Acta Poloniae Pharmaceutica – Drug Research, Vol. 74 No. 3 pp. 911–920, 2017 ISSN 0001-6837
Polish Pharmaceutical Society

NEUROPROTECTIVE EFFECT OF *BOSWELLIA SERRATA* AND ITS ACTIVE CONSTITUENT ACETYL 11-KETO-β-BOSWELLIC ACID AGAINST OXYGEN-GLUCOSE-SERUM DEPRIVATION-INDUCED CELL INJURY

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Abstract: Oxidative stress plays a key role in pathophysiology of brain ischemia. This study aimed to test whether *B. serrata* hydroalcoholic extract (BSE) and its active constituent 3-acetyl-11-keto-β-boswellic acid (AKBA) could protect neurons against ischemic condition induced by oxygen, glucose and serum deprivation (OGSD). First, PC12 neural cells were incubated with BSE (0-400 µg/mL) or AKBA (0-40 µg/mL) for 24 h to find non-cytotoxic concentrations of BSE and AKBA. Then, the cells were pre- (for 2 h) and co-treated with 1.5-6 µg/mL BSE or 0.5-2.5 µg/mL AKBA, and then exposed to OGSD condition for 6 h. The IC_{50} values of BSE and AKBA were 95 and 12.2 µg/mL, respectively. BSE (3 and 6 µg/mL) and AKBA (1 and 2.5 µg/mL) significantly increased viability of ischemic cells, in a concertation-dependent manner. The levels of intracellular oxygen free radicals, lipid peroxidation and oxidative DNA damage were also significantly and concentration-dependently decreased following treatment of ischemic cells with BSE or AKBA. Using HPLC analysis, the mount of AKBA in a sample of BSE was found to be 9.2%. In conclusion, *B. serrata* and AKBA reduce neuronal cell death induced by OGSD and this neuroprotective effect is mediated *via* attenuation of oxidative stress.

Keywords: *Boswellia serrata*, boswellic acid, ischemia; DNA damage, oxidative stress, PC12

In ischemic brain tissue, an insufficient supply of oxygen and nutrients results in activation of multiple death signaling pathways in neurons. Although the mechanisms of neuronal cell death following ischemia remained to be fully elucidated, oxidative stress obviously plays an important role by oxidizing cellular macromolecules and inducing mitochondrial dysfunction (1, 2). Neurons are particularly vulnerable to oxidative stress because of higher energy demands and having relatively low levels of endogenous antioxidants (3, 4). For example, neurons have limited capacity to produce glutathione for detoxifying reactive oxygen species (ROS) (3). Increased level of ROS following ischemia is an important inducer of neural apoptosis. Therefore, use of antioxidants derived from natural products might be a promising approach for protection of neurons against ischemia-induced cytotoxicity (5). Recent studies have shown that a number of natural compounds in food and medicinal plants, such as flavonoids and triterpenoids, protect neurons against ischemic insult (6-10).

Boswellia serrata, a medicinal plant of family Burseraceae, exhibited satisfactory antioxidant activity in several studies (11-13). This plant also displays several pharmacological activities including anti-inflammatory and immunomodulatory properties (14, 15). The pharmacological activities are mainly attributed to boswellic acids which are found in gum resin extracts of *B. serrata* (14, 16). Acetyl 11-keto-β-boswellic acid (AKBA), with pentacyclic triterpenoid structure, is one of the most abundant and active compounds among these acids, and presents antioxidant and cytoprotective properties (17, 18). For example, it has been reported that AKBA protects cardiomyocytes against ischemia reperfusion injury (18).

The present work aimed to test whether *B. serrata* hydroalcoholic extract (BSE) and AKBA may protect neuronal cells against oxygen, glucose and serum deprivation (OGSD)-induced cytotoxicity, an *in vitro* model which simulates neuronal damage during ischemia (7, 19, 20).

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EXPERIMENTAL

Materials

High glucose (25 mM) Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Gibco (Grand Island, NY, USA). Glucose-free DMEM, thiobarbituric acid, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), bicinchoninic acid kit, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) were purchased from Sigma (St. Louis, MO, USA). Trichloroacetic acid, triton X-100, dimethyl sulfoxide (DMSO), sodium lauroyl sarcosinate (sarkosyl), ethidium bromide, malondialdehyde bis-(dimethyl acetal) (MDA), and AKBA were obtained from Merck (Darmstadt, Germany).

Preparation of extract

The oleo-gum resin of *B. serrata* was provided from Medicinal Plants Division of Imam Reza Pharmacy (herbarium No. 92083). The plant material was washed, dried, and ground to fine powder with a blender. Then, the powder (10 g) was soaked in 100 mL of 56% ethanol, for 48 h at 40°C under gentle shaking. The resulting BSE was filtered and the solvent was removed using rotary evaporator. The residue (yield 30% w/w) was kept at -20°C until use. Using HPLC method, the mount of AKBA in a sample of BSE was found to be about 9.2%.

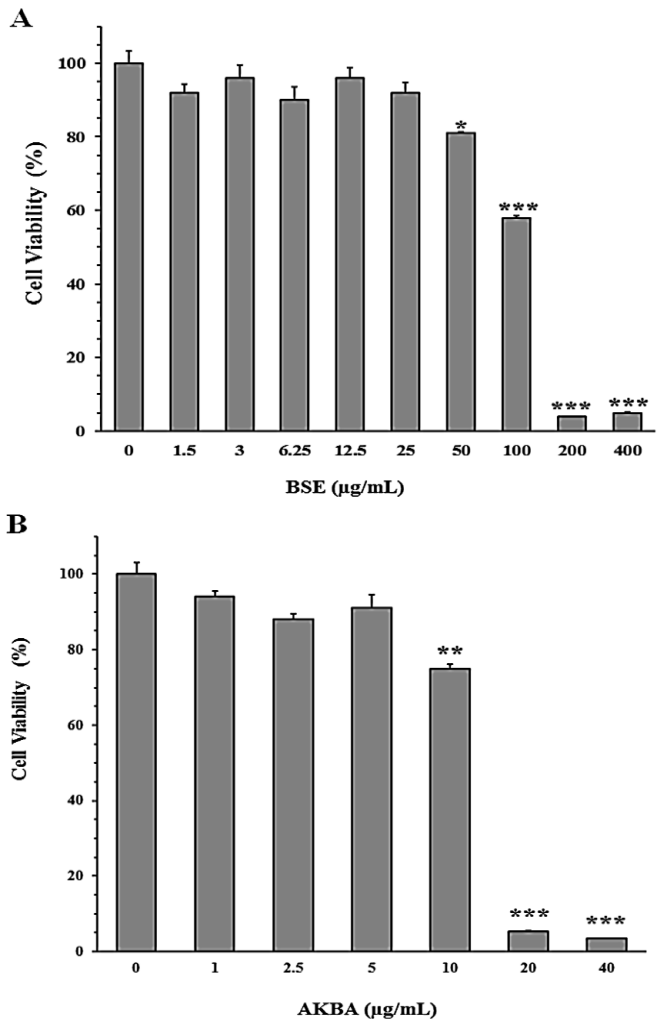

Figure 1. Effects of BSE (A) and AKBA (B) on viability of PC12 cells. The cells were cultured for 24 h in high-glucose DMEM/10% FBS medium containing different concentrations of BSE or AKBA. Data are the mean ± SEM (n = 9). *p < 0.05, **p < 0.01 and ***p < 0.001 *vs.* untreated control cells (0 µg/mL)

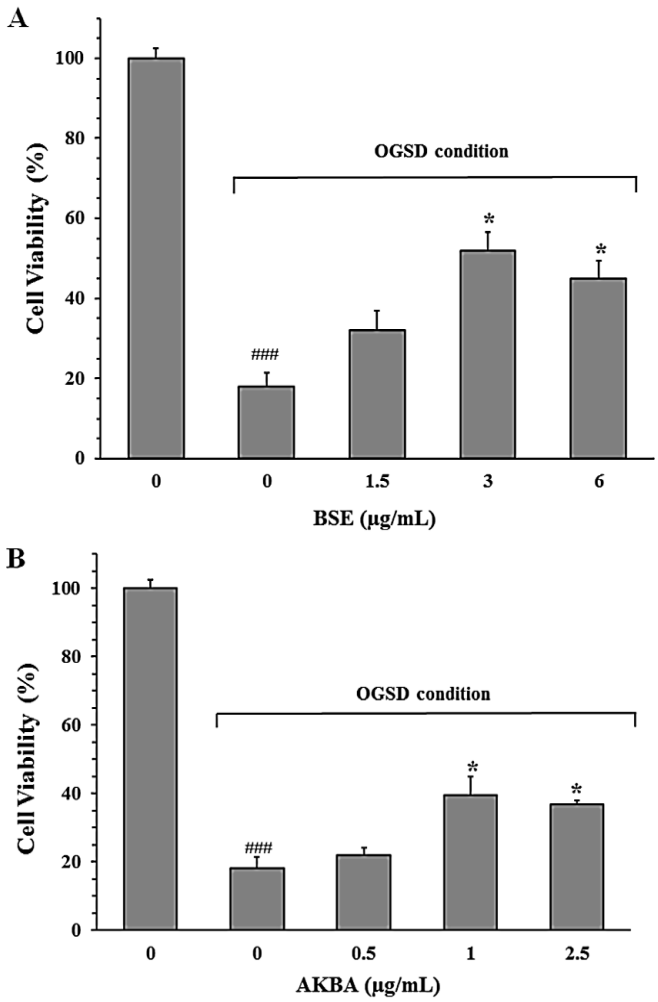

Figure 2. Effects of BSE (A) and AKBA (B) on viability of PC12 cells exposed to oxygen/glucose/serum deprivation (OGSD) condition. The cells were pretreated for 2 h with the drugs and then incubated in glucose- and FBS-free DMEM (containing BSE or AKBA) medium in an anoxic atmosphere for 6 h. Data are the mean ± SEM (n = 9). $^{###}p < 0.001$ *vs*. untreated control cells; $*p < 0.05$ *vs*. untreated cells cultured in OGSD condition

Cell culture and treatment

The rat pheochromocytoma-derived (PC12) cells were cultivated in 25 cm^2 tissue culture flasks containing high-glucose DMEM medium supplemented with 10% FBS. At sub-confluent stage, the cells were harvested using trypsin and seeded in 96-well plates (5000 cells/well) and grown to subconfluency. To find toxic concentrations of BSE and AKBA, the cells were treated with 3-400 µg/mL BSE or 1-40 µg/mL AKBA for 24 h. In a separate experiment, to evaluate neuroprotective effect of *B. serrata*, the cells were pretreated for 2 h with 1.5-25 µg/mL BSE or 0.5-5 µg/mL AKBA and then they were exposed for 6 h to glucose- and FBS-free DMEM (containing BSE and AKBA) in an anoxic atmosphere (95% N_2 and 5% CO_2).

MTT assay

The cell viability was evaluated by MTT assay as described previously (21, 22). In brief, MTT reagent was added to cell media at a final concentration of 5 mg/mL and incubated at 37°C for 2 h. Then, the supernatant was discarded and 100 µL DMSO was added to solubilize the formazan crystals. The absorbance of formazan dye was measured at wavelength 545 nm using a StatFAX303 plate reader.

Measurement of reactive oxygen species

At the end of incubation in OGSD condition, the cells were exposed to H_2DCFDA (10 µM) for 30 min at 4°C in the dark (23). The H_2DCFDA diffuses into the cells and is hydrolyzed by intracellular esterases to H_2DCF and then oxidized to 2',7'-dichlorofluorescein (DCF) in the presence of ROS. The fluorescence intensity of DCF was determined with excitation at 485 nm and emitted light monitored at 530 nm.

Lipid peroxidation assay

Lipid peroxidation was evaluated by measuring MDA which is a sensitive index of the peroxidation of cellular lipids and reacts with thiobarbituric acid to form a pink-colored complex (24). At the end of incubation in OGSD condition, the cells were scraped into trichloro acetic acid (2.5%, 1 mL) and centrifuged for 2 min at 13000 × g. Then, 800 µL of thiobarbituric acid (0.7%) and 400 µL of trichloroacetic acid (15%) were added to 500 µL of cell samples. After heating in a boiling water bath for 40 min, the samples were centrifuged at 2500 rpm for 10 min at 4°C. Then, a 200 µL supernatant was transferred to 96-well plate and fluorescence intensity was measured with excitation/emission of 480/530 nm. The MDA level was expressed as nmol/mg protein. Protein content was determined by the Lowry procedure using bicinchoninic acid kit.

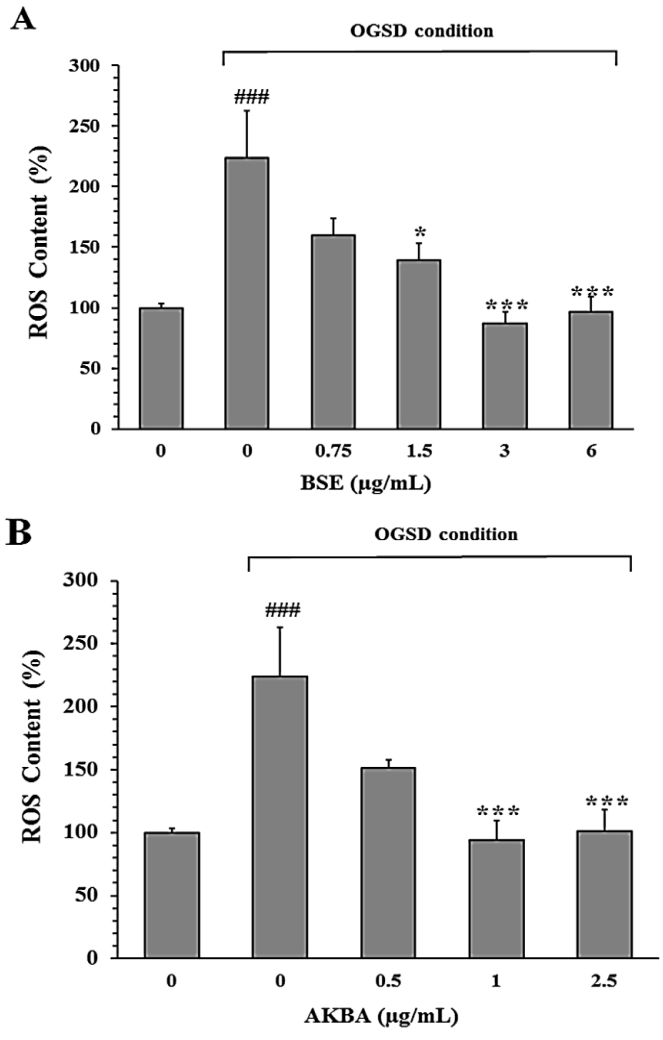

Figure 3. Effects of BSE (A) and AKBA (B) on intracellular reactive oxygen species (ROS) in PC12 cells exposed to oxygen/glucose/serum deprivation (OGSD) condition. The cells were pretreated for 2 h with the drugs and then incubated in glucose- and FBS-free DMEM medium (containing BSE and AKBA) in an anoxic atmosphere for 6 h. Data are the mean ± SEM (n = 9). $^{###}p < 0.001$ *vs.* the cells cultured in standard condition; $*p < 0.05$ and $***p < 0.001$ *vs.* untreated cells cultured in OGSD condition

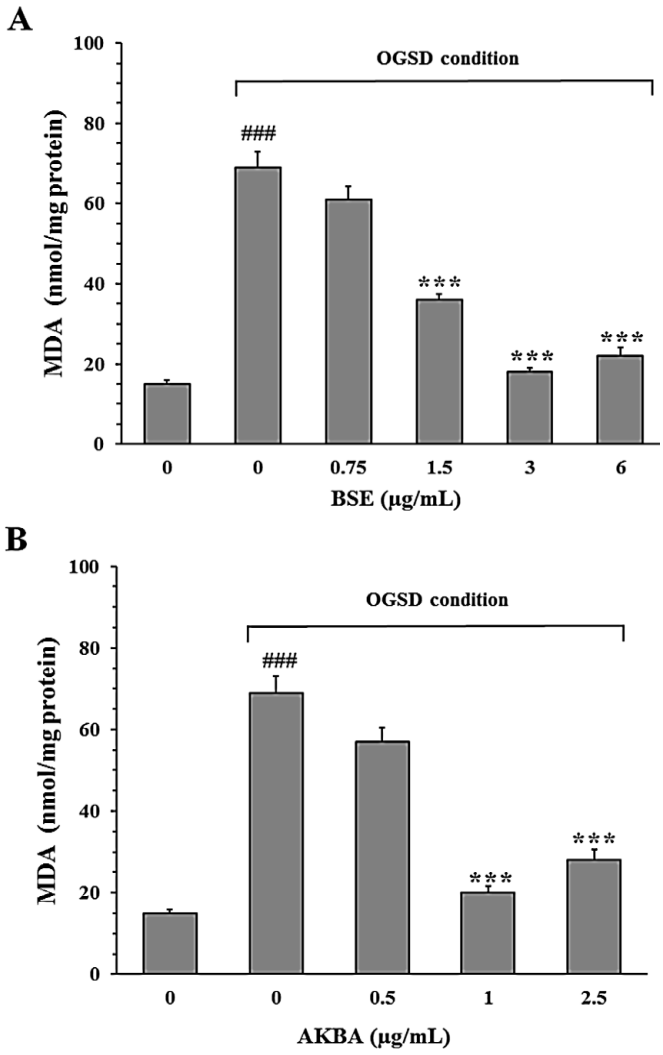

Figure 4. Effects of BSE (A) and AKBA (B) on lipid peroxidation in PC12 cells exposed to oxygen/glucose/serum deprivation (OGSD) condition. The cells were pretreated for 2 h with the drugs and then incubated in glucose- and FBS-free DMEM (containing BSE and AKBA) in an anoxic atmosphere for 6 h. The lipid peroxidation level was evaluated by measuring the level of malondialdehyde (MDA). Data are the mean ± SEM (n = 9). ###$p < 0.001$ *vs.* the cells cultured in standard condition; ***$p < 0.01$ *vs.* untreated cells cultured in OGSD condition.

Single cell gel electrophoresis (comet) assay

After incubation in OGSD condition, the cells were harvested and suspended in low melting point agarose and then layered over a microscope slide pre-coated with normal melting point agarose. The slides were covered with a cover slip and placed on ice to allow agarose to gel. Then, another layer of low melting point agarose was added on top and the slides were immersed in lysis solution (2.5 M NaCl, 10 mM Trizma, 100 mM Na_2EDTA, 10% DMSO, 1% sarkosyl, 1% Triton X-100, pH 10). After 24 h, the slides were placed for 40 min on a horizontal gel electrophoresis platform containing an alkaline solution (1 mM Na_2EDTA, 300 mM NaOH, pH > 13). Finally, they were electrophoresed (25 V, 300 mA, 30 min), rinsed with Trizma (pH 7.5), and stained with ethidium bromide. Using fluorescence microscope (excitation: 520-550 nm, barrier filter: 580 nm), 150 nuclei from 3 replicated slides were randomly selected and photographed. Undamaged cells have intact nucleus without a tail, while damaged cells show DNA fragmentation and have the appearance of a comet (25, 26). The percent of DNA in the comet tail was analyzed by an image analysis system (CASP software).

Statistical analysis

Data were analyzed by one-way analysis of variance followed by Tukey's *post hoc* test for multiple comparisons. Results were expressed as the mean ± standard error of the mean and the p-values less than 0.05 were considered to be statistically significant.

RESULTS

Effects of BSE and AKBA on neuron viability

As shown in Figure 1A, treatment with 1.5-25 µg/mL BSE had no significant effect on viability of PC12 cells. At concentrations of 50, 100, 200, and 400 µg/mL of BSE, cell viability significantly decreased from 100 ± 3.2% (control) to 81 ± 0.3% ($p < 0.05$), 58 ± 0.7% ($p < 0.001$), 4 ± 0.03% ($p < 0.001$), and 5 ± 0.07% ($p < 0.001$), respectively. The IC_{50} value for BSE was calculated to be 95.0 µg/mL. Also, while treatment with 1-5 µg/mL AKBA had no significant effect on viability of PC12 cells, at concentrations of 10, 20, and 40 µg/mL, cell viability decreased from 100 ± 3.2% (control) to 75 ± 1.2% ($p < 0.01$), 5.4 ± 0.2% ($p < 0.001$), and 3.4 ± 0.1% ($p < 0.001$), respectively (Fig. 1B). The IC_{50} value for AKBA was calculated to be 12.2 µg/mL.

Neuroprotective effects of BSE and AKBA

Exposure of PC12 cells to OGSD condition for 6 h reduced cell viability from 100 ± 2.6% (control) to 18 ± 3.4% ($p < 0.001$). The neurotoxic effect of OGSD condition was inhibited by both BSE and AKBA. The BSE enhanced the cell viability at concentrations of 3 µg/mL (52 ± 4.5%, $p < 0.05$) and 6 µg/mL (45 ± 4.5%, $p < 0.05$) as compared to untreated ischemic cells (Fig. 2A). Similarly, when the cells incubated with 1 and 2.5 µg/mL AKBA, percent of viable cells significantly increased from 18 ± 3.4% (untreated ischemic cells) to 39.5 ± 5.5% and 36.8 ± 1.2 %, respectively ($p < 0.05$ for both) (Fig. 2B).

Effect of BSE and AKBA on ROS content

The level of intracellular ROS in PC12 cells cultured in OGSD condition was significantly increased

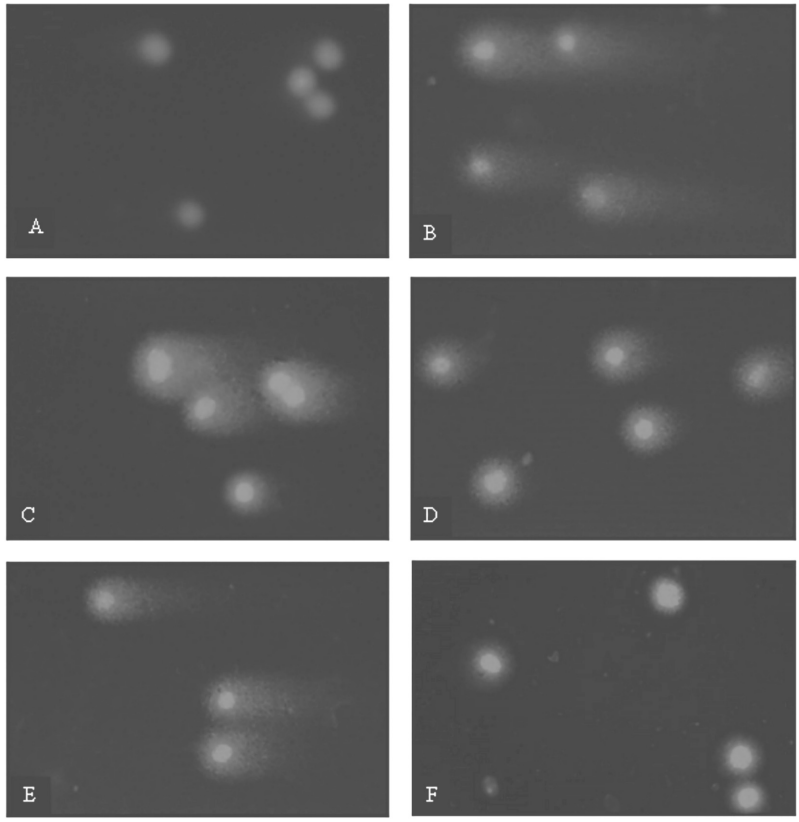

Figure 5. Comet images from PC12 cells cultured in standard medium (high-glucose DMEM supplemented with 10% FBS and in atmosphere of 5% CO_2/ 95% air) or oxygen/glucose/serum deprivation (OGSD, glucose- and FBS-free DMEM in an anoxic atmosphere of 5% CO_2/ 95% N_2) conditions or pretreated with BSE or AKBA for 2 h and then exposed to OGSD condition for 6 h

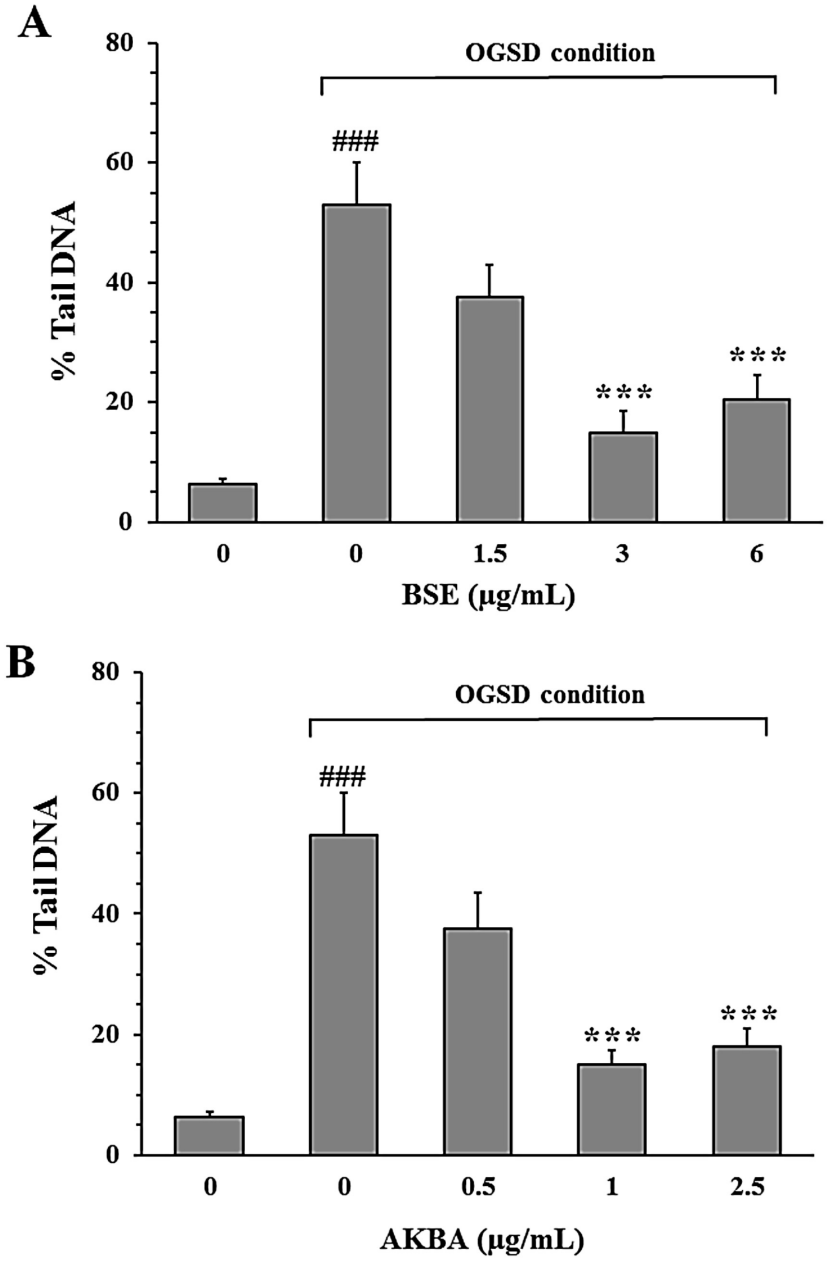

Figure 6. Bar graphs representing the effects of BSE (A) and AKBA (B) on DNA damage of PC12 cells cultured in oxygen/glucose/serum deprivation (OGSD) condition. The cells were pretreated for 2 h with the drugs and then incubated in glucose- and FBS-free DMEM (containing BSE and AKBA) in an anoxic atmosphere for 6 h. The level of DNA damage was expressed as the percentage of DNA in the comet tail (% tail DNA). Data are the mean ± SEM (n = 9). ###$p < 0.001$ *vs*. the cells cultured in standard condition; ***$p < 0.01$ *vs*. untreated cells cultured in OGSD condition

(224 ± 39%, $p < 0.001$), as compared to the cells cultured in standard medium (100 ± 3%). Incubation with 1.5, 3 and 6 µg/mL of BSE significantly decreased the OGSD-induced ROS accumulation to 139 ± 14% ($p < 0.05$), 87 ± 10% ($p < 0.001$) and 97 ± 12% ($p < 0.001$), respectively (Fig. 3A). Similarly, the presence of 1 and 2.5 µg/mL of AKBA in cell culture medium reduced the ROS content from 224 ± 39% (untreated cells) to 94 ± 16% ($p < 0.001$) and 101 ± 17% ($p < 0.001$), respectively (Fig. 3B).

Effect of BSE and AKBA on lipid peroxidation

Exposure of PC12 cells to OGSD condition resulted in a significant increase in the level of

MDA (69 ± 4 nmol/mg protein) as compared to the cells cultured in standard condition (15 ± 1 nM/mg protein, $p < 0.05$). As shown in Figure 4A, the content of MDA was significantly decreased in the cells treated with 1.5 µg/mL (52 ± 2.3 nM/mg protein, $p < 0.001$), 3 µg/mL (30 ± 4 nM/mg protein, $p < 0.001$), and 6 µg/mL (30 ± 4 nM/mg protein, $p < 0.001$) of the BSE. Likewise, in the cells treated with 1 µg/mL (20 ± 1.6 nM/mg protein) and 2.5 µg/mL (28 ± 2.6 nM/mg protein) of AKBA, the MDA level was significantly lower than untreated cells (69 ± 4 nM/mg protein, $p < 0.001$) (Fig. 4B).

Effect of BSE and AKBA on DNA damage

Comet images from cells cultured in standard condition and OGSD condition were shown in Figure 5. To quantify the amount of genomic DNA damage, the percent of DNA in the comet tail (% tail DNA) was determined (Fig. 6). As shown in Figures 5 and 6, the cells cultured in standard condition (no OGSD) showed only minor DNA fragmentation (6.3 ± 0.8%). However, OGSD condition led to DNA damage and the percent of DNA fragmentation significantly increased (53 ± 7%, $p < 0.001$). The BSE at concentrations of 3 µg/mL (54 ± 3.6%, $p < 0.001$) and 6 µg/mL (20 ± 4.2%, $p < 0.001$) significantly decreased the OGSD-induced DNA damage. Similarly, a significant decrease in DNA damage was seen following incubation with 1 µg/mL (15 ± 2%, $p < 0.001$) and 2.5 µg/mL (18 ± 3%, $p < 0.001$) of AKBA.

DISCUSSION AND CONCLUSION

At present, there are few treatment options available to inhibit neuronal injury following brain ischemia and there is therefore an urgent need for finding new therapeutic agents. This study was performed to examine the protective effects of BSE against neuronal injury induced by OGSD, which mimic the pathological features of cerebral ischemia (7, 19, 20, 25). Since the pharmacological effects of *B. serrata* are mainly attributed to the pentacyclic triterpenoid boswellic acids, especially AKBA, the possible neuroprotective effect of this compound was also examined. Our results demonstrated that incubation with BSE and AKBA can inhibit cell death under OGSD condition. The neuroprotective effect of BSE and AKBA was more obvious at concentration of 2.5 µg/mL and 1 µg/mL, respectively. For both BSE and AKBA, the effective concentrations were far away from their IC_{50} values.

The pathophysiology of cerebral ischemia is complex, and mainly involves oxidative stress, inflammation, excitotoxicity, blood-brain barrier disruption, and apoptosis (27). Oxidative stress obviously plays an important role in ischemia-induced neuronal cell death. Accumulation of intracellular ROS because of imbalance in generation and neutralization by antioxidants promotes peroxidation of membrane lipids, oxidation of proteins, and damage to DNA (28). It also promotes the mitochondrial release of cytochrome c, which subsequently activates caspase-3 and caspase-9, resulting in the DNA fragmentation and apoptosis (5, 28, 29). Our data showed that BSE and AKBA were able to prevent the increase of intracellular ROS and the enhanced lipid peroxidation in OGSD condition. They also significantly inhibited DNA fragmentation as evaluated by the comet assay, which suggests that BSE and AKBA may protect neurons against DNA damage following ischemia. This protective effect is most probably mediated thorough their antioxidant property. This is consistent with previous studies demonstrating that *B. serrata* has antioxidant activity in several tissues including colon, joints, and brain (11-13). In an animal model of ulcerative colitis, treatment with *B. serrata* significantly reduced the lipid peroxidation, while increased the glutathione peroxidase activity (11). Also in an experimental model of arthritis, *B. serrata* gum resin extract was able to increase glutathione level and superoxide dismutase activity as endogenous defense against hydrogen peroxide formation and superoxide anions, respectively (30). Similarly, it has been shown that AKBA has antioxidant and anti-apoptotic activities. This compound can protect cardiomyocytes against cytotoxic effects of doxorubicin and injury induced by ischemia-reperfusion (17, 18). In gouty arthritic mice, boswellic acids restore the lipid peroxidation, tumor necrosis factor-α, lysosomal enzymes activities, and the antioxidant status to near normal levels (31).

The neuroprotective effect of *B. serrata* observed in this study is in concert with reports from Jalili et al. that aqueous extract of this plant increase the number of neuronal processes in the hippocampus and improves learning deficit in both normal and kindled rats (32). An *in vitro* study also showed that β-boswellic acid could increase neurite outgrowth and branching of primary cells of hippocampal tissue in rats (33). In patients suffering from cerebral edema due to brain tumors, administration of *B. serrata* significantly reduced the volume of edema (34). This effect may be mediated in part by anti-inflammatory action of *B. serrata* (11, 14). Because inflammation is also known to play a pivotal role in the pathogenesis of cerebral ischemia

(27), it is reasonable to assume that anti-inflammatory action of BSE and AKBA be involved in their neuroprotective effect observed in this study. Consistently, it has been shown that *B. serrata* down regulates key inflammatory mediators; interleukin-1β, interleukin-6, and tumor necrosis factor-α in peripheral blood mononuclear cells (35). Also, among boswellic acids, AKBA is the most potent inhibitor of 5-lipoxygenase, an enzyme responsible for inflammatory responses (15). AKBA is able to cross blood-brain barrier, and it can be detected in the brain after oral administration of BSE (36).

In conclusion, results of the present study suggest that *B. serrata* and its active constituent AKBA can reduce neuronal cell death induced by OGSD. The potential mechanism for this neuroprotective effect of *B. serrata* and AKBA might be the inhibition of oxidative stress occurring following ischemia.

Acknowledgment

This work was a part of the Pharm. D. thesis of Fatemeh Arjmand and supported by a grant (No. 910427) from the Vice Chancellor for Research and Technology, Mashhad University of Medical Sciences. The authors declare that they have no conflict of interest.

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Received: 14. 06. 2016