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NEUROPROTECTIVE EFFECT OF PRETREATMENT WITH *MENTHA LONGIFOLIA* L. EXTRACT ON BRAIN ISCHEMIA IN THE RAT STROKE MODEL

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Abstract: Recent studies have suggested that *Mentha longifolia* L. extracts (ME) have anti-inflammatory and antioxidant activities. In this paper, we attempted to determine the effect of ME in a rat stroke model. Five groups of experimental animals were used (n=14); the first and second groups (control and sham, respectively) received intraperitoneal injections of daily-distilled water; the other three groups received 50, 100, and 200 mg/kg/day of ME for 21 days. Two hours after the last dose, each group was subdivided into a middle cerebral artery occlusion (MCAO) group operated for neuropathological assessment (neurologic deficit scores, infarct volume, brain edema, and blood–brain barrier (BBB) permeability), and an intact subgroup for the assessment of brain and serum antioxidant capacity and lipid peroxidation, respectively. Pretreatment with ME resulted in a significant reduction in total infarct volume, brain water content and Evans Blue extravasation in the ischemic hemisphere compared with the control. ME in doses of 100 and 200 mg/kg/day increased brain antioxidant capability in comparison with the control. The antioxidant capacity of the serum in the 100 mg/kg/day group was significantly higher than that of the control group. The level of malondialdehyde (MDA) in the brain was significantly elevated in the control group in comparison with other pretreated groups. Moreover, ME in doses of 100 and 200 mg/kg/day reduced the MDA level in the serum. This study showed that intraperitoneal injection of ME reduced infarct volume, brain edema, blood-brain barrier permeability, as well as lipid peroxidation, and improved antioxidant power.

Key words: Cerebral ischemia; *Mentha longifolia* L. extract; malondialdehyde; neuroprotection; pretreatment.

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INTRODUCTION

Transient brain ischemia is characterized by hypoxia during a short period of time followed by a period of hyperperfusion of the tissue with well-oxygenated blood. The reperfusion produces a considerable amount of free radicals in the tissue, which damage macromolecules, including proteins, nucleic acids and cell membranes. The pathophysiological end result is variable depending on the affected cell organelles or macromolecules (Beckman et al., 1990). The increased intracellular calcium levels cause cell death by various mechanisms, including activation of proteases and lipases, formation of free radicals, lipid peroxidation and formation of nitric oxide (NO) and arachidonic acid. The production of a high level of NO may result in the production of high levels of free radicals, which damage principal biomolecules and include membrane lipids, enzymes, and DNA (Dawson et al., 1991). When reperfusion occurs and oxygenation is restored, even further damage can be caused because of the generation of reactive oxygen species (ROS), which includes e.g. superoxide, hydroxyl radicals and hydrogen peroxide generated as side products in mitochondrial ATP synthesis. When oxygenation is restored, reactive oxygen species accumulate and cause damage to cellular macromolecules (Karolinska, 2007).

Phenolics are defined as a class of polyphenols that are important secondary metabolites in plants

(Williams et al., 2004). Based on their C-skeleton, polyphenols are classified as flavonoids and phenolic acids (Williams et al., 2004). These compounds have antioxidant action and exert various beneficial effects in a multitude of diseases (Shirzad et al., 2011; Kabiri et al., 2013). Flavonoids are known to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, cyclooxygenase and lipoxygenase enzyme activities. They exert these effects as antioxidants, free radical scavengers and chelators of divalent cations (Cook and Samman, 1996; Hosseinpour et al., 2013; Rafieian-Kopaie and Baradaran, 2013). The powerful antioxidant activities of flavonoids against free radicals are attributed to their hydrogen-donating ability (Rafieian-Kopaie, 2012). It has been proposed that plant antioxidants can prevent or improve brain injury due to ischemia/reperfusion**.** It is therefore essential to study the effects of antioxidants, free radical scavengers, or trapping agents for use as potential cerebroprotective agents of various brain injuries, including ischemia/reperfusion-mediated brain injury (Baradaran et al., 2012).

Mentha longifolia L. has medicinal and aromatic properties (Gulluce et al., 2007). It grows wild in various regions of Iran (Omidbaigi, 2005) and has been used in Iranian traditional medicine as a stomach pain-relieving, antispasmodic, digestive and carminative agent (Zargari, 1990). Aerial parts of *M. longifolia* possess medicinal properties. The extract of *Mentha* species has been reported to have fungicidal, anti-inflammatory, antimicrobial and antioxidant activities (Mimica-Dukic et al., 2003; Gulluce et al., 2007; Mkaddem et al., 2009).

Stanisavljevic et al. (2012) showed that the highest content of phenolic compounds in *Mentha longifolia* L. was found in the extract obtained from naturally dried plant materials. The dominant phenolic component in this extract was kaempferol 3-O-glucoside and the highest value of antioxidant activity was found in the extract obtained from the naturally dried herbs.

Kaempferol (3, 4′,5,7-tetrahydroxyflavone) is one of the most commonly found dietary flavonols, the biological and pharmacological effects of which may depend upon its behavior as either an antioxidant or a prooxidant (Leung et al., 2007). Based on these results, it may be concluded that *Mentha longifolia* L. extract is a well-recognized source of secondary metabolites, like flavonoids, which are considered as an excellent antioxidant.

However, the neuroprotective effect of ME on ischemia/reperfusion-induced brain injury has not been evaluated thus far. Hence, this study was designed to examine the effect of pretreatment with various doses of intraperitoneal injections of ME in the rat model of transient focal cerebral ischemia on brain infarct volume, brain edema and blood-brain barrier (BBB) permeability. In addition, the impact of intraperitoneal injection of ME on the total antioxidant capacity of the brain and serum, as well as lipid peroxidation in rat brain and serum were characterized.

MATERIALS AND METHODS

Plant material

Aerial parts of *M. longifolia* were collected at the full flowering stage from the city of Shahrekord, Chaharmahal and Bakhtiari Province, Iran, and dried in shade for 14 days. The plant was authenticated in the Herbarium Center of the Shahrekord University of Medical Sciences (Shahrekord, Iran). Fifty g of dried herbal materials was chopped into small pieces and macerated in 250 ml ethanol (80 v/v) for 2 days. Then, it was filtered and evaporated on a rotary evaporator at 40°C. The extract was dried in an oven at 37°C until constant mass and kept in well-sealed glass vessels in a dry, cold and dark place.

Animals

Male Wistar rats, weighing 300-350 g, were used for the study. The rats were obtained from the Central Animal House of Shahid Beheshti University, Iran. Experiments were carried out in accordance with the official regulations approved by Animal Ethics Committee of Shahrekord University of Medical Sciences.

Experimental protocol

Four main groups of animals (n=14 per group) received intraperitoneal injections of different doses of plant extract (50, 100, and 200 mg/kg/day, between 10:00 and 11.00 am) for 21 days. These doses were identified using previously published data (Hosseinzadeh et al., 2011). A control group received the same volume of distilled water. Two hours after administration of the last dose, each group was subdivided into a middle cerebral artery occlusion (MCAO) group that subsequently underwent neuropathological assessment (neurologic deficit scores, infarct volume, brain edema and BBB permeability), and intact subgroups for assessing antioxidant capacity and lipid peroxidation of the brain and serum, respectively. Animals from a sham-operated group (n=7) underwent surgical procedure but without MCAO.

Ischemia induction

Middle cerebral artery occlusion was induced as described by Longa et al. (1989) with minor modifications. The rats were weighed and anesthetized by intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (6 mg/kg). Briefly, the right common carotid artery, external carotid artery and internal carotid artery were isolated through a ventral midline incision. A 3-0 nylon monofilament, with its tip rounded by heating near a flame, was introduced through the external carotid artery stump. The occlude was advanced to the internal carotid artery 20-22 mm beyond the carotid bifurcation until mild resistance indicated that the tip was lodged in the anterior cerebral artery and blocked the blood flow to the middle cerebral artery. One hour after middle cerebral artery occlusion, reperfusion was started by withdrawing the suture. Body temperature of the animals was maintained at 37°C by surface heating and cooling during the surgery.

Assessment of neurobehavioral impact

After the suture was withdrawn, the rats were returned to their separate cages. Twenty four h later, the rats were evaluated neurologically by an observer blind to the animal groups. The neurobehavioral scoring was performed using a six-point scale as previously described (Longa et al., 1989): normal motor function=0; flexion of contra-lateral forelimb upon suspension vertically by tail or failure to extend forepaw=1; circling to the contra-lateral side, but normal posture at rest=2; loss of righting reflex=3; no spontaneous motor activity=4. Death with the score 5 was only considered when a large infarct volume was present in the absence of subarachnoid hemorrhage. If the rats died due to subarachnoid hemorrhage or pulmonary insufficiency and asphyxia, they were eliminated from the study.

Measurement of infract volume

After decapitation, the brains were quickly removed, placed in saline at 4°C for 15 min, and then cut into seven 2-mm coronal slices in a rodent brain matrix (RBM 4000C; Activational Systems, Warren, MI, U.S.A.). The slices were immersed in 2% solution of 2, 3, 5-triphenyltetrazolium chloride (Merck, Germany) and kept at 37°C in a water bath for 15 min. The slices were then digitally photographed (Lumix-Panasonic camera, Japan, connected to a computer). Unstained areas were defined as infarct and measured using image analysis software (UTHSCSA Image Tool). The infarct volume was calculated by measuring the unstained and stained areas in each hemisphere slice, multiplying by slice thickness (2 mm), and then summing all seven slices. To compensate for brain swelling in the ischemic hemisphere (Swanson et al., 1990), infarct volume was corrected in each rat by computing the volume of the left and right hemispheres and applying the following formula: corrected infarct volume = left hemisphere volume − (right hemisphere volume−infarct volume).

Assessment of brain water content

After decapitation, the brains were removed, the cerebellum, pons and olfactory bulbs were separated, and their wet weights (WW) were measured (Bigdeli et al., 2007). After drying in a desiccating oven at 120°C for

24 h, dry weights (DW) were assessed. Brain water content (BWC) was expressed as percent H_2O, which was calculated using [(WW−DW)/WW] ×100.

Evaluation of BBB permeability

BBB disruption was measured as a function of Evans Blue (EB, Sigma Chemicals, USA) extravasation. BBB is normally impermeable to Evans Blue, which can only cross the barrier when it is disrupted. Briefly, the rats received 4 ml/kg of 2% EB solution in saline by tail vein injection 30 min after MCAO. Twenty-four h after reperfusion, each rat was transcardiacally perfused with 250 ml of physiological saline under anesthesia. After decapitation, the brains were removed and the hemispheres were separated and weighed. The right and left hemispheres were separately homogenized in 2.5 ml of phosphate-buffered saline to extract the EB; to precipitate protein; 2.5 ml of 60% trichloroacetic acid was added and mixed by vortex for 3 min. The samples were then placed at 4°C for 30 min and centrifuged for 30 min at 1000 g. The absorbance was read at 610 nm by a spectrophotometer (Perkin-Elmer, Illinois, USA). EB levels were expressed as ng/g of brain tissue against a standard curve (Bigdeli et al., 2007).

Determination of antioxidant power in the brain and serum

Antioxidant power of the brain or serum was determined by measuring the ability to reduce Fe^{3+} to Fe^{2+}, referred to as the ferric reducing antioxidant power (FRAP) test (Heidarian et al., 2013a). For assessing antioxidant power in the brain, the animals were decapitated and the brains were rapidly removed from the dead animals within 30 s from decapitation and immersed in liquid nitrogen. After weight determination, they were homogenized in the buffer (3 g Tris base in 450 ml dd H_2O, 38.52 g sucrose, 186 mg EDTA (ethylene diamine tetra acetic acid) and scaled up to 500 ml with dd H_2O, pH=7.4). The homogenate was centrifuged (10 min at 4000 rpm) and the supernatant was then separated. The reagents included 300 mM acetate buffer solution (pH=3.6) with 16 ml of acetic acid per 1 l of buffer solution, 10 ml of TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM $FeCl_3$. Working FRAP reagent was prepared as required by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3$. Fifty µl of supernatant was added to 1.5 ml of freshly prepared reagent warmed at 37°C. After 10 min, the complex between Fe^{2+} and TPTZ had a blue color with absorbance at 593 nm. Data were expressed as µM per g of wet tissue. For assessing antioxidant power in the serum, fifty µl of serum was added to 1.5 ml of freshly prepared FRAP reagent warmed at 37°C. After 10 min, the complex between Fe^{2+} and TPTZ had a blue color with absorbance at 593 nm. Data were expressed as µM per l of serum.

Assessment of lipid peroxidation in the brain and serum

Analysis of malondialdehyde MDA by the thiobarbituric acid (TBA) assay has been widely employed, initially as a measure of rancidity in food and in biological systems for assessing lipid peroxidation (Heidarian and Rafieian-Kopaei, 2013). We used the TBA to assess the ischemia/reperfusion damage. TBA is a spectrophotometric assay based upon sample heating under acidic conditions to form an adduct MDA-$(TBA)_2$. Specificity of the assay has been questioned, and high-performance liquid chromatography (HPLC) of the MDA-$(TBA)_2$ complex has been introduced to improve the specificity of the reaction (Bird et al., 1983). For assessing lipid peroxidation in the brain, the animals were decapitated and the brains were rapidly removed from the dead animals within 30 s from decapitation and immersed in liquid nitrogen. After weight determination, they were homogenized in the buffer (3 g Tris base in 450 ml dd H_2O, 38.52 g sucrose, 186 mg EDTA and scaled up to 500 ml with dd H_2O, pH=7.4). The homogenate was centrifuged (10 min at 4000 rpm) and the supernatant was separated. The TBA assay in the brain was adapted from Heidarian et al. (2013b). The optimized TBA assay was as follows: 100 µl of supernatant was treated with 10 µl of 0.5 M BHT (butylated hydroxytoluene) (in absolute ethanol) followed by the addition of 200 µl of 8.1%

SDS (sodium dodecyl sulfate) and 1.5 ml of 0.8% TBA (0.8 g TBA, 19 ml of 20% acetic acid, and scaled up to 100 ml with dd H_2O, pH=3.5). Then, the samples were heated for 60 min in a boiling-water bath. After cooling to 4°C, 3 ml of butanol-pyridine was added to the samples and centrifuged at 4000 g for 10 min. Finally, an aliquot (120 µl) was directly injected into the HPLC. Data were expressed as µM per g of wet tissue.

Serum MDA was determined by TBA method as described by Asadi et al. (2013). In this assay, 50 µl of serum was treated with 50 µl 0.05% BHT (in absolute ethanol) followed by the addition of 400 µl 0.44 M H_3PO_4 (aqueous) and 100 µl 42 M TBA (aqueous), which was mixed by vortex. Then, the samples were incubated for 60 min at 100°C. After cooling at 4°C, 250 µl of butanol-pyridine was added to the samples, vortexed, and centrifuged at 13000 g for 7 min. Finally, an aliquot (120 µl) was directly injected into the HPLC. Data were expressed as µM per l of serum.

HPLC separation

Separation of the MDA-$(TBA)_2$ adduct was performed using the automated Waters™ HPLC system (510 Pump,717 Autosampler and 486 Detector (at 532 nm), driven by the Millennium 2010 Chromatography Manager software (Waters Ltd., Watford, Hertfordshire, England). The analytical column was a reverse phase silica-based C_{18} column (Spherisorb 5ODS 2, HPLC Technology Ltd. (Warrington, Cheshire, England)) with the column dimensions of 25 cm×4.6 mm and a C_8 guard column (HPLC Technology Ltd.). The elution buffer was determined to be 50 mM KH_2PO_4-KOH, pH=7.0, 35% methanol, which was degassed under vacuum through a 0.45-µM filter (Millipore, type HAWP). The sample run was 10 min with a flow rate of 1.2 ml/min, injection volume of 120 µl and visible detection of 532 nm. All the standards and samples were run and data were analyzed automatically using the Millennium software package. The mean peak area was determined for each sample run in duplicate and the levels of MDA were calculated directly from the calibration of the TEP (1,1,3,3-tetraethoxypropane) standards (Heidarian et al., 2013b).

Determination of the antioxidant activity of the extracts

The free radical scavenging activity of ME was measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical using Heidarian and Rafieian-Kopaei's (2013) method. Briefly, a 0.1-mM solution of DPPH in methanol was prepared and 2 ml of this solution was added to 2 ml of the sample solution of the extract in methanol at different concentrations (10-100 μg/ml). After 15 min of incubation at room temperature in the dark, the absorbance was measured at 517 nm. BHT was used as a positive control. The control was prepared like the test samples and methanol was used for baseline correction. Inhibition of free radicals by DPPH (%) was calculated using the following formula:

$$\text{Inhibition of free radical by DPPH (\%)} = \frac{(A_{0-A_1})}{A_0} \times 100$$

where A_0 is the absorbance of the control reaction and A_1 is absorbance in the presence of the standard sample or extract.

Determination of total phenol contents

The total phenol content in the extracts was determined according to the Folin-Ciocalteu (FC) reagent (Sharafati et al., 2011) using gallic acid (GA) as the standard. The extract solution in 70% ethanol (0.2 ml, 1 mg/ml) was mixed with the FC reagent (1 ml) and aqueous solution of Na_2CO_3 (0.8 ml, 7.5%). After 30 min of incubation at room temperature, the absorbance of the reaction compound at 765 nm was measured by spectrophotometer. The overall phenol content was expressed as mg of gallic acid/g of extract and calculated using the equation of the standard curve.

Determination of total flavonoid contents

The total flavonoid content in the extracts was determined by spectrophotometry, based on the production of complex compounds of flavonoids with aluminum chloride (Quettier et al., 2000). Each plant

extract (2.0 ml, 1.0 mg/ml) in 70% ethanol was mixed with 0.10 ml of 10% aluminum chloride, 0.10 ml of (1.0 M) potassium acetate and 2.8 ml of distilled water. After 30 min of incubation at room temperature, the absorbance of the reaction mixture was measured at 415 nm in relation to distilled water. Rutin (RU) was chosen as the standard and the total flavonoid content was expressed as mg of rutin/g of extract and counted using the equation of the standard curve.

Statistical analysis

Graph Pad Prism 5.0 (Graph Pad Software Inc., California, USA) software was used to analyze the data and the results were expressed as mean±SEM. All the variables, except the neurological score, were analyzed using one-way ANOVA and post-hoc LSD. The neurologic deficit scores were analyzed using Mann-Whitney U test. Differences were considered statistically significant at $p<0.05$.

RESULTS

Assessment of free radical scavenging activity

The free radical scavenging effect is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50% (IC_{50}). IC_{50} indicates the potency of scavenging activity. In this investigation, BHT was found to have the IC_{50} of 35.64±0.76 µg/ml. Compared to BHT, ME showed an IC_{50} of 62.27±0.72 (Table 1).

Content of total phenols and flavonoids in the extract

The total phenol content in the examined extract was 112.17±1.9 mg of GA/g of extract, while the concentration of total flavonoid in the plant extract from ME was 89.71±0.5 mg RU/g of extract (Table 2).

Effects of ME on neurologic deficit scores

Median neurologic deficit scores (NDS) were reduced by ME, and were as follows: 1 (range: 0-4), 0 (range: 0-2), 1 (range: 0-4), and 2 (range: 2-4) in the 50, 100 and 200 mg/kg/day and control groups, respectively (Table 3). Putative beneficial effects of ME were confirmed by a reduction in infarct volume (Fig. 1).

Table 1. DPPH radical scavenging activity of ME and BHT as the positive control.

IC50 (µg/ml)	Sample
62.27±0.72	ME
35.64±0.76	BHT

Each value is the mean ± standard error.

Table 2. Total phenol and flavonoid contents in the *M. longifolia* extract.

Sample	Total phenol (mg of GA/g of extract)	Total flavonoid (mg of RU/g of extract)
M. longifolia extract	112.17±1.9	89.71±0.5

Each value is the average of three analyses ± standard errors.

Effects of ME on infarct volume

Pretreatment with 100 mg/kg/day ME for 21 days and 2 h before MCAO resulted in the reduction of total infarct volume ($p=0.04$), while lower and higher doses (50 and 200 mg/kg/day, respectively) had no effects (Fig.1). The neuroprotection exerted by ME was mainly seen in the penumbra (cortex), while slightly observed in the infarct core. Beside pretreatment with 50 and 200 mg/kg/day, ME reduced infarct volume in the penumbra but not in core ($p=0.001$ and $p=0.000$, respectively).

Effects of ME on brain edema

Focal cerebral ischemia resulted in a significant increase in the brain water content of the ischemic hemisphere in the control compared with the sham group ($p=0.004$). Pretreatment with ME in a dose of 100 mg/kg/day for 21 days and 2 h before MCAO induced a statistically significant ($p=0.024$) reduction in brain water content in the ischemic hemisphere, while in the other groups (50 and 200 mg/kg/day) it had no effects. In addition, the brain water content of the ischemic and non-ischemic hemispheres in the control group was significantly different ($p=0.029$) (Fig. 2).

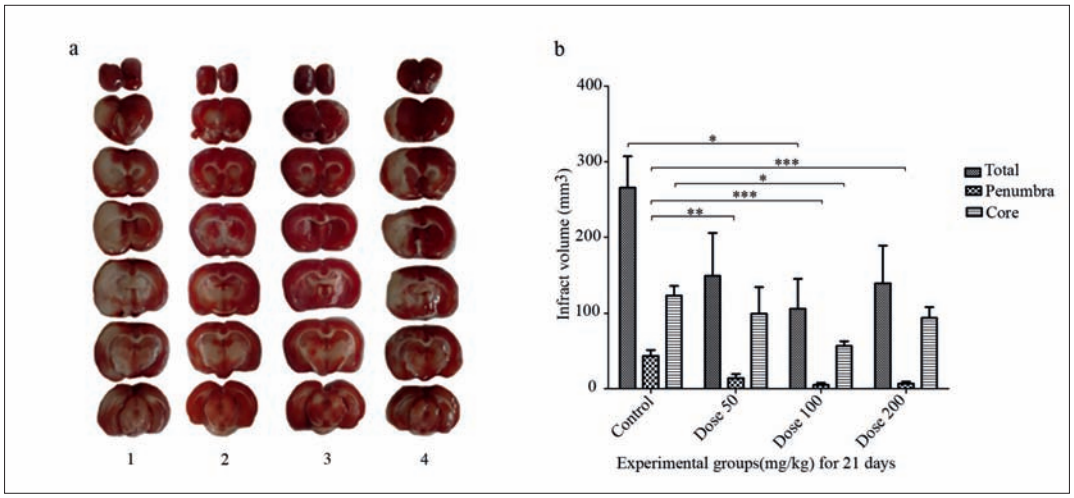

Fig. 1. (a) Photographs showing the neuroprotective effect of *M. longifolia* extract (ME) on focal cerebral ischemia. All the sections of the brain were stained with TTC 24 h after 60 min of ischemia. Each column represents the series of rat brain coronal sections: (1) Brain sections of control rats; (2), (3), and (4) brain sections of rats pretreated with 50, 100 and 200 mg/kg/day of ME, respectively. (b) The graph shows the effects of various doses (control, 50, 100 and 200 mg/kg) of ME on total infarct volume in different experimental groups (*p<0.05; n=7).

Table 3. Distribution of neurologic deficit score in each group.

Statistical results (*P* value)	Median	Total (*N*)	NDS in each group					Experimental groups	No
			4	3	2	1	0		
1:3 sig (0.008)	2	7	1	0	6	0	0	Control (dose 0)	1
1:2, 1:4, 2:3, 2:4 and 3:4 nonsig	1	7	1	0	2	1	3	Dose 50	2
	0	7	0	0	2	1	4	Dose 100	3
	1	7	1	0	2	3	1	Dose 200	4
	-	28	3	0	12	5	8	Total (*N*)	-

NDS: Neurologic deficit score; *N*: Number of cases per group; sig: Significant; nonsig: Non-significant

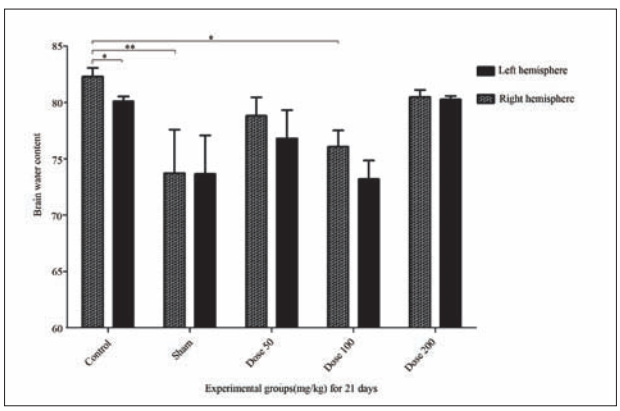

Fig. 2. Brain water content in the left and right hemisphere of control, sham-operated and groups pretreated with 50,100 and 200mg/kg/day of ME (*p<0.05; **p<0.01; n=7).

Effects of ME on BBB permeability

Brain edema formation was associated with increased BBB permeability at 24 h. In the control group, EB concentration in ischemic cerebral tissue was 85.98±9.93 and 59.85±4.52 ng/g tissue in the non-ischemic left hemisphere. Pretreatment with 50, 100 and 200 mg/kg/day of ME was associated with the significant reduction in EB extravasation in the ischemic hemisphere compared with the control group (p=0.001, p=0.000, and p=0.002, respectively). BBB permeability was not significantly higher in the ischemic right hemisphere scale compared to the non-ischemic left hemisphere in the groups pretreated with ME (Fig. 3).

Effects of ME on brain and serum antioxidant capacities

In the brain, antioxidant capacity was significantly decreased in the control group compared with sham-operated animals (p=0.000). Extract of *M. longifolia* at the doses of 100 and 200 mg/kg/day increased antioxidant capacity in the brain (p=0.000 and p=0.001; respectively) compared with the control group. At the dose of 50 mg/kg/day, the extract did not significantly modify the brain's antioxidant power (Fig. 4).

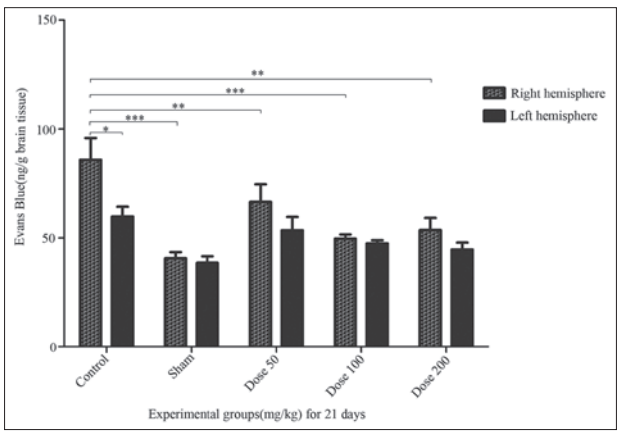

Fig. 3. EB extravasations in various experimental groups including left and right hemisphere of control, sham-operated, and groups pretreated with 50, 100 and 200 mg/kg/day of ME (*p<0.05; **p<0.01; ***p<0.001; n=7).

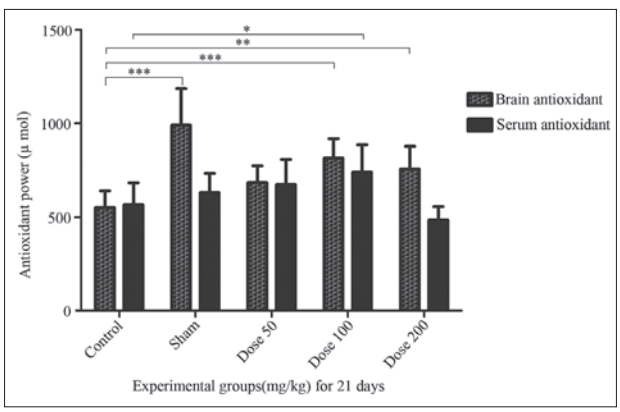

Fig. 4. Effects of ME on brain and serum antioxidant power in rats subjected to transient cerebral ischemia. The extract was administered intraperitoneally at different doses of 50, 100 and 200 mg/kg/day. (*p<0.05; **p<0.01; ***p<0.001; n=7).

The antioxidant capacity of serum in the 100-mg/kg/day group was significantly higher than that of the control group (p=0.01). Sham operation and pretreatment with doses of 50 and 200 mg/kg/day of ME had no statistically significant impact on the antioxidant power of serum compared to the control group (Fig. 4).

Effects of ME on lipid peroxidation in the brain and serum

The MDA level in the brain was significantly elevated in the control compared with the sham group. Alternatively, pretreatment with all investigated doses of ME (50, 100 and 200 mg/kg/day) considerably lowered the brain level of MDA (p=0.001, p=0.000, and p=0.000, respectively). Moreover, the extract of *M. longifolia* at the doses of 100 and 200 mg/kg/day reduced the MDA level of serum (p=0.002 and p=0.002, respectively), while the lower dose (50 mg/kg/day) had no effect (Fig. 5). As in the brain, the serum MDA level was significantly elevated in the control compared to the sham groups (p=0.033).

DISCUSSION

The results of this study show that transient cerebral ischemia induces lipid peroxidation and decreases the antioxidant power of the brain, as previously

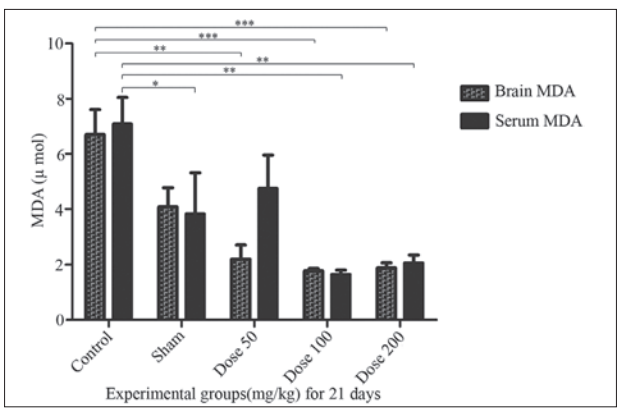

Fig. 5. Effects of ME on brain and serum MDA levels in rats subjected to transient cerebral ischemia. The extract was administered intraperitoneally at different doses of 50, 100 and 200 mg/kg/day. (*p<0.05; **p<0.01; ***p<0.001; n=7).

described (Asgary et al., 2013a; Akbari et al., 2013; Calapai et al., 1993). Other studies have shown the neuroprotective property of ME by multi-mechanisms, such as antioxidation and effects on the lipid metabolism (Gulluce et al., 2012; Lopes et al., 2010) Therefore, it can be speculated that ME might play a key role in the treatment of ischemic disorders of the cerebrovascular system. Pretreatment with ME markedly reduced the infarct size induced by MCAO and produced significant protection against neuronal damage. This effect was in harmony with other studies showing that antioxidant agents could reduce various brain injuries, including ischemia/reperfusion-mediated brain injury (Tanaka et al., 2007; Rabiei et al. 2014). Interestingly, we have shown that all doses of ME radically reduced the volume of ischemic penumbra that surrounds the infarct core, which may contribute to the better neurological recovery noticed in the ME-treated groups. Our observation is in agreement with the fact that there is a high correlation between the extent of spontaneous neurological recovery and the volume of penumbra that escapes infarction (Guadango et al., 2003).

The beneficial effects of naturally occurring antioxidants have been reported in rodent models of stroke. For example, pretreatment with antioxidants, such as vitamin E (Khanna et al., 2005), *Ginkgo biloba* extract and α-lipoic acid (Clark et al., 2001) reduced ischemic brain damage in rodents. The protective effects of antioxidants have been also reported in clinical observations. For example, the intake of an antioxidant-enriched diet was associated with lower risk for cerebral infarction in male patients (Hirvonen et al., 2000). Similarly, low antioxidant activity in plasma is associated with greater lesion volumes and neurological impairments in stroke patients (Leinonen et al., 2000). Taken together, these data suggest that compounds that possess antioxidant properties can reduce ischemia-mediated neurodegeneration in the brain.

In the present study, the highest dose of ME (200 mg/kg) had no better effect on median neurologic deficit scores (NDS) than the lower doses (50 and 100 mg/kg). Pretreatment with 100 mg/kg/day of ME resulted in the reduction of total infarct volume ($p=0.04$), while the lower and higher doses (50 and 200 mg/kg/day, respectively) had no such effects. Focal cerebral ischemia resulted in a significant increase in the brain water content of the ischemic hemisphere in the control compared with the sham and 100-mg/kg/day groups; however, this was not the case in the 50- and 200-mg/kg/day groups. Also, pretreatment with 100 mg/kg/day of ME had a better effect on BBB integrity in the ischemic hemisphere and the antioxidant power of the brain and serum compared with the 200-mg/kg/day group. The lack of positive effect of 200 mg/kg/day was somewhat surprising, because 100 mg/kg/day positively affected all the above-mentioned examinations. The exact mechanism is not clear, but pro-oxidant activity of ME in high dose might have caused the reduced effect in this dose. In the sham group, surgery stress led to the production of antioxidant enzymes. These enzymes can increase antioxidant power and decrease lipid proxidation (Halliwell, 1994). Although antioxidants have been shown to possess protective effects on various diseases such as diabetes (Bahmani et al. 2014; Asgary et al., 2014), atherosclerosis (Mirhosseini etal., 2014; Asgary et al., 2013b), nephrotoxicity (Baradaran et al., 2013; Nasri et al., 2013), cardiovascular diseases (Khosravi-Boroujeni et al., 2012; Sarrafzadegan et al., 2013), as well as brain ischemia, under certain circumstances, they may act as pro-oxidant inducing oxidative stress. It has been suggested that the pro-oxidant properties of antioxidants are mostly concentration-dependent. The generation of the superoxide anion radical and products of lipid peroxidation is increased with increasing the concentrations of hesperetin, quercetin, naringenin or morin in human lymphocytes. Moreover, these compounds induce DNA strand breakage at high concentrations (Yen et al., 2003).

BBB selectively limits the passage of solutes to the brain, thereby maintaining homeostasis and protecting the brain from toxins. Focal cerebral ischemic injury results in the disruption of BBB in rodents (Yang et al., 1994), as well as in humans (Latour et al., 2004). The data presented in this study indicated that ME may influence brain water content and brain water homeostasis by directly increasing BBB integrity, modulating cell volume of neurons and astrocytes.

Mohagheghi et al. (2010) showed that pretreatment with dietary virgin olive oil can reduce infarct volume, brain edema, BBB permeability and neurobehavioral deficit scores in a reliable and reproducible animal model of stroke followed by reperfusion. Oxidative stress, via the increased production of ROS and lipid oxidation, induces inflammatory response. The primary reason for the particular vulnerability of the brain in cerebral ischemia is the fact that the inter- and intracellular signalling mechanisms crucial for the normal functions of the brain become harmful under ischemic conditions. In addition, energy failure is accelerated, enhancing the final pathways underlying ischemic cell death, including free radical production, activation of catabolic enzymes, membrane failure, inflammation and apoptosis (Centonze et al., 2001). Lack of ATP causes the cells to enter a state of anoxic depolarization, which opens up voltage sensitive ion channels and allows the pathological entry of calcium, sodium and chloride ions into the cells. Passive and excessive entry of water into the cells subsequently results in cytotoxic edema (Siesjö, 1992).

M. longifolia phenols with high antioxidant capacity can thus inhibit lipid oxidation and block inflammatory responses (Karimian et al., 2013). Flavonoids also have antiapoptotic activity. The fact that they increase cell survival in an oxidative stress model where scavenging antioxidants fail to protect cells from the oxidative insult suggests that these molecules have a specific cell survival-increasing activity in addition to their scavenger activity. Cytoprotective capacity of flavonoids may be linked to their ability to activate intracellular molecules (kinases, phosphatases, gene promoters), which in turn activates the components of intracellular cascades and promotes the expression of survival signals. This issue would be specifically true for cellular protection in cases where apoptosis is an important component of cell death (Dajas et al., 2003).

This study was focused on the effects of ME on the antioxidant potential and lipid peroxidation, which are important in neuroprotection assessments. Although the mechanism of neuroprotection induced by ME requires further elucidation, it has been shown that polyphenolic compounds in ME exert their antioxidant activities in the following ways: by giving out an H-atom, by directly connecting free oxygen and nitrogen radicals, by chelating prooxidant metal ions (Fe or Cu), and by inhibiting prooxidant enzymes (lipoxygenase, myeloperoxidase, xanthine oxidase, NAD(P)H oxidase, and cytochrome enzymes P-450) (Cook and Samman, 1996).

The presented experimental data showed that, in the brain, pretreatment with ME doses of 100 and 200 mg/kg/day significantly increased antioxidant power in comparison with the control group. In addition, the antioxidant power of serum in the 100-mg/kg/day group was significantly higher than in the control group. Compelling evidence implicates free radicals act as major contributors to ischemic and excitotoxic tissue injury in the CNS. The mechanisms of calcium-dependent free radical (FR) generation caused by ischemia reoxygenation injury might include xanthine/xanthine oxidase reaction and activation of NO or phospholipase A_2 (Shaheen et al., 2009).

The presented results show that there was a significant decrease in the level of lipid peroxidation in the brain and serum after treatment with ME. The extract reversed the increase of MDA levels to a considerable extent, thereby confirming its probable antioxidant role in ischemia. Peroxidation of lipids is a binding process connected to the formation of MDA (Niedworok and Bielaszka, 2007), which has been utilized as a suitable biomarker for lipid peroxidation (Atip et al., 2010). Besides, in the ischemic brain, a substantial elevation in the lipid peroxidation along with depletion in the activity of various protective antioxidant enzymes was observed, which could be validated by many earlier reports (Gupta et al., 2003; Sugawara and Chan, 2003). However, treatment with ME resulted in decreased MDA level and increased antioxidant power when compared with the control group, suggesting the decreased formation of ROS or radical scavenging activity of ME.

In another study, the protective role of oleuropein, a phenolic compound present in olive leaf extract, in the midbrain of aged rats has been investigated by Sarbishegi et al. (2013). The finding showed

that the lipid peroxidation level was decreased after oleuropein treatment in the experimental group, which could be due to the antioxidant effect of oleuropein phenolic compounds. Researchers have suggested that the MDA levels, as a result of lipid peroxidation in damaging brain, are overestimated and one of the beneficial effects of oleuropein is the reduction of MDA in these patients.

Since the antioxidant effects of some species of *Pistacia* have been reported (Weia et al., 2002), the protective effects of *Pistacia vera* L. gum extract on oxidative damage following cerebral ischemia were studied in rats. Phytochemical analysis revealed that the gum extract of *P. vera* had flavonoids, tannins and saponins. The MDA levels increased significantly following cerebral ischemia-reperfusion injury, but the extract reversed the increase of MDA levels to a considerable extent, confirming its antioxidant role in the ischemia. Researchers have also evaluated the antioxidant or reducing potential of hippocampus homogenate samples following ischemia reperfusion injury using FRAP assay. As expected, following the reperfusion, the extract increased the antioxidant power of homogenate samples of the hippocampus (Mansouri et al., 2005).

Phytochemical studies have revealed that ME has natural antioxidants such as flavonoids. Recently, the antioxidant properties of various flavonoids and related phenolic compounds have been studied extensively. In addition, antihypoxic and anti-ischemic activities have been reported for some of these components (Calapai et al., 2000; Nishimura et al., 2000).

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Conflict of interest disclosure: Farhad Fathi, Shahrbanoo Oryan, Mahmoud Rafieian, and Akram Eidi declare that they have no conflict of interests. All the institutional and national guidelines for the care and use of laboratory animals were followed.

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