Aqueous and Ethanol Extracts of *Boswellia serrata* Protect Against Focal Cerebral Ischemia and Reperfusion Injury in Rats

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Oxidative stress and cell apoptosis play major roles in neuronal injury after ischemia–reperfusion (I-R) injury. *Boswellia serrata* is a medicinal plant with antioxidant properties. Acetyl-11-keto-β-boswellic acid (AKBA) is an active triterpenoid compound from *B. serrata*. In the current study, the neuroprotective effects of aqueous and ethanol extracts of *B. serrata* (named ABS and EBS, respectively) and AKBA were investigated against middle cerebral artery occlusion-induced cerebral I-R injury in rats. ABS and EBS with doses of 125, 250 and 500 and AKBA with a dose of 50 mg/kg were administered (intraperitoneally) just after middle cerebral artery occlusion induction for 30 min and reperfusion for 24 h. HPLC analysis suggested that ABS and EBS had AKBA of 8.8% and 9.5% (w/w), respectively. *B. serrata* and AKBA significantly improved neurological deficit and reduced brain infarction, neuronal cell loss and apoptosis and also attenuated lipid peroxidation while increasing glutathione content and superoxide dismutase activity in the cerebral cortex following a stroke. Apoptosis suppression was found to be mediated through regulating caspase-3 and bax/bcl-2 expressions. In conclusion, our results demonstrated that *B. serrata* and AKBA attenuate oxidative damage and inhibit cell apoptosis, subsequently protecting cerebral I-R injury in rats. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: *Boswellia serrata*; acetyl-11-keto-β-boswellic acid (AKBA); middle cerebral artery occlusion (MCAO); oxidative stress; apoptosis; neuroprotection.

INTRODUCTION

Stroke is the third leading reason of death and one of the main causes of disability in the world (Strong et al., 2007; Feigin et al., 2009). Because of its high incidence and life-threatening nature, it is a major challenge to public health (Rossi et al., 2007). About 80% of stroke is ischemic (Della-Morte et al., 2012), a consequence of a transient or permanent decrease in cerebral blood flow that is restricted to the territory of a main brain artery (Mahajan et al., 2004).

Numerous recent studies have presented that oxidative stress plays a major role in neuronal injury after ischemic–reperfusion (I-R) insult (Allen and Bayraktutan, 2009; Niizuma et al., 2009; Thaakur and Sravanthi, 2010; Sharma et al., 2014). Oxidative metabolism is necessary for survival of brain and besides is associated with the generation of reactive oxygen species (Albarracin et al., 2012). In physiologic conditions, reactive oxygen species is regulated via an endogenous antioxidant defense mechanism and maintains homeostasis; if any disturbance in this homeostasis occurs, it lead to oxidative stress, which is the key factor for initiation of inflammation and apoptotic cascades that cause neuronal damage (Rao and Balachandran, 2002; Yuan et al., 2013). *Boswellia serrata* is a tree of the Burseraceae (frankincense) species that abundantly grows in India, Africa and the Middle East (Dalla Libera et al., 2014). The oleogum resin of *B. serrata* has been traditionally used in folk medicine for a variety of therapeutic purposes such as headache (Dalla Libera et al., 2014), hyperlipidemia (Gerhardt et al., 2001), colitis (Gupta et al., 2001), arthritis (Sharma et al., 1989), cancer (Shao et al., 1998) and inflammation (Singh and Atal, 1986; Darshan and Doreswamy, 2004; Sferra et al., 2012).

Recently, numerous publications reported the encouraging results of *B. serrata* on a variety of inflammatory disorders, such as asthma (Gupta et al., 1998), inflammatory bowel disease (Poockel and Werz, 2006), osteoarthritis (Kimmatkari et al., 2003) and rheumatoid arthritis (Sander et al., 1998). Moreover, it reduced peritumoral brain oedema accompanying glioma (Winking et al., 2000). Also, it has been shown that *B. serrata* resin possesses strong antioxidant and immunomodulatory properties (Ammon, 2010; Kokkiripati et al., 2011).

Based on the antioxidant and antiinflammatory effects of *B. serrata*, the current study was carried out to estimate the effects of *B. serrata* extract on ischemic cerebral reperfusion injury in rats.
MATERIALS AND METHODS

Preparation of ABS and EBS. The oleo-gum resin of B. serrata was obtained from the Medicinal Plants Division of Imam Reza Pharmacy (herbarium no. 92083). They were washed, dried and crushed to powder with an electric micronizer. For preparation of aqueous and ethanolic extracts from B. serrata (named ABS and EBS, respectively), 10 g of the powder was soaked and gently agitated in 100 mL of ethanol (96%) or distilled water at 40°C for 48 h. After that, the extracts were filtered and the solvents were removed using a rotary evaporator. The residues were then maintained at −20°C until use. The percent yield of the procedure was about 30%.

Animals. Male Wistar rats weighing 200–230 g were obtained from the animal facilities of the School of Medicine, Mashhad University of Medical Sciences (Mashhad, Iran). The animals were housed five per cage with a 12 h light/dark cycle at 21±2°C and had free access to food and water, ad libitum. All animals were treated in accordance with the National Institutes of Health Guidance for the Care and Use of Laboratory Animals, and their use was approved by the Animal Ethics Committee of Mashhad University of Medical Sciences.

Chemicals. 2, 2′-Dinitro-5, 5′-dithiodibenzoic acid, 2-thiobarbituric acid (TBA), dimethyl sulfoxide, potassium chloride, hydrochloric acid (HCl) and trichloroacetic acid (TCA) were bought from Merck (Darmstadt, Germany). A terminal deoxynucleotidyl transferase–dinitro-5, 5′-dithiodibenzoic acid–Dinitro-5, 5′-dithiodibenzoic acid–dithiodibenzoic acid, sodium deoxycholate, complete protease inhibitor cocktail (P8340), phenyl-β-galactosidase, sodium orthovanadate (Na3VO4), triphenyl tetrazolium chloride (TTC), chloral hydrate, Tris-HCl, ethylenediaminetetraacetic acid (EDTA), sodium fluoride, sodium orthovanadate (Na3VO4), β-glycerol phosphate, sodium deoxycholate, complete protease inhibitor cocktail (P8340), phenylmethylsulfonyl fluoride, sodium dodecyl sulfate (SDS), mercaptoethanol, bromophenol blue, glycerol, β-actin (4967), caspase 3 (9665), bax (2870) and horseradish peroxidase-conjugated goat anti-rabbit IgG (7074) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-acetyl-11-keto-β-boswellic acid quantification was carried out by HPLC on a reversed-phase C18 analytical column (25 × 94.6 mm, 4.6 mm particle size), using an isocratic mobile phase of acetonitrile–water (90:10% v/v) adjusted to pH 4 with glacial acetic acid at a flow rate of 1 mL/min. Ultraviolet monitoring was carried out at 260 nm (Shah et al., 2008). The chromatograms of a sample of ABS, EBS and standard AKBA were shown in Fig. 1A–C.

INDUCTION OF FOCAL CEREBRAL ISCHEMIA–REPERFUSION INJURY

The right MCAO was induced by the intraluminal filament model with some modifications, according to the method described by Longa et al. (1989). Each animal was anaesthetized with chloral hydrate (360 mg/kg) intraperitoneally (i.p.), and through a ventral cutaneous exposure, a sterile silicone-coated 3/0 nylon suture was introduced into the internal carotid artery and advanced intracranially to a distance of 17.5 mm from the carotid bifurcation to temporarily occlude the blood flow into the middle cerebral artery. After 30 min ischemia, the silicone-coated 3/0 nylon suture was carefully removed to establish reperfusion for 24 h. Rectal temperature was maintained at 37±0.5°C with use of a heat lamp during the surgery. The cutaneous wound was then sutured and disinfected. After recovering from the anaesthesia, the animals were returned to their cages.

Experimental protocol

The rats were randomly divided into nine groups, each with 6–8 rats: Group 1, the sham group, was the only group that underwent surgery without the induction of ischemia. Group 2 was the control group that underwent the MCAO operation and received a saline solution (10 mL/kg) i.p. Moreover, in groups 3–8, ABS and EBS (125, 250 and 500 mg/kg dissolved in saline) and in group 9 AKBA at a dose of 50 mg/kg dissolved in 0.5% dimethyl sulfoxide in saline solution were i.p. injected just after MCAO induction (Chen et al., 2016; Ding et al., 2014b). The doses and time were selected based on previous studies and earlier experiments (Ding et al., 2014a, 2014b; Hosseini-Sharifabad and Esfandiari, 2015).

Neurological assessment

The neurological score of each rat was carefully evaluated 24 h after MCAO induction (Vafaee et al., 2012). For observing the symmetry in the movements of the four limbs, the rats were kept in the air by the tail. The neurological scores were as follows: (3) when all four limbs extend symmetrically, (2) when the limbs on the left side extended fewer or extra slowly than those on the right side, (1) when the limbs on the left side had the minimum motion and (0) when there is no motion on the forelimb on the left side. Neurological examinations were carried out in 3 to 5 min.

QUANTIFICATION OF AKBA IN ABS AND EBS

Acetyl-11-keto-β-boswellic acid quantification was carried out by HPLC on a reversed-phase C18 analytical column (25 × 94.6 mm, 4.6 mm particle size), using an isocratic mobile phase of acetonitrile–water (90:10% v/v) adjusted to pH 4 with glacial acetic acid at a flow rate of 1 mL/min. Ultraviolet monitoring was carried out at 260 nm (Shah et al., 2008). The chromatograms

DETERMINATION OF CEREBRAL INFARCT VOLUME

After behavioural observations, rats were sacrificed and the brains were removed and sliced into 2-mm
sections using a brain matrix. The brain slices were then incubated in 2% TTC solution for 30 min at 37 °C. The slides were then photographed (Canon PowerShot SX120 IS) and the infract regions were analysed using an image processing software (NIH Image J 1.48). The infarct volume was then calculated with the following formula:

\[
\text{Corrected infarct volume} = \frac{\text{infarct volume}}{\text{right hemisphere volume}} - \frac{\text{left hemisphere volume}}{\text{right hemisphere volume}}
\]

(Vafaee et al., 2012).

Histopathological examinations

The animals were deeply anaesthetized with chloral hydrate (400mg/kg) and transcardially perfused with 100mL of heparinized phosphate buffered saline (PBS), followed by 100mL of 4% paraformaldehyde in phosphate buffer (pH7.4). Brains were carefully removed and postfixed in the same fixative for 24h, dehydrated and embedded in paraffin using an automated tissue processor. A total number of six coronal brain sections (5μm) per rat were used. The sections were then stained with haematoxylin and eosin (H&E) and examined under a Leica DMRB microscope (Leica, India) (Ghorbani et al., 2016). Photographs were then

Figure 1. (A–C) HPLC analysis for quantification of 3-acetyl-11-keto-β-boswellic acid (AKBA) in plant extracts. (A) An HPLC chromatogram of AKBA dissolved in mobile phase (10μg/mL). (B) A chromatogram of purified aqueous extract of *Boswellia serrata* (ABS) sample (100μg/mL). (C) A chromatogram of purified ethanolic extract of *B. serrata* (EBS) sample (100μg/mL). Detection was carried out at λ 260 nm.
taken using a Canon PowerShot S70 digital camera (Canon, Japan) at 400× magnification, and cell counts in both the ipsilateral and contralateral sides of the cortex areas were scored in a blinded manner.

Apoptotic cell detection

To detect apoptotic cells, DNA fragmentation in apoptotic cell nuclei was determined using the TUNEL reaction by means of a TUNEL POD kit (Roche, Germany). First, tissue sections were deparaffinized with xylene, rehydrated through descending concentrations of ethanol and rinsed for 10 min in 0.1 M PBS and then treated with 20 μg/mL protease K for 20 min at room temperature. The specimens were then treated with 3% H₂O₂ in methanol for 10 min to inactivate endogenous peroxidase. After washing with PBS, specimens were incubated in the labelling reaction mixture containing terminal deoxynucleotidyl transferase and biotinylated dUTP at 4 °C overnight. After incubation, all the sections were rinsed in PBS and incubated with horseradish peroxidase (1:500) for 30 min at room temperature. Then, the sections were washed extensively with PBS for 3 min and treated with 3,3′-diaminobenzidine solution (30 mg 3,3′-diaminobenzidine and 200 μL H₂O₂/100 mL PBS) for 15 min at room temperature in dark. After being washed under running water, all the sections were counterstained with haematoxylin for 1 min. Finally, the sections were dehydrated in increasing graded ethanol, cleared in xylene and mounted with a cover slip. In this method, apoptotic nuclei were identified by the presence of dark brown staining (Ataei and Ebrahimzadeh-Bideskan, 2014).

Sample preparation

A portion of the ipsilateral frontoparietal cortex corresponding to the penumbra region (underlying cortex of middle cerebral artery territory immediately distal to occlusion, i.e. the peri-infarct cortex) was dissected and used for the subsequent experiments.

WESTERN BLOTTING

At the time of experiment, the right peri-infarct cortex from each experimental group was homogenized in the lysis buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate (Na₃VO₄), 10 mM β-glycerophosphate, 0.2% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Roche, Mannheim, Germany). The homogenate was sonicated on ice with three 10s bursts at high intensity with a 10s cooling period between each burst. The samples were centrifuged at 10000 g for 10 min at 4 °C to remove cellular debris. Protein concentrations were determined using the Bradford method (Amin et al., 2014), and then samples with adjusted protein content were mixed 1:1 with 2× SDS blue buffer, boiled, aliquoted and kept in a −80 °C freezer.

One hundred micrograms of each protein extract was separated on a 12% SDS–polyacrylamide gel by electrophoresis and transferred onto polyvinylidene fluoride membranes. Then, blots were blocked with 5% skim milk in TBST (20 mM Tris-HCl pH 7.6, 137 mM NaCl and 0.05% Tween-20) at 4 °C overnight. Rabbit polyclonal anti-caspase-3, rabbit polyclonal anti-bax, rabbit polyclonal anti-bcl2 and rabbit polyclonal anti-β-actin antibodies were used as a primary antibody with an incubation time of about 1–2 h at room temperature, washing three times with TBST and 1 h incubation by rabbit horseradish peroxidase-conjugate anti-rabbit IgG. Enhanced chemiluminescence (Pierce) was used to visualize the peroxidase-coated bands and Alliance 4.7 Gel Doc (UK). Densitometric analysis for protein bands was performed using the NIH image J program. The protein levels were normalized with respect to corresponding β-actin band and expressed as fold of control (Sadeghnia et al., 2014).

THIOBARBITURIC ACID REACTIVE SPECIES MEASUREMENT

The lipid peroxidation level was measured as malondialdehyde (MDA), which is the final product of lipid peroxidation process and reacts with TBA, as a thiobarbituric acid reactive substance (TBARS), to produce a red-coloured complex that has a peak absorbance at 532 nm (Silva et al., 2006). The ipsilateral penumbra region was homogenized in ice-cold PBS (pH 7.4) to give a 10% homogeneity. Then, 1 mL of homogenate sample was mixed with 2 mL of TCA–TBA–HCl reagent (15% TCA, 0.67% TBA and 0.25 N HCl) and heated for 45 min in a boiling water bath. After cooling, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was collected, and the absorbance was read against blank at 532 nm. The amount of MDA produced was calculated, using a molar absorption coefficient of 1.56 × 10⁻⁵ M⁻¹ cm⁻¹ and expressed as nmol/mg protein. Protein contents were determined using the Bradford method.

ESTIMATION OF REDUCED GLUTATHIONE

Glutathione (GSH) was measured based on the Ellman method. Equal quantity of the homogenate was mixed with 10% TCA and centrifuged at 2500 g for 10 min. To 0.01 mL of this supernatant, 2 mL of phosphate buffer (pH 8.4), 0.5 mL of 2, 2′-dinitro-5, 5′-dithiodibenzonic acid and 0.4 mL of double-distilled water were added. The mixture was vortexed, and the absorbance was recorded at 412 nm. The concentration of GSH was expressed as nmol/mg of protein (Ellman, 1959).

DETERMINATION OF SUPEROXIDE DISMUTASE ACTIVITY

Superoxide dismutase (SOD) activity was evaluated using a spectrophotometric method previously described (Marklund and Marklund, 1974). Briefly, the homogenate sample was incubated in a solution containing

Tris-HCl buffer (pH 8.2, 50 mM) and EDTA (1 mM). Reaction was initiated by the addition of 0.2 mM pyrogallol. Oxidation of pyrogallol was measured at 420 nm for 10 min, at intervals of 1 min. The percentage inhibition of pyrogallol autoxidation was determined using the following equation:

\[
\% \text{ inhibition of pyrogallol autoxidation} = \left[ 1 - \frac{\Delta A}{\Delta A_{\text{max}}} \right] \times 100,
\]

where \( \Delta A \) is absorbance change due to pyrogallol autoxidation in the sample reaction system and \( \Delta A_{\text{max}} \) is absorbance change due to pyrogallol autoxidation in the control (without homogenate sample). A 50% inhibition was defined as one unit (U) of SOD activity, and the results were normalized by protein content and expressed as units/min/mg protein (Marklund and Marklund, 1974).

**STATISTICAL ANALYSIS**

Data were expressed as mean ± SEM. Statistical analysis was carried out using one-way analysis of variance, followed by the Tukey–Kramer post hoc test for multiple comparisons. A statistical difference was determined with \( p \)-values less than 0.05.

**RESULTS**

**Acetyl-11-keto-β-boswellic acid quantification**

Under the experimental conditions, retention time of standard compound AKBA was 6.61 min (Fig. 1A). HPLC analysis of ABS and EBS showed peaks corresponding to standard AKBA with retention times of 6.83 and 6.84 min, respectively (Fig. 1B and C). Using a calibration curve (0, 5, 10, 20, 40 and 80 \( \mu \)g/mL), the amounts of AKBA in a sample of ABS and EBS were quantified and were about 8.8% and 9.5% w/w, respectively.

**B. serrata increased neurological score following ischemic insult**

Middle cerebral artery occlusion decreased neurological score from 3 ± 0.0 to 1 ± 0.0, as compared with the sham animals (\( p < 0.001 \)) (Fig. 2). Treatment with ABS or EBS at a dose of 500 mg/kg or AKBA at a dose of 50 mg/kg significantly increased the neurological score to 3 ± 0.0 (\( p < 0.001 \)), 3 ± 0.0 (\( p < 0.001 \)) and 2.75 ± 0.25, respectively (Fig. 2).

**B. serrata decreased brain infarction volume**

Obvious infarction was observed in the right cortex of ischemic rats as compared with sham-operated animals, while ABS, EBS or AKBA remarkably decreased brain infarction (Fig. 3A). MCAO significantly increased infarct volume from 5.7 ± 1.2 mm\(^3\) (sham group) to 65.3 ± 3.7 mm\(^3\) (\( p < 0.001 \)) (Fig. 3B). The ABS at doses of 250 and 500 mg/kg significantly decreased brain infarct volume to 29.1 ± 6 mm\(^3\) (\( p < 0.01 \)) and 17.8 ± 6.2 mm\(^3\) (\( p < 0.01 \)), respectively. Similarly, a significant decrease in brain infarction was observed following treatment with EBS at doses of 250 and 500 mg/kg (20.4 ± 7.0, \( p < 0.01 \) and 8 ± 2, \( p < 0.001 \), respectively). AKBA also significantly decreased the brain infarct volume (15.50 ± 5.7, \( p < 0.001 \)), as compared with ischemic rats (Fig. 3B).

**B. serrata decreased cortical cell loss**

Figure 4A shows representative photomicrographs of coronal brain sections staining with H&E from ipsilateral cortical areas of sham-operated, MCAO-operated and ABS-treated, EBS-treated or AKBA-treated stroke rats. Statistical analysis revealed significant neuronal cell loss in the ipsilateral (\( p < 0.001 \)), but not the contralateral, cortical regions of stroke animals compared with the sham group. In addition, there were significant differences in the ipsilateral cortical neuronal cell loss between ABS-treated, EBS-treated and AKBA-treated stroke animals and the MCAO group (\( p < 0.001 \)) (Fig. 4B).

**B. serrata reduced neuronal apoptosis**

A few positive TUNEL stainings (brown) were detected in the brain sections of sham animals. However, a large number of TUNEL positive cells in the ischemic penumbra of rat brain were seen in the MCAO group (Fig. 5A). The quantitative analysis of the number of TUNEL positive cells exhibited that MCAO significantly enhanced apoptotic cell death in the right cortical regions (84.6 ± 9.8) as compared with the sham group (5.3 ± 1.6, \( p < 0.001 \)). As shown in Fig. 5B, ABS at a dose of 500 mg/kg significantly decreased the number of TUNEL positive cells to 18.8 ± 6.2, as compared with the ischemic group (\( p < 0.001 \)). Similarly, EBS (125 and 500 mg/kg) significantly decreased the number of TUNEL positive cells (51.3 ± 9.4, \( p < 0.05 \) and 11.9
± 6.7, \( p < 0.001 \), respectively) as compared with the ischemic group.

Also, apoptosis rate was significantly decreased in AKBA-treated stroke rats in comparison with the ischemic group (21.9 ± 4.7 vs 84.6 ± 9.8, \( p < 0.001 \)) (Fig. 5B).

**B. serrata** decreased cleaved caspase-3 and bax/bcl-2 expressions

As illustrated in Fig. 6A–C, MCAO led to a substantial increase in the expression of cleaved caspase-3 (2.9-fold) and bax/bcl-2 ratio, as compared with the sham group (\( p < 0.001 \)). Treatment with ABS and EBS (500 mg/kg) or AKBA (50 mg/kg) markedly decreased cleaved caspase-3 expression to 1.7 and 1.5 (\( p < 0.01 \) for both) or 1.3 (\( p < 0.001 \)) fold of control value, respectively. In addition, the bax/bcl-2 ratio was significantly decreased in ABS-treated and EBS-treated (500 mg/kg) or AKBA-treated (50 mg/kg) ischemic rats (Fig. 6 A–C, \( p < 0.001 \) for all).

**B. serrata** significantly decreased lipid peroxidation

The MCAO group showed a significant enhanced level of MDA in the right cortical region, 13.6 ± 0.1 nmol/mg protein, as compared with the sham group (7.4 ± 0.3 nmol/mg protein, \( p < 0.001 \)) (Fig. 7). ABS at doses of 125, 250 and 500 mg/kg significantly and dose-dependently decreased the MDA levels to 10.2 ± 0.06 nmol/mg protein (\( p < 0.05 \)), 8.6 ± 0.2 nmol/mg protein (\( p < 0.001 \)) and 7 ± 0.1 nmol/mg protein (\( p < 0.001 \)), respectively (Fig. 7). TBARS levels were also
Figure 4. (A–B) Effects of aqueous (ABS) and ethanolic (EBS) extracts of Boswellia serrata and 3-acetyl-11-keto-β-boswellic acid (AKBA) on cell density and percentage of degenerated cells in the ipsilateral cortical region of rats subjected to the middle cerebral artery occlusion (MCAO) for 30 min and reperfusion for 24 h. (A) Photomicrographs are representative coronal brain sections staining with haematoxylin and eosin from cortical areas of sham-operated (a), MCAO-operated (b), 125 mg/kg ABS-treated (c), 500 mg/kg ABS-treated (d), 125 mg/kg EBS-treated (e), 500 mg/kg EBS-treated (f) and 50 mg/kg AKBA-treated (g) stroke rats. The scale bar shows 100 μm. (B) The bar graph represents the percentages of neuronal loss in the cortical area of rats treated with ABS, EBS and AKBA just after the MCAO induction. Values are expressed as mean ± SEM (n = 8). ***p < 0.001 as compared with the MCAO group.
Figure 5. (A–B) Effects of aqueous (ABS) and ethanolic (EBS) extracts of *Boswellia serrata* and 3-acetyl-11-keto-β-boswellic acid (AKBA) on neuronal cell apoptosis at 24 h after reperfusion in the rats with 30 min of middle cerebral artery occlusion (MCAO). Cell apoptosis assayed by transferase dUTP nick end-labelling (TUNEL) staining method. (A) Photomicrographs are representative TUNEL-stained coronal brain sections from the ipsilateral cortical region of sham-operated (a), MCAO-operated (b), 125 mg/kg ABS-treated (c), 500 mg/kg ABS-treated (d), 125 mg/kg EBS-treated (e), 500 mg/kg EBS-treated (f) and 50 mg/kg AKBA-treated (g) stroke rats. TUNEL-positive cells were manifested as a marked appearance of dark brown apoptotic cell nuclei. The scale bar shows 100 μm. (B) The bar graph represents the percentages of TUNEL-positive cells in the cortical areas of rats treated with ABS, EBS and AKBA just after the MCAO induction. Values are expressed as mean ± SEM (*n = 8*). *p < 0.05, **p < 0.001 as compared with the MCAO group.
significantly and dose-dependently attenuated following treatment with EBS at doses of 125, 250 and 500 mg/kg (10.7 ± 0.6 nmol/mg protein, \( p < 0.01 \); 8.3 ± 0.2 nmol/mg protein, \( p < 0.001 \); and 6.4 ± 0.3 nmol/mg protein, \( p < 0.001 \), respectively), as compared with the MCAO group (Fig. 7).

Similarly, the lipid peroxidation level was significantly decreased following AKBA administration (6.3 ± 0.4, \( p < 0.001 \)) (Fig. 7).

**B. serrata** significantly increased GSH content

Middle cerebral artery occlusion decreased the right cerebral GSH level from 145.3 ± 1.4 nmol/mg protein to 9.3 ± 4.6 (\( p < 0.001 \), Fig. 8). ABS at doses of 250 and 500 mg/kg significantly increased GSH level to 60.4 ± 3.8 nmol/mg protein (\( p < 0.05 \)) and 145.1 ± 4.3 nmol/mg protein (\( p < 0.001 \), respectively (Fig. 8). As illustrated in Fig. 8, a significant increase in the right
cerebral GSH content was seen following treatment with EBS (73.7 ± 5.3 nmol/mg protein, \( p < 0.01 \) and 139.5 ± 5.7 nmol/mg protein, \( p < 0.001 \) for doses of 250 and 500 mg/kg, respectively) or AKBA (126.4 ± 17.2), as compared with the ischemic group.

**B. serrata** significantly increased SOD activity

The MCAO rats showed decreased SOD activity as compared with the sham group (7.4 ± 0.05 vs 14.2 ± 0.9 units/min/mg protein, \( p < 0.001 \), Fig. 9). Whereas, as shown in Fig. 9, ABS and EBS at a dose of 500 mg/kg and AKBA at a dose of 50 mg/kg significantly \( (p < 0.001) \) increased the SOD activity to 12.8 ± 0.2, 8.3 ± 0.9 and 12.7 ± 0.7, respectively, as compared with the MCAO rats (Fig. 9).

**DISCUSSION**

Our results revealed the neuroprotective potentials of **B. serrata** extracts and the biologically active constituent, AKBA, against focal cerebral ischemia in rats in a dose-dependent manner, which were confirmed by improvement in neurological deficits, reduced cerebral infarction, neuronal cells loss and apoptosis, and also improved cerebral redox status. The experimental results also showed that ABS and EBS provide approximately equal therapeutic efficacy against MCAO injury, which might be because of approximately the same content of AKBA.

The largest vessel branching from the internal carotid artery is the middle cerebral artery, which is the most commonly occluded vessel and is responsible for embolic and thrombotic stroke at clinical level. Therefore, a transient MCAO model is still the most appropriate model for studying I-R injury and mimics most of the human stroke cases (Durukan and Tatlisumak, 2007; Tabassum et al., 2013). Under I-R insult, excess generation of free radicals causes oxidative stress, excitotoxicity and neuroinflammation, which results in neuronal damage and disturbed functional outcomes (Vaibhav et al., 2012; Vakili et al., 2014).

It has been reported that neuronal damage induced by I-R injury in the hippocampal and cortical regions leads to neurological deficits in MCAO rats (Garcia et al., 1995; Vafae et al., 2012; Tabassum et al., 2015).

By evaluating the symmetry in the movements of the four limbs, we found that the **B. serrata** extracts significantly improved neurological scores in comparison with the ischemic group.

It is well documented that histological outcomes provide direct evidences to measure the amount of...
neuronal damage and also protection by therapeutic means (Durukan and Tatham, 2007; Hartmann et al., 2012). Using TTC and H&E staining methods, remarkable cerebral infarction and neuronal cell loss were revealed in the ischemic group, whereas treatment with B. serrata significantly decreased unstained areas.

It is well documented that cerebral I-R injury may cause neurological impairments and neuronal apoptosis associated with the activation of caspase-3 and bax and the downregulation of bel-2 (Liu et al., 2013). Our results showed that B. serrata and AKBA significantly reduced the number of TUNEL-positive cells, suggesting prevention of neuronal apoptosis after MCAO in rats. Furthermore, western blot analysis showed that the expression of anti-apoptotic protein bel-2 was enhanced, while the pro-apoptotic proteins, bax and caspase-3, were downregulated by B. serrata and AKBA, suggesting the anti-apoptotic effect. These findings are consistent with a previous study demonstrating that B. serrata possesses antiinflammatory and anti-apoptotic effects against neurodegeneration characterizing Alzheimer’s disease in a rat model (Ahmed et al., 2014).

It is generally believed that oxygen-free radicals are closely related to cerebral I-R injury by inducing lipid peroxidation, glutathione depletion, SOD dysfunction and apoptosis. Therefore, antioxidants have been considered in prevention and treatment of stroke (Ding et al., 2014a, 2014b; Tabassum et al., 2015). The results of our study indicated that B. serrata extracts prevent the increase of MDA levels, a marker of lipid peroxidation, and also increase GSH content and SOD activity in the cortex, which suggests antioxidant properties for B. serrata after cerebral ischemia. B. serrata resins have potent antioxidant and immunomodulatory properties as proved by earlier published evidences (Ammon, 2010; Kokkiri et al., 2011; Hartmann et al., 2012; Umar et al., 2014). In an experimental model of acute ulcerative colitis, treatment with the extract of B. serrata caused a significant reduction in lipid peroxidation while significantly increased glutathione levels (Hartmann et al., 2012). Also, in an animal model of arthritis, Umar et al. (2014) showed that treatment with B. serrata extract significantly inhibited GSH depletion and an increase in TBARS levels. Togni et al. (2015) reported that a boswellia-based cream is effective in preventing adjuvant radiotherapy skin damage in patients with mammary carcinoma. In gouty arthritic mice treated with boswellic acids, the activities of lysosomal enzymes, lipid peroxidation, TNF-α levels, paw volume and antioxidant status were modulated to near normal levels (Sabina et al., 2012).

The pharmacological effects of B. serrata have been mainly attributed to the pentacyclic triterpenoid boswellic acids, especially 11-keto-β-boswellic acid and AKBA, with antioxidant and antiinflammatory properties (Abdel-Tawab et al., 2011; Lakshmi and Rajendran, 2013). EBS and ABS contained AKBA of 9.5% (w/w) and 8.8% (w/w), respectively. This finding was similar to the finding of Kokkiri et al. (2011), who reported that EBS and ABS extracts of B. serrata contained 9% (w/w) and 7.8% (w/w) AKBA, respectively. The neuroprotective effects of B. serrata described here could be attributed to its biologically active phytochemical, AKBA. In this study, both ABS and EBS extracts of B. serrata were standardized to contain approximately 10% of AKBA. Therefore, an additional experimental group receiving 50 mg/kg AKBA (corresponding to 500 mg/kg B. serrata extracts) was used. The experimental results showed that AKBA (50 mg/kg) effectively attenuated stroke-induced neurological deficits, cerebral infarction, apoptosis and oxidative damages. The same magnitude of protection was also observed in rats treated with 500 mg/kg ABS/EBS, concerning all parameters tested. These observations suggest that the neuroprotective effects of ABS/EBS could be attributed to AKBA. It is important to note that the yield content of AKBA from B. serrata is about 30%. In addition to boswellic acids and their respective O-acetyl derivatives, B. serrata is also characterized by the presence of euphane and tirucallane triterpenes, especially tirucallic acids, as inhibitors of Akt/mTOR signalling pathway with potential immunomodulatory properties (Badria et al., 2003; Estrada et al., 2010; Zhang et al., 2013; El Gaafary et al., 2015). The well-known inhibitors of Akt/mTOR signalling such as tacrolimus or sirolimus (rapamycin) have been shown to exert neuroprotective properties against cerebral I-R injury (Furuchi et al., 2003; Chauhan et al., 2011). Therefore, other constituents of B. serrata may also contribute to the neuroprotection described here.

For the more appropriate conversion of drug doses from animal studies to human studies, the body surface area (BSA) normalization method should be used. BSA correlates well across several mammalian species with several parameters of biology, including oxygen utilization, caloric expenditure, basal metabolism, blood volume, circulating plasma proteins and renal function. Based on the BSA normalization method, the animal doses (125, 250 and 500 mg/kg for B. serrata extracts and 50 mg/kg for AKBA) used in this study could be extrapolated to human equivalent doses by multiplying those by 0.16, which are 20, 40 and 80 mg/kg for ABS and EBS and 8 mg/kg for AKBA (Reagan-Shaw et al., 2008).

Several previous studies described the neuroprotective, antioxidant, anti-apoptotic and antiinflammatory properties of B. serrata or its active compounds against numerous neurological disorders. Jalili et al. (2014) showed that aqueous extract of B. serrata dramatically increased the number of neuronal processes in the hippocampal CA1 region and improves passive-avoidance learning in both control and pentylenetetrazol-kindled rats. Moein et al. (2013) also suggested that B. serrata might improve cognitive outcome of patients with diffuse axonal injury. It was shown that treatment with AKBA (20 mg/kg) significantly decreased the apoptotic cell death and infarct volumes and also improved neurologic scores via elevating the nuclear factor erythroid-2-related factor 2/heme oxygenase-1 pathway in brain tissues of rats with MCAO at 48 h post-reperfusion (Ding et al., 2014b). The same authors showed that 11-keto-β-boswellic acid (25 mg/kg) applied 1 h after reperfusion significantly reduced infarct volumes, MDA levels and apoptotic cells as well as increased neurologic scores at 48 h after reperfusion (Ding et al., 2014a). An in vitro study also revealed that beta-boswellic acid could significantly enhance neurite outgrowth and branching of primary cells of hippocampal tissue in rats (Karima et al., 2010). Recently, Ding et al. (2016) showed that nanoformulation of AKBA leads to enhanced protection of AKBA against cerebral ischemic
insult by improving antioxidant defence (SOD and glutathione peroxidase) and diminishing inflammation (TNF-α and IL-1β), as well as the improvement activation of the nuclear factor erythroid-2-related factor 2/heme oxygenase-1 pathway and inhibition of inflammatory protein expressions (NF-kB and 5-lipoxygenase). Sayed and El Sayed (2016) showed that co-administration of AKBA and celecoxib reversed the behavioural and molecular changes caused by lipopolysaccharide-induced cognitive dysfunction in mice. A recent study showed that long-term administration of Boswellia resin (100 mg/kg/day for 8 weeks) can attenuate age-related dendritic regression in CA1 pyramidal cells in rat hippocampus (Hosseini-Sharifabad and Esfandiari, 2015). The same authors showed that chronic treatment with B. serrata (100 mg/kg/day for 8 weeks) improved the spatial learning capability in aged rats. The study revealed that B. serrata-treated aged rats had greater dentate gyrus with increased dendritic complexity in the dentate granule cells than that in control rats. Hippocampal granule cells of B. serrata-treated aged rats had also more dendritic segments, larger arbors, more numerical branching density and more dendritic spines in comparison with control animals (Hosseini-Sharifabad et al., 2016).

This study has several limitations. First, the current study period was only 24 h; therefore, the long-term efficacy of treatment with B. serrata on improvement of neurobehavioural functions, such as learning and memory, remains to be elucidated. Second, in this study, the drug was given shortly after stroke was induced. Treatment immediately following MCAO induction is clinically less interesting, as in most clinical cases, immediate treatment is difficult and impossible (Green, 2008; Stocchetti et al., 2015). On the other hand, preclinical neuroprotective agents are generally ineffective more than 3–4 h from stroke onset because of the diminishing efficacy of neuroprotection with time. Therefore, the design should permit the studied drug to be given as early as possible (Tymianski, 2013).

In conclusion, based on the results from this study, it was concluded that B. serrata extracts and AKBA are a potential neuroprotectant and that the possible mechanisms may occur partly through the diminished oxidative stress and apoptosis. However, other mechanisms may be also involved and needed further investigations.

Acknowledgements

This work was supported by a grant (922902) from the Vice-Chancellor for Research and Technology, Mashhad University of Medical Sciences (MUMS), Iran.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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