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Original article

Neuroprotective effects of *Salvia aristata* Aucher ex Benth. on hydrogen peroxide-induced apoptosis in SH-SY5Y neuroblastoma cells

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Abstract

Background and objectives: Oxidative stress is implicated in the neuronal damage associated with Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis and cerebral ischemic stroke. The present work was designed to establish the neuroprotective effects of *Salvia aristata* extract on H_2O_2-induced apoptosis in human dopaminergic SH-SY5Y cells. **Methods:** The total phenol and flavonoids contents of the plant extracts were quantified by colorimetric methods. The antioxidant activity was assessed using DPPH free radicals scavenging activity assay, and the neuroprotective effect on H_2O_2-induced oxidative stress was also investigated using human dopaminergic SH-SY5Y cells by MTT assay and western blotting techniques. **Results:** The highest scavenging activity was found for methanol extract of *S. aristata* roots (85.28 ± 2.61 µg/mL), with the highest total phenolic and flavonoids content (90.28 mg total phenols as gallic acid and 250.12 mg total flavonoids as rutin, respectively). Our results also, showed that H_2O_2-induced cytotoxicity in SH-SY5Y cells was suppressed by treatment with *S. aristata*. Moreover, *S. aristata* root extract was effective in attenuating the disruption of mitochondrial membrane potential and apoptotic cell death has induced by H_2O_2. *S. aristata* suppressed the down-regulation of Bcl-2, upregulation of Bax, and the release of mitochondrial cytochrome *c* to cytosol. In addition, *S. aristata* attenuated caspase-3, and -9 activation, and eventually protected the cells against H_2O_2-induced apoptosis. **Conclusion:** The results of the present study suggest that treatment of SH-SY5Y cells with *S. aristata* could block H_2O_2-induced apoptosis by regulating Bcl-2 family members and by suppressing caspase cascade activation.

Keywords*:* apoptosis, human neuronal SH-SY5Y cells, neuroprotective, *Salvia aristata*

Introduction

Oxidative stress is implicated in the neuronal damage associated with Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis and cerebral ischemic stroke. The damage is thought to be mediated by reactive oxygen species (ROS) including superoxide anion ($O2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) [1]. H_2O_2-induced cell death involves apoptosis and necrosis in a concentration dependent manner in SH-SY5Y cells, as shown by flow cytometric analysis of Annexin V-fluorescein isothiocyanate

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(FITC) and propidium iodide staining, PS translocation [2], decrease in SH-SY5Y cell viability and mitochondrial membrane depolarization [3]. In addition, cell cycle is a highly regulated process with numerous checkpoints to ensure homeostatic balance between cell proliferation and cell death under appropriate environmental signals [4], thus these results suggest therapeutic strategies aimed at preventing and delaying apoptosis might be a reasonable choice for the treatment of the disease. However, the removal of free radicals and ROS is probably one of the most effective defenses of body to maintain the oxidative–antioxidant balance.

Nowadays, there is an increasing interest on natural products with antioxidant capacities that may be promising therapeutics for Parkinson's disease (PD). The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs from plant origin in developing countries [5]. Phenolic compounds such as flavonoids, phenolic acids, diterpenes and tannins have received attention for their high antioxidative and antibacterial activity. Dietary intake of such phytochemicals may be an important strategy for inhibiting or delaying pathological conditions and disease prevention [6].

The genus *Salvia*, with about 700 species, is one of the most widespread members of the Lamiaceae family. An unusually large number of useful secondary metabolites, belonging to various chemical groups, such as essential oils, terpenoid compounds and phenolic derivatives, have been isolated from this genus, which features prominently in the pharmacopoeias of many countries throughout the world for wound healing, in alleviating stomach, liver, and rheumatism pains and for treating common cold [7,8]. Some members of this genus are of economic importance since they have been used as flavouring agents in perfumery and cosmetic industries. Sage (*Salvia officinalis*) with high antioxidant compounds such as carnosic acid, carnosol and rosmarinic acid has shown excellent antimicrobial activity as well as scavenging activity of reactive oxygen, inhibiting lipid peroxidation and antioxidant activity [9]. Nevertheless, no information is available regarding the effect of *S. aristata* extract against the pathogenesis of PD. Therefore, this study was designed to evaluate the neuroprotective effects of *S. aristata* extract against H_2O_2-induced neuronal damage.

Experimental

Chemicals

The cell culture medium (DMEM/F12) and penicillin-streptomycin were purchased from Gibco BRL (life technologies, Paisley, Scotland). The culture plates were obtained from Nunc (Denmark). Butylated hydroxytoluene (BHT), catechin, and Folin–Ciocalteu's reagent (FCR) were obtained from Sigma (St. Louis, MO, USA). The compound 1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Fluka (Buchs, Switzerland). Antibodies directed against caspase-3, caspase 9, Bax, Bcl-2, cytochrome c, and β-actin were obtained from Cell Signaling (Technology Beverly, MA, USA). All other reagents were of analytical reagent (AR) grade.

Plant material

The roots and aerial parts of *Salvia aristata* were collected at full flowering stage from Qazvin province, Iran in June, and were identified by Dr. Ali Sonboli. A Voucher specimen (MPH-1038) has been deposited in Medicinal Plants and Drugs Research Institute Herbarium of Shahid Beheshti University, Tehran, Iran. The plant aerial parts and roots were air-dried, protected from direct sunlight, and then powdered. The aerial and root parts powder (50 g) was passed through a sieve in order to maintain particle size unity and was extracted by maceration method with methanol (150 mL) at room temperature overnight. The methanol extract was filtered, concentrated under reduced pressure using a rotary evaporator and used for future studies.

Determination of total phenols and flavonoids levels

The total phenolics content of the plant extract was determined according to the Folin and Ciocalteu procedure [10] and the results were expressed as gallic acid equivalents (mg gallic acid equivalents/g dried extract). The flavonoid content of the plant was determined by using the colorimetric method as described Maksimovic and colleagues [11]. The concentrations of flavonoids were deduced from a standard curve and calculated as mg rutin equivalents.

DPPH free radicals scavenging activity assay

Radical scavenging capacity was determined according to the technique reported by Blois [12]. An aliquot of 1.5 mL of 0.25 mM DPPH solution in ethanol and 1.5 mL of the extract with various concentrations were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the absorbance at 517 nm with a spectrophotometer (Elx 800 Microplate Reader, Bio-TEK, Winooski, Vermont, USA). The DPPH radicals scavenging activity was calculated according to the following equation:

Scavenging activity = $[(A_0-A_1/A_0)\times 100]$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract or standard sample. Butylated hydroxytoluene (BHT) was used as positive control.

Cell culture and cell viability

SH-SY5Y neuroblastoma cells obtained from Pasteur Institute (Tehran, Iran) were grown in DMEM/F12 (1/1) medium with L-glutamine (Gibco), supplemented with 10 % fetal bovine serum and 1 % antibiotic mixture comprising penicillin–streptomycin, in a humidified atmosphere at 37 °C with 5 % CO_2. Cell viability was estimated using the MTT assay. The cells were seeded in 96-well culture plates at a concentration of 10^5 cells/mL for 24 h and then treated in duplicate with plant extract at different concentrations. After various time intervals, 10 µL MTT (5 mg/mL) were added to each well 4 h before harvesting. The reaction was stopped by adding DMSO and the absorbance values at 570 nm were determined with a multi-well plate reader (Elx 800 Microplate Reader, Bio-TEK, Winooski, Vermont, USA).

Cell treatments

For evaluating the protective effect of plant extract against an oxidative insult, the cells were pretreated with different concentrations of the plants extracts for 16 h and then, the medium was discarded and fresh medium containing 300 µM H_2O_2 were added followed by incubation for an additional 3 h [13]. Cell viability was estimated using the MTT assay, and the cellular viability was expressed in percentage of survival relative to the control cell samples.

Hoechst staining

To quantify and assess nuclear morphology, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, and then stained with 10 µg/mL fluorescent DNA-binding dye Hoechst 33342 (Invitrogen, H3570) for 10 min to reveal nuclear condensation. The cellular morphology was evaluated by fluorescence microscope (Zeiss, Germany).

Acridine orange/ethidium bromide (AO/EB) double staining

SH-SY5Y cells were seeded in a 96-well plates and were treated with different concentrations of the plant extract for 16 h followed by adding H_2O_2 (300 µM) for 3 h. AO/EB solution (1:1 v/v) was added to the cell suspension with the final concentration of 100 µg/mL. The cellular morphology was evaluated by fluorescence microscope (Zeiss, Germany).

Quantification of apoptotic cells by flow cytometry

Apoptosis was measured with annexinV-FITC/PI apoptosis detection kit (Invitrogen, CA, USA) according to the manufacture's instruction.

Treated cells were harvested for 24 h, washed twice with PBS, gently re-suspended in annexin-V binding buffer and incubated with annexin-V-FITC/PI in dark for 10 min and analyzed by flow cytometry using FloMax software. The fraction of cell population in different quadrants was analyzed using quadrant statistics.

SDS–PAGE and Western blot analysis

SH-SY5Y neuroblastoma cells were initially seeded at a density of 1×10^6 in 100-mm^2 dishes. After treatment with selected doses of H_2O_2 and plant extract for the indicated times, adherent cells were collected for protein extract preparation. Briefly, treated and control cells were lysed with RIPA buffer then nuclear and cytoplasmic extracts were separated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Waltham, MA, USA). Equal amounts of lysate (based on the protein contents) were then separated using SDS–PAGE, blotted onto polyvinylidene di-fluoride membranes, reacted with specific primary antibodies, and then visualized with appropriate conjugated secondary antibodies. Immunoreactive polypeptides were detected by chemiluminescence using enhanced Electro Chemi Luminescence (ECL) reagents (Amersham Bioscience, USA) and subsequent autoradiography. Quantification of the results was performed by densitometric scan of films. Data analysis was done by Image J, measuring integrated density of bands after background subtraction.

Measurement of mitochondrial membrane potential (MMP)

The mitochondrial membrane potential (MMP) was measured using the mitochondria-specific lipophilic cationic fluorescence dye JC-1. As a monomer, JC-1 is capable of selectively entering the mitochondria. Under normal conditions, JC-1 aggregates within the mitochondria and emits red fluorescence, but when the MMP collapses during apoptosis, JC-1 emits green fluorescence. The ratio of red to green JC-1 fluorescence reflects the change in MMP. Cells were seeded into 96-well polystyrene culture plates and treated with different concentrations of *S. aristata* extract. After 24 h, 100 µL of medium was removed from each well and 100 µL of 5 µg/mL JC-1 was added to the cells for 1 h. Finally, cells were washed twice with PBS and then analyzed by fluorescence microplate reader (Molecular Devices Co., CA, USA).

Statistical analysis

The results represent means± standard deviation (SD) from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. All comparisons were made relative to untreated controls and significance of difference in means was measured at $p< 0.01$

Results and Discussion

Oxidative stress, an imbalance in antioxidant levels, has been implicated to play a crucial role in the pathogenesis of a number of diseases including neurodegenerative disorders [1]. Among all the body organs, the brain is particularly vulnerable to oxidative damage because of its high utilization of oxygen, increased levels of polyunsaturated fatty acid (that are readily attacked by free radicals), relatively high levels of redox transition metal ions and low levels of antioxidants. Thus, treatment with antioxidants appears to be an alternative approach for slowing brain diseases progression. It is well known that plant phenolic compounds are highly effective free radical scavengers and antioxidants. They exhibit considerable free radical scavenging activities, through their reactivity as hydrogen- or electron-donating agents and metal ion chelating properties [14]. Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties [15]; therefore, it would be valuable to determine the total phenolic and flavonoid content of the plant extracts. Total phenolic and flavonoid contents of plant extracts were determined and expressed in terms of gallic

acid and rutin equivalents (table 1). As shown in table 1, the methanol extract of *S. aristata* roots has showed the highest total phenolic and flavonoids (90.28 mg phenols as gallic acid and 250.12 mg flavonoids as rutin, respectively) compared to *S. aristata* areal parts (70.43 mg phenols as gallic acid and 33.46 mg flavonoids as rutin, respectively.

Table 1. Antioxidant activity and total phenolic and flavonoid contents of *Salvia aristata* roots and aerial parts in DPPH assays as compared to BHT and rutin

Samples	DPPH (IC_{50})[a]	Total phenolics[b]	Total flavonoids[c]
S. aristata roots	85.28 ± 2.61	90.28±4.63	250.12±8.57
S. aristata aerial parts	157.18 ± 2.24	70.43±5.04	33.46±4.21
BHT	124.32	-	-

[a]: IC_{50} values were represented as µg/mL. Each value represents the mean ± SD

[b] Total phenolics content was expressed as mg gallic acid equivalents/g dried extract.

[c] Total flavonoids content was expressed as mg rutin equivalents/g dried extract

A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods [16]. It has also been used to quantify antioxidants in complex biological systems in recent years. The IC_{50} values (defined as the concentration of test compound required to produce 50% inhibition) for DPPH scavenging by *S. aristata* roots and areal parts are shown in table 1. According to the findings presented in table 1, the highest scavenging activity was found for methanol extract of *S. aristata* roots (85.28 ± 2.61 µg/mL), followed by *S. aristata* areal parts (157.18 ± 2.24 µg/mL). Based on these cumulative data, it is clear that the methanol extract of *S. aristata* roots possess high antioxidant activity. Therefore, we used *S. aristata* roots extract in further studies for neuroprotective experiments.

SH-SY5Y is a human derived cell line used in scientific research. The original cell line, called SK-N-SH, was isolated from a bone marrow biopsy taken from a four-years-old female with neuroblastoma. SH-SY5Y cells are often used as in vitro models of neuronal function and differentiation. They are adrenergic in phenotype but also express dopaminergic markers and have been used to study Parkinson's Disease [17]. H_2O_2-induced cytotoxicity is the common method employed for the measurement of potential neuroprotective antioxidants [18,19]. The SH-SY5Y cells exposed to H_2O_2 were distinctively low in viability and prevented the gap junction intercellular communication. However, the cells pretreated with *S. aristata* increased cell viability and protected from H_2O_2-Induced oxidative stress (figure1).

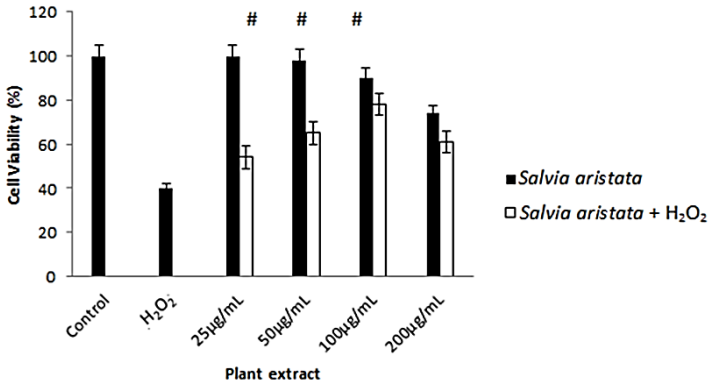

Figure 1. Effect of f *S. aristata* extract on cell growth treated with H_2O_2. Each value represents the mean ±SD (n = 3). # significantly different from H_2O_2-treated cells

We further investigated the neuroprotective effect of *S. aristata* roots on cell cycle and on inducing programmatic cell death. Morphological changes, DNA fragmentation and phosphatidyl serine externalization were analyzed. The nuclear morphology of cells exposed to *S. aristata* alone was intact and similar to that of untreated control cells. However, exposing SH-SY5Y cells to H_2O_2 for 3 h resulted in nuclear condensation, heterogeneity in the shape and fragmentation and characteristics of apoptosis (figure 2a).

In contrast, *S. aristata* pretreatment significantly blocked the H_2O_2 induced nuclear damage. Nuclear fragmentation and apoptotic bodies formation observed in SH-SY5Y cells treated with H_2O_2 that consequently could be the cause of significant decrease in cell viability. Genomic DNA laddering is a hallmark of apoptosis that was apparent in SH-SY5Y cells incubated with H_2O_2 for 3 h (figure 2b). This method assesses the total number of cells that had been exposed to phosphatidyl serine during apoptosis and/or lost plasma membrane integrity. As detected by annexinV(+), PI(-) % cells using flow cytometry, H_2O_2 led to a concentration-dependent externalization of phosphatidylserine, a hallmark of apoptosis. AnnexinV(+)/PI(-) and annexinV(+)/PI(+) represent the cells in early and late stages of apoptosis/necrosis, respectively. The lower left quadrant in figure 3 shows intact cells, the lower right quadrant shows the early-apoptotic and the In contrast, *S. aristata* pretreatment significantly reduced the formation of apoptotic bodies in SH-SY5Y cells. Apoptotic cell death was further determined by annexin V staining of phosphatidyl serine exposure on the cell surface, and assessed by flow cytometry (figure 3). upper right quadrant show late-apoptotic or necrotic cells. We compared *S. aristata* pretreated cells with H_2O_2 treated controls and expressed the percentages of late apoptotic cells that were decreased from 25.36% to 10.82% in the pretreated cells with 100 µg/mL of plant extract (figure 3).

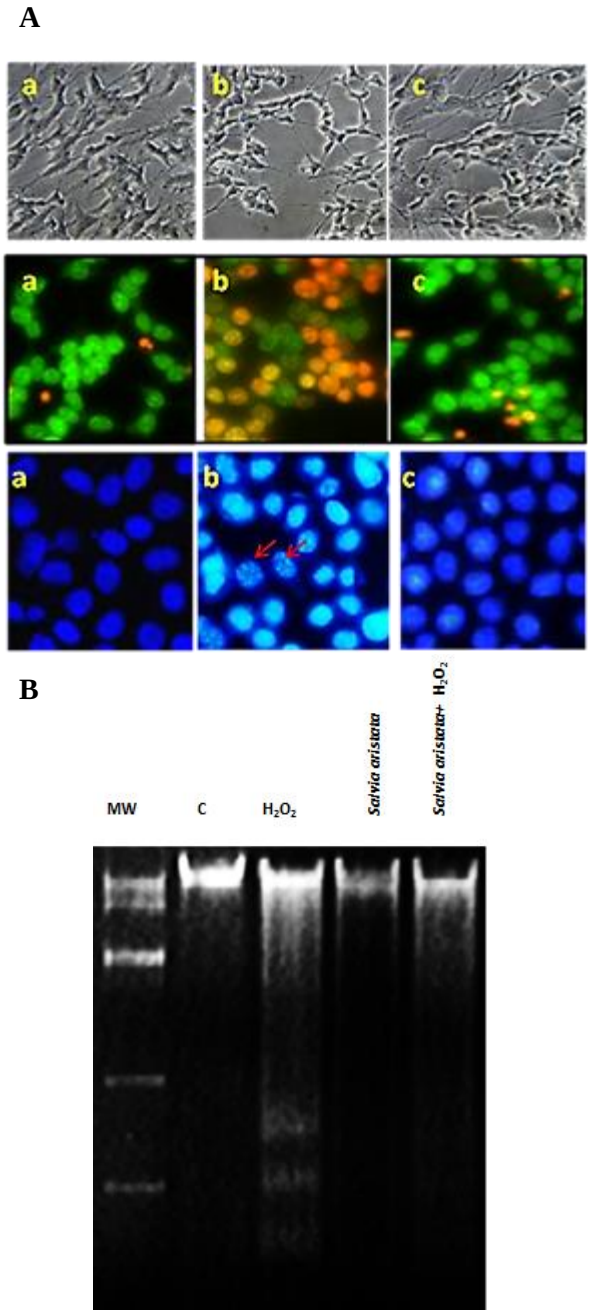

Figure 2. A) Phase contrast and fluorescent micrographs of acridine orange (AO, green) and ethidium bromide (EB, red) double-stained human SH-SY5Y cells. (a) Untreated control cells. (b) Treatment with 300 µM H_2O_2 for 3 h. (c) Cells pretreated with 100 ug/mL of plant extract and subsequent treatment with 300 µM H_2O_2 for 3 h. Condensed nuclei could be observed in the H_2O_2-treated group as indicated by the arrows. B) DNA fragmentation profile of control and treaded cells.

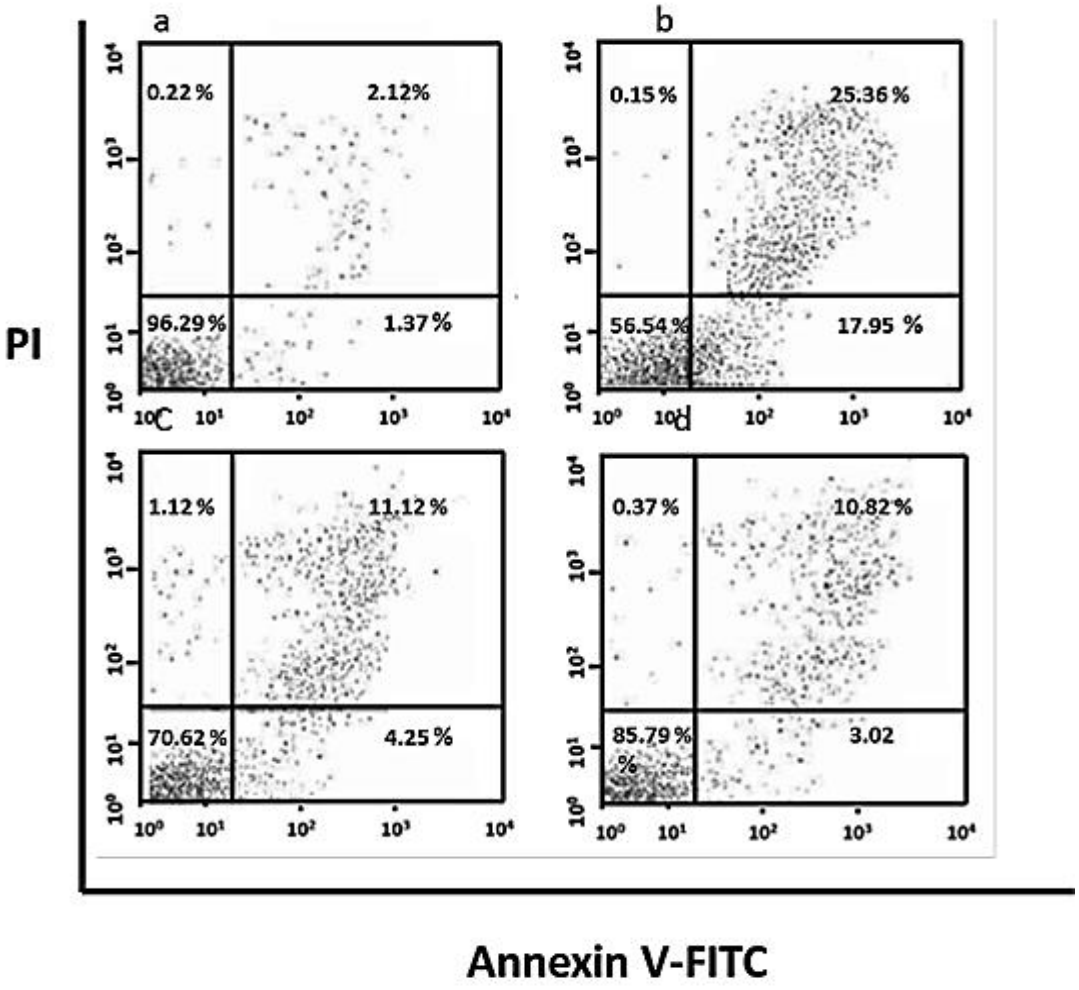

Figure 3. Flow cytometric determination of apoptotic and necrotic cell death after exposure of SH-SY5Y cells to M H_2O_2. a) Control, untreated SH-SY5Y cells; b) SH-SY5Y cells treated with H_2O_2; c and d) exposure of SH-SY5Y cells to H_2O_2 in the presence of pre-treated 50 and 100 µg/mL *S. aristata*, respectively

These results showed major hallmarks of apoptotic cell death, and demonstrated the neuroprotective effect of *S. aristata* root extract against H_2O_2-induced cell death in SH-SY5Y neuroblastoma cells.

Apoptosis is an active process of cell death characterized by distinct morphological features such as chromatin condensation, cell and nuclear shrinkage, membrane blebbing and oligonucleosomal DNA fragmentation [20]. The biochemical activation of apoptosis occurs through two main pathways, i.e. the intrinsic apoptotic pathway that involves the mitochondria, and the extrinsic apoptotic pathway that involves death receptors. The mitochondrial pathway of apoptosis is regulated by members of the Bcl-2 family of proteins. Two of the major proteins of this family responsible for regulating apoptotic cell death are Bcl-2 and Bax. Bcl-2 is a 28 kDa protein found in the nuclear envelope, parts of the endoplasmatic reticulum and the outer mitochondrial membrane that regulates the antioxidant pathway at the site of free radical generation [21]. An elevated intracellular ratio of Bax to Bcl-2 occurs during

apoptotic cell death. Indeed, the ratio of pro-apoptotic proteins of the Bcl-2 family, such as Bax, versus anti-apoptotic proteins such as Bcl-2, determines the sensitivity or resistance of cells to various apoptotic stimuli [22]. Moreover, it has been shown that cells overexpressing Bcl-2 have a higher mitochondrial potential than wild–type cells which is responsible for the enhanced survival of the cells after challenges. In contrast, following activation of multi domain pro-apoptotic proteins, the integrity of the outer mitochondrial membrane is broken which generates an efflux pathway for cytochrome c and other pro-apoptotic factors stored in the inner membrane space of the mitochondria [23,24].

Under normal conditions, cytochrome c acts as an electron shuttle between respiratory complexes in the mitochondria. Its oxidation is due to its ability to catalyze the oxidation of superoxide radicals to molecular oxygen. During the depolarization, the mitochondrial cytochrome c is released to the cytoplasm as an oxidized form which is capable of activating the apoptosome and caspase cascade[24,25]. In the present study, we found that pretreatment with *S. aristata* decreased the cytosolic levels of cytochrome c, which was significantly elevated in H_2O_2-treated cells. Thus, it seems that part of neuroprotective effect of *S. aristata* exert by inhibition of mitochondrial cytochrome c release to the cytosol. Increase of cytochrome c initiates apoptotic pathway by activating series of cysteine proteases called caspases. Among this family, caspase-3 has been known as a key executor protease in apoptotic cell death pathways. In this study, we detected that, incubation of SH-SY5Y cells with H_2O_2 for 3h induced the cleavage of Caspase-3 from its native 35 kDa form to an active 17 kDa fragment, indicative of cells undergoing apoptosis. However, the cells pre-incubated with *S. aristata* prior to the addition of H_2O_2, showed a marked attenuation of Caspase-3 and 9 activation (figure 4a). Substantial evidence supports the idea that Bcl-2 family members regulate the release of mitochondrial proteins [26,27].

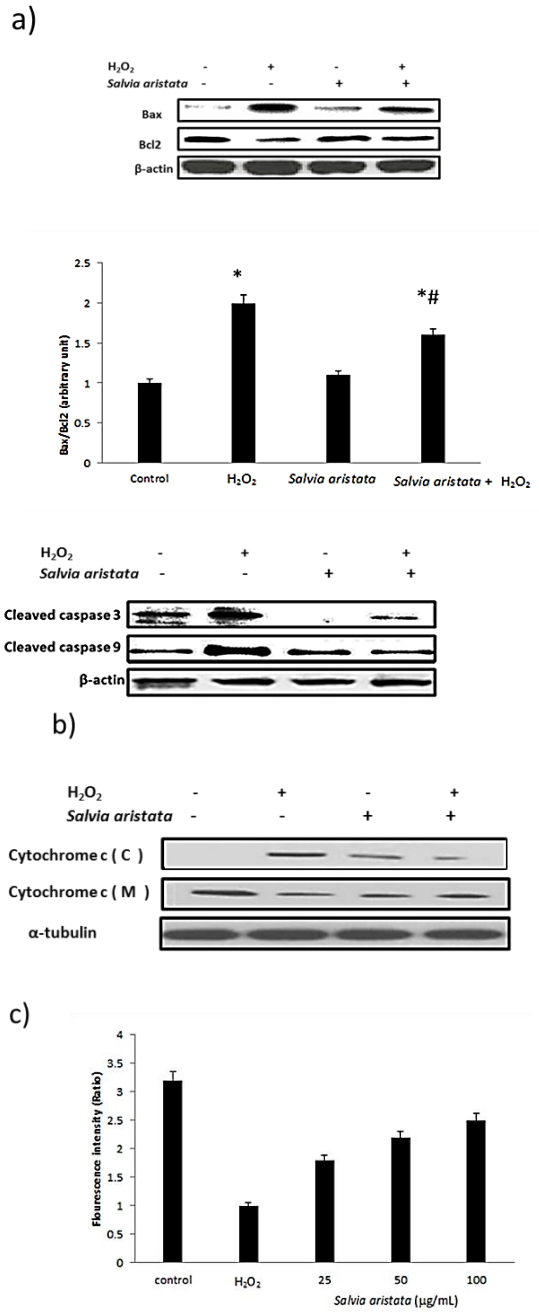

Figure 4. (a, b) The expression of apoptosis-related proteins in SH-SY5Y cells treated with *S. aristata* detected by immunoblotting, (c) Rearrangement of mitochondrial membrane potential (Δψm) after plant extracts treatment in SH-SY5Y neuroblastoma cells

In the present study, we found that H_2O_2 treatment significantly increased the level of the pro-apoptotic protein Bax (figure 4a). In contrast, the level of the anti-apoptotic protein Bcl-2 decreased (figure 4a). Our data consistently demonstrates that *S. aristata* suppressed H_2O_2 induced apoptosis takes place via the mitochondrial pathway (intrinsic apoptotic pathway) and activation of the caspase cascade, including caspase-9 and caspase-3. These suggest that the protective effect of *S. aristata* is mediated via an, at least in part, anti-apoptotic pathway.

Changes in mitochondrial membrane potential ($\Delta\psi$) are one of the early events leading to mitochondrial functional alterations. Incubation of SH-SY5Y cells with *S. aristata* for 16 h, followed by H_2O_2 treatment, resulted in a dose dependent increase in the mitochondrial membrane potential (figure 4c). The dose-dependent increase of the OD585/OD538 ratio indicated that the mitochondrial membrane potential had been repaired (figure 4c). *Salvia aristata* (100 µg/mL), inhibited a significant release of cytochrome c from mitochondria to the cytosol fraction as compared to the H_2O_2 treated cells (figure 4b).

In summary, these results show that *S. aristata* decreased H_2O_2-induced cell death, morphological change of nuclei, apoptosis-related gene expressions (Bax, Caspase-3, and Caspase-9), and increased Bcl-2 expression, in human dopaminergic cells, SH-SY5Y. Based on these results, we concluded that *S. aristata* exhibits its neuroprotective effect thorough the inhibition of apoptosis-related gene expression. This study may offer a new therapeutic strategy in treating Parkinson's disease, although further research into the neuroprotective mechanisms of *S. aristata* will be necessary.

Acknowledgments

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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