAC
iNOS-R	CTCCAATCTCTGCCTATCCGTCT

*Forward (F)
**Reverse (R)

absorbance of the wells was read by an ELISA reader at the wavelength of 540 nm, and the nitric oxide (NO) content of the samples was identified by making a comparison with a standard nitrite curve.

3.10. Detection of IL-1 β , TNF- α , iNOS, Caspase 3, and Bcl-2 coding gene expression

Adequate volumes of guanidium thiocyanate solution were used to freeze the brain samples. They were then kept at -80°C until the RNA extraction, and total cellular RNA was extracted by the phenol/chloroform method. In addition, the concentration of the total tissue RNA was estimated based on the light absorbance at the wavelength of 260 nm. Moreover, the quality of the extracted RNA was verified by the agarose gel electrophoresis method. The purified total RNA was entered the reverse transcription process included the incubation of RNA (1 μ g) with oligo (dT) primer (1 μ M) and 200 units of Moloney murine leukemia virus reverse transcriptase from a Clontech first-strand complementary DNA (cDNA) synthesis kit.

In the next stage, an aliquot (5 μ l of a 1/10 dilution) of each synthesized cDNA was utilized for

the reverse transcription-polymerase chain reaction (RT-PCR) method. Table 1 tabulates the PCR primers.

The amplification of DNA was performed in the solution containing 1 \times Taq polymerase buffer, 1.5 mM MgCl₂, 50 μ M dNTPs, 0.25 μ M of each 5' and 3'-specific primers, 1 μ Ci [α -32p], and 2 units Taq DNA polymerase (Promega C) in a final volume of 50 μ l. The mixture was overlaid with mineral oil and amplified for 30 cycles. The steps included denaturation at 94°C for 1 min, primers annealing at 60°C for 1 min, and extension at 72°C for 1 min. The reaction was then terminated by a final extension at 72°C for 7 min, and the PCR products were stored at 4°C. Following that, the products of PCR were size-separated by 10% acryl/bis-acrylamide gel electrophoresis, followed by ethidium bromide staining (15 μ g/ml). Table 1 displays the excision of each band from the gel and the measurement of the incorporated 32p quantity in a scintillation counter.

3.10. Statistical analysis

The data were analyzed in the GraphPad Prism 5 statistical software and expressed by mean \pm SEM. Moreover, a one-way ANOVA and multiple comparison Turkeys' test were utilized to determine the significant differences between the treated and control groups. A p-value less than 0.05 was considered statistically significant.

4. Results

Figures 1A and 1B indicate a remarkable improvement in the MDA levels in serum and brain tissues (cortex) among the Isc-induced rats, compared to the control group (P<0.001). Nonetheless, Isc-mediated lipid peroxidation in serum and brain tissues (cortex) decreased in rats treated with EC essential oil (25, 50, and 75 mg/kg), compared to the control group (P<0.001). Furthermore, the

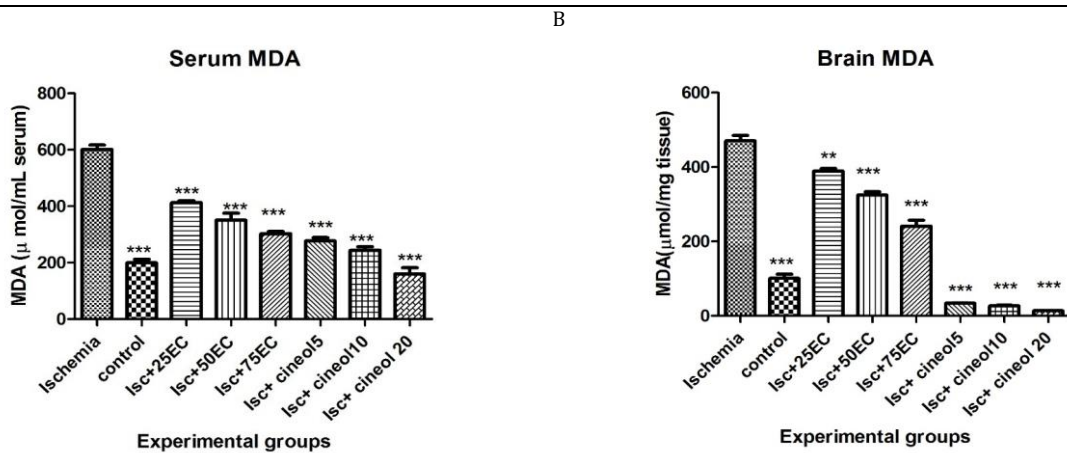


Figure 1. Effects of *Elettaria cardamomum* essential oil and 1,8-cineole on lipid peroxidation in serum (A) and brain tissues (B) following cerebral hypoperfusion, compared to the ischemia group. Values are presented as mean \pm SEM (n=7).

Isc and EC show ischemia and *Elettaria cardamomum*, respectively.

*** Represents the significant differences at P<0.001.

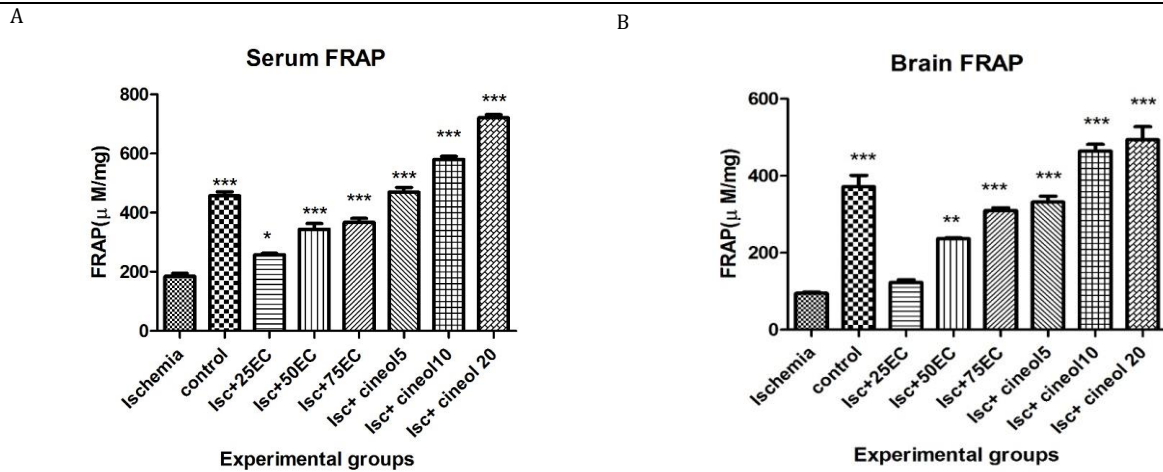


Figure 2. Effects of *Elettaria cardamomum* essential oil and 1,8-cineole on ferric reducing antioxidant capacity in serum (A) and brain tissues (B) following cerebral hypoperfusion, compared to the ischemia group. Values are presented as mean±SEM (n=7). lsc and EC show ischemia and *Elettaria cardamomum*, respectively. ***, **, and * represent the significant differences at P<0.001, P<0.01, and P<0.05, respectively.

administration of 1,8-cineole (5, 10, and 20 mg/kg) led to a decrease in the level of MDA in the serum and brain tissues (cortex) of the groups under treatment, compared to the lsc group (P<0.001).

As shown in Figures 2A and 2B, the lsc group obtains a significant decrease in antioxidant capacity according to the ferric reduction in brain cortex and serum, compared to the control group (P<0.001). However, treatment with EC essential oil (25, 50, and 75 mg/kg) enhanced the ferric reducing antioxidant capacity in the brain cortex, compared to the lsc group (P<0.001). Moreover, the administration of EC essential oil (25, 50, and 75 mg/kg) improved serum ferric reducing antioxidant capacity, compared to the lsc group (P<0.001). In addition, 1,8-cineole (5, 10, and 20 mg/kg) led to the enhancement of the ferric reducing antioxidant capacity in the serum and brain tissue, compared to the lsc group (P<0.001).

As can be seen in Figures 3A and 3B, BCCA occlusion for 30 min followed by 24 h reperfusion induces a significant increase in the NO level in serum and brain tissues, compared to the control group (P<0.001). Moreover, treatment with EC essential oil (25, 50, and 75 mg/kg) resulted in a significant decrease in the NO levels in serum and brain tissues. On the other hand, the rats that received 1,8-cineole (5, 10, and 20 mg/kg) showed an lsc-induced increase in the NO levels in serum (P<0.001) (Figure 3A) and 1,8-cineole (5 and 10 mg/kg) pretreatment leading to a decrease in the NO level in the brain tissue (Figure 3B).

The results from RT-PCR for the detection of Caspase 3, IL1-β, inducible nitric oxide synthase (iNOS), TNF-α, and bcl2 mRNAs in the brain of rats after 24 h reperfusion are illustrated in Figure 4. An up-regulation was also observed in caspase-3

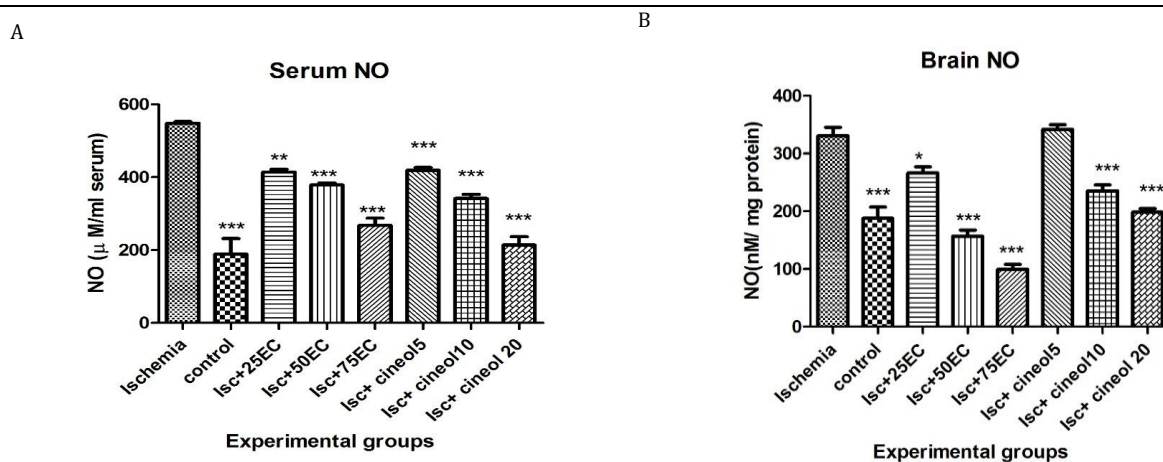


Figure 3. Effects of *Elettaria cardamomum* essential oil and 1,8-cineole on the NO levels in serum and brain tissues in the experimental groups, compared to the ischemia group. Data are represented as mean±SEM (n=7). lsc and EC show ischemia and *Elettaria cardamomum*, respectively. ***, **, and * represent the significant differences at P<0.001, P<0.01, and P<0.05, respectively.

expression after cerebral hypoperfusion in the Isc group, compared to the control group ($P < 0.01$) (Figure 4A). Furthermore, treatment with EC essential oil (25, 50, and 75 mg/kg) caused the suppression of the Caspase 3 expression levels in the cerebral cortex of the animals. In addition, Caspase 3 expression in the cerebral cortex was notably reduced by 1,8-cineole treatment (5, 10, and 20 mg/kg) (Figure 4A). Cerebral hypoperfusion also resulted in a significant and more sustained elevation of TNF- α mRNA in the cerebral cortex of the animals, compared to the control group ($P < 0.001$) (Figure 3B).

Pretreatment with EC essential oil (25, 50, and 75

mg/kg) and 1,8-cineole (5, 10, and 20 mg/kg) reduced TNF- α mRNA, compared to the Isc group (Figure 4B). Cerebral hypoperfusion also caused a significant increase in the expression of iNOS and IL-1 β in the cerebral cortex, compared to the control group (Figures 4C and 4D). Moreover, treatment with EC essential oil (25, 50, and 75 mg/kg) and 1,8-cineole (5, 10, and 20 mg/kg) reduced the iNOS and IL-1 β expression in the cerebral cortex of the Isc-induced rats, compared to the control group. Cerebral hypoperfusion also resulted in a decrease in Bcl2 expression of the cerebral cortex among the rats, compared to the control group ($P < 0.05$) (Figure 4E).

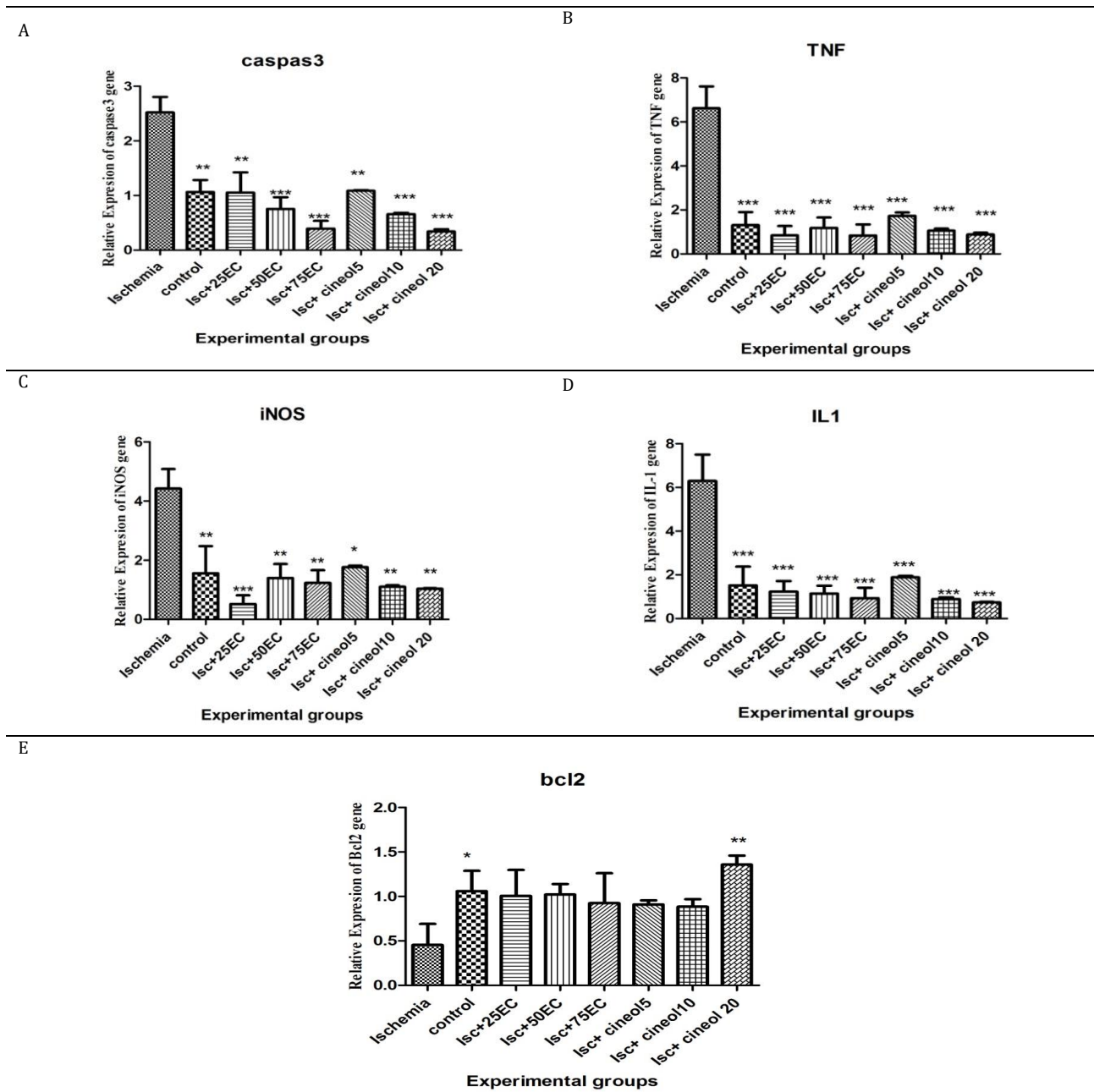


Figure 4. Effects of *Elettaria cardamomum* essential oil and 1,8-cineole on the expression of active caspase-3, IL1- β , iNOS, TNF- α , and Bcl2 in the cerebral cortex of the rats homogenizing after 24 h reperfusion. Data are presented as mean \pm SEM. Isc and EC show ischemia and *Elettaria cardamomum*, respectively. ***, **, and * represent the significant differences at $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively.

and amyloid β cells treated with PC12 pheochromocytoma (19). Furthermore, 1,8-cineole has reduced the release of inflammatory cytokines IL-6 and IL-8 as well as NO, thereby alleviating the inflammatory phenotype of human umbilical vein endothelial cells (20).

The 1,8-cineole that has been detected among the major components of *Achillea millefolium* L. essential oil has been shown as a suppressor for the inflammatory responses of lipopolysaccharide-stimulated RAW 264.7 macrophages according to its ability to decrease the level of NO and down-regulate iNOS, TNF- α , and IL-6 (21). The TNF- α induces the local production of reactive nitrogen species through the induction of nitric oxide synthase leading to the elevation of oxidative stress responsible for brain injury. In addition, TNF- α , as a pro-inflammatory cytokine, has different effects on the pathological processes, such as the induction of edema after Isc (22). The findings in this study showed that cerebral hypoperfusion enhanced the expression of TNF- α and IL-1 β mRNA in the cerebral cortex. The down-regulation of increased hippocampal TNF- α and IL-1 β that was observed in the transient bilateral carotid artery occlusion rats treated with EC essential oil and 1,8-cineole may be responsible for the protective effects on cerebral hypoperfusion via the inhibition of the inflammatory response.

It should be noted that the obtained results are in line with those of the earlier studies on cerebral hypoperfusion injury (23). Apoptosis that occurs following cerebral hypoperfusion is one of the major pathways leading to cell death (24). The outer membranes of mitochondria become more permeable in response to the oxidation load resulting in the translocation of Bax from the cell cytoplasm to the mitochondria. This process leads to the release of cytochrome c that normally existed in the mitochondrial inter-membrane space (25). This pro-apoptotic translocation of Bax protein is under the control of the Bcl-2 protein family. The entrance of cytochrome c into the cytoplasm results in the formation of the apoptosome that is a complex containing apoptotic-protease activating factor-1, procaspase-9, and ATP.

The apoptosome allows the auto-activation of procaspase-9, which is followed by the expression of procaspase-3, and the activated caspase-3 catalyzes DNA fragmentation (26). According to the findings in the present study, the expression of caspase-3 was up-regulated after cerebral hypoperfusion injury. Moreover, the EC essential oil and 1,8-cineole pretreatment significantly suppressed the level of Caspase 3 expression in the cerebral cortex of the Wistar rats.

6. Conclusion

The results of the current study demonstrated the

protective effects of EC essential oil and its major component (i.e., 1,8-cineole) against brain hypoperfusion in an animal model. Since chronic brain Isc and reperfusion induce the attenuation of oxidative stress and release of proinflammatory cytokines to the cerebral cortex, EC essential oil and 1,8-cineole can be proposed as potential natural therapeutics for the treatment of brain Isc and reperfusion.

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Footnotes

Authors' Contribution: Mehrdad Farivar conceived and extracted the data; Zahra Hooshmandi designed the study, analyzed the data, and wrote the manuscript; Mahbubeh Setorki revised the paper and had full access to all of the data in the study; Sabrieh Amini who is the guarantor took the responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interests: The authors declare that there is no conflict of interest.

Ethical Approval: The study protocol was approved in 2019 by the Ethics Committee of Sanandaj Branch, Islamic Azad University, Sanandaj, Iran.

Informed consent: Written informed consent was obtained from legally authorized representatives before the study.

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