S. choloroleuca, *S. mirzayanii* and *S. santolinifolia* protect PC12 cells from H₂O₂-induced apoptosis by blocking the intrinsic pathway

Shabnam Zeighamy Alamdary · Fariba Khodaghohi ·
Fatemeh Shaerzadeh · Niloufar Ansari · Ali Sonboli ·
Solaleh Khoramian Tusi

Received: 5 September 2011 / Accepted: 7 December 2011 / Published online: 31 December 2011
© Springer Science+Business Media B.V. 2011

Abstract Several studies have shown that neuronal cell death due to apoptosis is the major reason for cognitive decline in Alzheimer's disease. In this study, we report the anti-apoptotic effects of three *Salvia* species from Iran—*S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia*—against H₂O₂-induced cytotoxicity in neuron-like PC12 cells. We showed that these antioxidant species could interfere with the intrinsic pathway of apoptosis by attenuating Bax/Bcl-2 ratio, decreasing outer mitochondrial membrane break and decreasing cytochrome *c* release to cytoplasm. Interestingly, we found that these species were able to replenish reduced glutathione level which affects cellular redox status and cytochrome *c* activity. Moreover, the decreased level of caspase-3, the executioner caspase, resulted in decrease of PARP-1 cleavage. Anti-apoptotic effects of these species along with their antioxidant effects, may represent a promising approach for treatment of neurodegenerative diseases.

Keywords Apoptosis · Oxidative stress · PC12 cells · *Salvia*

Introduction

Oxidative stress refers to the undue oxidation of biological molecules by intracellular reactive oxygen species (ROS) such as the superoxide radical (O₂^{•−}), the hydroxyl radical (HO[•]) and hydrogen peroxide (H₂O₂) that are normally byproducts of cellular metabolism (Touyz and Schiffrin 2004). Under certain conditions, the balance between the generation of ROS and their detoxification is upset and their overproduction overwhelms antioxidant defense systems and initiates a cascade of events which leads to impaired cellular function or cell death (Götz et al. 1994; Halliwell and Aruoma 1991; Valko et al. 2007). Apoptosis, which plays a critical role in the normal development and maintenance of tissue homeostasis, plays an important role in neurodegenerative diseases and aging (Zawia et al. 2009; Fadeel et al. 1999). Since mitochondria are the main site of free radical production (Raha and Robinson 2001), their involvement in neuronal degeneration has been hypothesized (Beal 1992; Schapira 1996; Wallace et al. 1992). In mammalian cells, apoptosis has been divided into two major pathways: the extrinsic pathway, activated by pro-apoptotic receptor signals at the cellular surface; and the intrinsic pathway, which involves the disruption of mitochondrial membrane integrity. Several

S. Z. Alamdary · F. Khodaghohi (✉) · F. Shaerzadeh ·
N. Ansari · S. K. Tusi
Neuroscience Research Center, Shahid Beheshti
University of Medical Sciences, Tehran, Iran
e-mail: khodaghohi@sbmu.ac.ir

A. Sonboli
Department of Biology, Medicinal Plants and Drugs
Research Institute, Shahid Beheshti University,
G.C. Tehran, Iran

molecular changes have been investigated during intrinsic pathway of apoptosis including activation of anti-apoptotic factors, such as Bcl-2, and inactivation of pro-apoptotic effectors, such as Bax (Gaumer et al. 2000), that result in disruption of outer mitochondrial membrane and efflux of cytochrome *c* and other pro-apoptotic factors into cytoplasm (Caroppi et al. 2009). Cytochrome *c* initiates a cascade of caspase activation that brings about known features of apoptotic cell death (Suto et al. 2005).

The genus *Salvia* (*Lamiaceae*) includes nearly 900 species spread throughout the world, of which 17 are endemic to Iran including *S. mirzayanii* (Mozafarian 1996). The phytochemical analysis of *Salvia* species shows the presence of many compounds belong mainly to the group of phenolic acids, phenolic glycosides, flavonoids, anthocyanins, coumarins, polysaccharides, sterols, terpenoids and essential oils (Lu and Foo 2002). Several lines of studies from our laboratory indicated the high antioxidant and neuro-protective effects of various *Salvia* species (Asadi et al. 2010, 2011; Shaerzadeh et al. 2011). Traditional medicinal uses and antioxidant properties of *Salvia* (Asadi et al. 2010, 2011; Shaerzadeh et al. 2011) prompted us to investigate intracellular signaling triggered by three species of this genus, *S. cholero-leuca*, *S. mirzayanii* and *S. santolinifolia*, against H₂O₂-induced oxidative stress in rat pheochromocytoma (PC12) cells.

Materials and methods

Materials

Antibodies directed against caspase-3, Bax, Bcl-2, cytochrome *c*, poly (ADP-ribose) polymerase-1 (PARP-1) and β -actin were obtained from Cell Signaling Technology (Danvers/MA, USA). All the other reagents, unless otherwise stated, were from Sigma Aldrich (St. Louis, MO).

Plant extraction

Aerial parts of plants were collected from different areas of Iran. These plants were deposited in Medicinal plant and Drug research Herbarium of Shahid Beheshti University. The plant aerial parts were air-dried, protected from direct sunlight, and then powdered.

The powder was kept in a closed container in cold room. Powdered plants (50 g) were extracted four times with methanol at room temperature overnight. Methanolic extracts were combined and concentrated under reduced pressure on a rotary evaporator, filtered and then lyophilized. For cell treatment, lyophilized powder was solved in distilled water.

Cell culture, differentiation and treatment conditions

Rat pheochromocytoma cells (PC12) obtained from Pasteur Institute (Tehran, Iran) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% horse serum, 5% fetal bovine serum (FBS) and 1% antibiotic mixture comprising penicillin–streptomycin, in a humidified atmosphere at 37 °C with 5% CO₂. Growth medium was changed three times a week. The cells were differentiated by treating with nerve growth factor (NGF; 50 ng/mL) every other day for 6 days.

To evaluate the neuroprotective effects of *Salvia* species, differentiated PC12 cells were pretreated with different concentrations of plant extract (10, 25, 50 and 100 μ g/mL). After 24 h, 150 μ M H₂O₂ was added as an oxidative agent. All the experiments were performed 24 h after addition of H₂O₂.

Measurement of neurite outgrowth in differentiated PC12 cells

For morphological analysis, random images were acquired from each well, two images per well. A minimum of 50 cells per treatment were quantified. Criteria for selection were that the cell body and processes were completely within the field of view, and that the cell body was distinct from neighboring cell bodies. Cells fitting these criteria were analyzed and their cell body area, average neurite length, average neurite width, number of primary neuritis and bipolar morphology were quantified. Cell body area was defined as the area of the cell exclusive of neurite processes. Neurite length was calculated by summing the lengths of the primary process and all associated branches. To establish the average neurite width, the outlines of individual primary neurites were traced, the area calculated and then divided by the length of the neurite. Primary neurites were defined as clear protrusions from the cell body greater than 10 μ M

length. Cells were considered “bipolar” if they displayed a cell body with one process at either end. To evaluate neurite networks, images were analyzed using the cell counter plugin to score all branching nodes in each image. Nodes were defined as sites at which individual neurites branched or separate neurites contacted each other.

All measurements expressed as proportions used the number of cells displaying the characteristic as a sub-population of the total number of cells that met selection criteria described above.

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

Apoptosis was determined morphologically after staining the cells with AO/EB followed by fluorescence microscopy inspection. Briefly, PC12 cells were seeded in a 6-well plate and were treated with different concentrations of extract (10, 25, 50 and 100 µg/mL). After 24 h, 150 µM H₂O₂ was added as an oxidative agent. After 24 h incubation, the cells were harvested and washed three times with phosphate buffered saline (PBS) and were adjusted to a density of 1×10^6 cells/mL of PBS. AO/EB solution (1:1 v/v) was added to the cell suspension in a final concentration of 100 µg/mL. Cellular morphology was evaluated by fluorescence microscope (Zeiss, Germany).

Hoechst staining

PC12 cells (1×10^6 cells/mL) were treated with different concentrations of *Salvia* species for 24 h followed by adding H₂O₂ (150 µM) for 24 h. Nuclear morphological changes were assessed using the Hoechst dye 33342 (Invitrogen, H3570). Cells were washed by PBS and incubated with Hoechst 33342 (1:1,000) for 5 min at room temperature. Nuclei were visualized using an Olympus Microscope.

Measurement of intracellular ROS

The fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA) was used to monitor intracellular accumulation of ROS. For this purpose, DCFH-DA solution (10 µM) was added to the suspension of the cells (1×10^6 /mL) and the mixture was incubated at 37 °C for 1 h. Cells were then washed twice with PBS and the fluorescence intensity was measured by the

Varian Cary Eclipse spectrofluorometer with excitation and emission wavelengths of 485 nm and 530 nm, respectively.

Measurement of mitochondrial membrane potential (MMP)

MMP was estimated by a fluorescence assay with fluorescent dye Rhodamin 123 (Poppe et al. 2001). Rhodamin 123 (Rh123) is a lipophilic cation and could be transported into mitochondria by negative MMP and concentrated within mitochondria matrix. After treatment, differentiated-PC12 cells were incubated for 30 min at 37 °C with PBS containing 5 µM Rh123. After being washed with PBS, cells were trypsinized at room temperature and suspended in PBS. The fluorescence intensity was measured by the Varian Cary Eclipse spectrofluorometer with excitation and emission wavelengths of 485 and 530 nm, respectively.

Western blot analysis

Total proteins were electrophoresed in 12% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. Immunoreactive polypeptides were detected by chemiluminescence using enhanced ElectroChemiluminescence reagents (Amersham Bioscience, USA) and subsequent autoradiography. Quantification of results was performed by densitometric scan of films. Data analysis was done by Image J after background subtraction. Protein concentration was determined by Bradford method (Bradford 1976), using bovine serum albumin (BSA) as a reference standard.

Isolation of mitochondria from the PC12 cells

All manipulation steps were carried out at 4 °C. Mitochondrial and cytosolic fractions were prepared as described previously (Mihara and Moll 2003), with minor modifications. Briefly, cells were harvested and re-suspended in buffer A (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 68 mM sucrose, 220 mM mannitol and 0.1% BSA), supplemented with protease inhibitors (1 mM PMSF, 2 µg/mL aprotinin, and 0.1 mM leupeptin). After 30 min incubation on ice, cells were disrupted with a 27 gauge syringe (25–40 strokes), and the homogenates were centrifuged at 200g for 2 min to eliminate unbroken cells.

The supernatants (500 µl) were then centrifuged at 7,000g for 15 min to obtain the heavy membrane pellet enriched with mitochondria. The resultant supernatants pre-cleared at 16,000g for 30 min were collected as the cytosolic fractions. Mitochondrial fractions were subsequently solubilized in TBSTDS (10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% SDS, 0.02% NaN₃ and 0.0004% NaF) supplemented with protease inhibitors (1 mM PMSF, 2 µg/mL aprotinin, and 0.1 mM leupeptin). All fractions were stored at −70 °C until used.

Measurement of glutathione levels

The concentration of glutathione (GSH) was determined in whole cell lysate using dithionitrobenzoic acid (DTNB) method at 412 nm (Ellman 1959) and GSH concentrations were expressed as µmol/mg protein.

Statistical analysis

All data are represented as the mean ± SEM (standard error mean). Comparison between groups was made by one-way analysis of variance (ANOVA) followed by an appropriate post hoc test to analyze the difference. The statistical significances were achieved when $P < 0.05$ (* or $^{\#}P < 0.05$, ** or $^{##}P < 0.01$ and *** or $^{###}P < 0.001$).

Results

The effect of *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* on neurite growth in differentiated PC12 cells

Three criteria, cell body area, average neurite length and average neurite width, were selected to monitor cell growth. As shown in Fig. 1a, b, average cell body area was increased in H₂O₂ exposed cultures, compared to control cultures, whereas pretreatment of cells with *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* significantly decreased this value. Effect of *Salvia* extracts on neurite length contrasted with the results of cell body area (Fig. 1c). Moreover, neurites exposed to 150 µM H₂O₂ were not only shorter but even wider than those in control cultures. Because of variability of the width of the neurite, the total area of the neurite was

divided by the neurite length to calculate average neurite width. This ratio was significantly decreased in *Salvia* extracts-treated cells (Fig. 1d).

The effect of *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* on neurite complexity in differentiated PC12 cells

Specific parameters of morphological complexity were also evaluated. First, the number of primary neurites (>10 µm) emanating from individual cell bodies was measured. As shown in Fig. 1e, the number of primary neurites per cell was decreased in H₂O₂-treated cells. In contrast, the proportion of cells with the very simple bipolar morphology of a cell body and only two neurites was increased in H₂O₂-treated cells, compared to control cultures (Fig. 1f). Pretreatment of cells with *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* significantly and dose-dependently increased the number of neurites per cell, and thus decreased the proportion of bipolar cells (Fig. 1e, f).

As a final parameter of complexity, we calculated the ratio of total neurite branching nodes to the total number of primary neurites. As shown in Fig. 1g, the ratio of nodes to primary neurites decreased in H₂O₂-treated cells, whereas *Salvia* extracts increased this ratio significantly.

Morphological evaluation of apoptosis

AO/EB staining discriminates live cells from dead ones on the basis of membrane integrity. AO is a cell-permeable nucleic acid selective cationic dye which is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double stranded nucleic acid. EB intercalates and stains DNA, providing a red-orange fluorescence. Although it does not stain healthy cells, it can be used to identify cells that are in the early or final stages of apoptosis which have much more permeable membranes. Thus, the extent of apoptosis is determined based on fluorescence emission and morphological aspect of chromatin condensation in stained nuclei. Apoptotic cells have orange to red nuclei with condensed or fragmented chromatin, whereas viable cells have uniform bright green nuclei with organized structure. The result obtained from AO/EB double staining is represented in Fig. 2. Analysis of the stained cells indicated that pretreatment of cells with *Salvia* extracts had significantly and

Fig. 1 Effect of *Salvia* species on H_2O_2 -induced disruption of neurite outgrowth in differentiated PC12 cells. **a** The criteria of PC12 differentiation is shown on two neurons (left image) of a sample image. The letter 'P' on right image indicates the primary neuritis of neuron 1. The two ways arrow shows the length of a neurite, extent elongated and membrane-enclosed protrusions of cytoplasm. The circle on the right image shows the cell body of neuron 1. Neurite width is not equal in all parts of neurons, thus the average neurite width must be calculated by dividing cell body area to average neurite length. The arrows show to bipolar cells. The letter 'N' indicates the nodes, the sites at which individual neurites branched or separate neurites

contacted each other. **b** NGF-differentiated PC12 cells were pretreated with different concentrations of *Salvia* (10, 25, 50 and 100 $\mu\text{g/mL}$) in the presence of H_2O_2 (150 μM). Criteria were quantified at 24 h; **c** cell body area; **d** average neurite length; **e** average neurite width; **f** primary neurites per cell; **g** percent of bipolar cells and **h** the ratio of nodes to primary neurites. Each value indicates the mean $\pm$ SEM from three independent experiments and three replicates ($n = 3$). $^{\#}P < 0.05$; $^{##}P < 0.01$ significantly different from control cells. $^*P < 0.05$; $^{**}P < 0.01$ significantly different from H_2O_2 -treated cells

Fig. 1 continued

dose-dependently decreased the extent of cell apoptosis compared to the cells exposed solely to H₂O₂.

In addition, Hoechst 33342 is a DNA stain that binds preferentially to A–T base-pairs. Hoechst staining showed that DNA fragmentation and condensation of chromatin, which cause bright fluorescence, were decreased in the presence of *Salvia* species, compared to H₂O₂-treated cells (Fig. 3).

S. choloroleuca, *S. mirzayanii* and *S. santolinifolia* inhibited H₂O₂-induced ROS production

Reactive oxygen species levels produced by H₂O₂ were determined based on DCF fluorescence. The fluorescence intensity of DCF in cells treated with

H₂O₂ (150 μM) was increased by 5.07-fold, compared to untreated control cells. Pretreatment of the cells with different concentrations of *Salvia* extracts (10, 25, 50 and 100 $\mu\text{g/ml}$) prevented the accumulation of ROS relative to H₂O₂-treated cells (Fig. 4).

S. choloroleuca, *S. mirzayanii* and *S. santolinifolia* decreased Bax/Bcl-2 ratio in neuron-like PC12 cells

The Bcl-2 family of proteins can be separated into two functionally distinct groups: anti- and pro-apoptotic proteins. Bcl-2, is a 28 kDa membrane-bound anti-apoptotic protein that blocks neuronal death in the developing brain (Farrow and Brown 1996).

Fig. 2 Analysis of apoptosis by using AO/EB double staining. **a** The cells were exposed to different concentrations of *Salvia* for 24 h followed by exposure to 150 µM of H₂O₂ for 24 h. The morphological patterns of apoptotic cells are described in the text. **b** The number of stained cells was counted in 10 randomly

selected fields. Viability was calculated as the percentage of living cells in treated cultures compared to those in control cultures. *Significantly different from untreated cells. #Significantly different from H₂O₂-treated cells (** or ## $P < 0.01$ and *** or ### $P < 0.001$)

According to these findings, to determine whether these *Salvia* species have anti-apoptotic effects or not, we measured the Bax/Bcl-2 ratio by Western blot analysis in the presence of different concentrations of these species (10, 25, 50 and 100 µg/mL). As shown in Fig. 5, H₂O₂ markedly increased Bax/Bcl-2 ratio, while in those cells pretreated with *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* at their highest concentration (100 µg/mL), this ratio was attenuated

by about 4.17-, 4.13- and 1.82-fold, compared to H₂O₂-treated cells, respectively.

Salvia extracts restored H₂O₂-induced MMP collapse

The loss of MMP is the result of the opening of permeability transition pores. As shown in Fig. 6,

Fig. 3 Analysis of apoptosis by using Hoechst staining. The cells were exposed to different concentrations of *Salvia* for 24 h followed by exposure to 150 µM of H₂O₂ for 24 h. The

morphological patterns of apoptotic cells are described in the text. All experiments were repeated three times and the number of stained cells was counted in 10 randomly selected fields

Fig. 4 Intracellular ROS levels in PC12 cells pretreated with *Salvia* species. The PC12 cells were incubated 24 h with or without *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* (10, 25, 50 and 100 µg/mL) and then exposed to H₂O₂ (150 µM) for 24 h. Intracellular levels of ROS were measured by using DCFH-DA. The mean of three independent experiments is shown.
*Significantly different from control cells.
#Significantly different from H₂O₂-treated cells (* or #*P* < 0.05, ** or ##*P* < 0.01)

H₂O₂ (150 µM) induced a significant loss of MMP in H₂O₂-treated cells, whereas pretreatment of PC12 cells with 10, 25, 50 and 100 µg/mL of *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* rescued markedly the loss of MMP.

Salvia species extracts inhibited H₂O₂-induced cytochrome *c* release

Cytochrome *c* is located in the inner membrane space of mitochondria. Changes in MMP lead to release of

Fig. 5 Bax and Bcl-2 levels in PC12 cells pretreated with *Salvia* species. **a** PC12 cells were pretreated with (10, 25, 50 and 100 $\mu\text{g}/\text{mL}$) of *Salvia* species, for 24 h and then exposed to H_2O_2 (150 μM) for 24 h. 20 μg proteins were separated on SDS-PAGE, Western blotted, probed with anti-Bax and/or anti-Bcl-2 antibodies and reprobed with anti- β -actin antibody (one representative Western blot is shown; $n = 3$). **b** The densities of the corresponding bands in the presence of *S. choloroleuca*, *S. mirzayanii*, *S. santolinifolia*, were measured and the ratio was calculated. The mean of three independent experiments is shown. *Significantly different from untreated cells. #Significantly different from H_2O_2 -treated cells (* or # $P < 0.05$, ** or ## $P < 0.01$)

cytochrome *c* into cytosol that is a key initiation step in the apoptotic mitochondria pathway (Cai et al. 1998). To study the role of the mitochondrial apoptotic pathway in mediating H_2O_2 -induced apoptosis, we examined the distribution of cytochrome *c* within PC12 cells undergoing H_2O_2 -induced apoptosis and treated with *Salvia* species. As shown in Fig. 7, we found a dose-dependent accumulation of cytochrome *c* in the mitochondria observe in PC12 cells pretreated with *Salvia* species extracts while in cells treated only

with H_2O_2 , this accumulation of cytochrome *c* was observed in cytosol.

Salvia species extracts inhibited caspase-3 cleavage in neuron-like PC12 cells

Caspases are a family of cysteine-proteases, which play a central role in apoptotic cell death. All caspases are produced as proenzymes and undergo proteolytic activation resulting in cleaved forms (Chen and Chang

Fig. 6 Effect of *Salvia* species on MMP level. PC12 cells were incubated 24 h with or without *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* (10, 25, 50 and 100 $\mu\text{g/mL}$) and then exposed to H_2O_2 (150 μM) for 24 h. MMP level was measured with Rhodamine 123. The mean of three independent experiments is shown. *Significantly different from control cells. #Significantly different from H_2O_2 -treated cells (* or # $P < 0.05$, ** or ## $P < 0.01$)

1997). To demonstrate whether these *Salvia* species have anti-apoptotic effect, we measured the level of cleaved caspase-3 by Western blot analysis in the presence of different concentrations of these species. As shown in Fig. 8, H_2O_2 induced the appearance of cleaved caspase-3, while in those cells pretreated with different doses of *Salvia* species, bands of cleaved (active) caspase-3 became weaker along with increasing concentration, compared to H_2O_2 -treated cells.

S. choloroleuca, *S. mirzayanii* and *S. santolinifolia* decreased H_2O_2 -induced PARP-1 cleavage in PC12 cells

The nuclear enzyme PARP-1 is activated in response to DNA damage. Single and/or double strand DNA breaks induce the production of branched chain ADP ribose polymers that are covalently attached to numerous nuclear proteins like histones or the PARP-1 itself and this process represents an early event in DNA repair (Kaufmann et al. 1993; Virág 2005). We examined the level of PARP cleavage by Western blot analysis. As shown in Fig. 9, in PC12 cells treated with H_2O_2 , accumulation of the 89- and 24-kDa PARP-1 cleavage fragments is evident, confirming the activation of caspase-3 as the main protease, responsible for PARP-1 cleavage. While, in PC12 cells pretreated with *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* bands of cleaved PARP-1, 89-kDa at the highest

concentration (100 $\mu\text{g/mL}$), were weaker by about 3.23-, 3.08- and 2.61-fold, respectively, compared to H_2O_2 -treated cells.

S. choloroleuca, *S. mirzayanii* and *S. santolinifolia* increased GSH level

GSH is an abundant cellular thiol which plays critical roles as an antioxidant, enzyme cofactor, cysteine storage form, the major redox buffer and a neuromodulator in the central nervous system (Boesch-Saadatmandi et al. 2008). As shown in Table 1, we found that H_2O_2 reduced cellular GSH by 2.23-fold, while pretreatment of cells with *Salvia* species increased GSH level in a dose-dependent manner.

Discussion

Oxidative stress, an imbalance in prooxidant and antioxidant levels, has been implicated to play a crucial role in the pathogenesis of a number of diseases including neurodegenerative disorders (Götz et al. 1994). 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,

Fig. 7 Cytochrome *c* levels in PC12 cells pretreated with *Salvia* species. **a** PC12 cells were pretreated with (10, 25, 50 and 100 $\mu\text{g}/\text{mL}$) of *Salvia* species for 24 h and then exposed to H_2O_2 (150 μM) for 24 h. Twenty μg proteins were separated on SDS-PAGE, western blotted, probed with anti-Cytochrome *c* antibody and reprobed with anti- β -actin antibody (one representative Western blot is shown; $n = 3$). **b** The densities of the corresponding bands in the presence of *S. choloroleuca*, *S. mirzayanii*, *S. santolinifolia*, were measured and the ratio was calculated. The mean of three independent experiments is shown. *Significantly different from untreated cells. #Significantly different from H_2O_2 -treated cells (* or # $P < 0.05$, ** or ## $P < 0.01$)

treatment with antioxidants appears to be an alternative approach for slowing disease progression. Oxidative stress also occurs in the central nervous system during aging and Alzheimer disease (AD) as a consequence of imbalance between the formation of cellular oxidants and the anti-oxidative processes. Oxidative stress disrupts a broad array of signal transduction pathways that regulate various biological processes including gene expression, cell growth and differentiation.

Salvia is the largest genus of the family of “*Lamiaceae*”, commonly referred to as “sage”. A multitude of biological activities have been described for *Salvia* spp. including antioxidant, anticholinesterase, anti-inflammatory, antimicrobial, antinociceptive and anti-apoptotic effects (Asadi et al. 2011; Shaerzadeh et al. 2011; Hohmann et al. 1999; Zupko et al. 2001; Savelev et al. 2004). Our previous study showed that *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* protect PC12 cells against H_2O_2 -induced cell death.

Fig. 8 Caspase-3 levels in PC12 cells pretreated with *Salvia* species. Cells were pretreated with *Salvia* species (10, 25, 50 and 100 $\mu\text{g}/\text{mL}$) for 24 h and then exposed to H_2O_2 (150 μM) for 24 h. Twenty μg proteins were separated on SDS-PAGE, Western blotted, probed with anti-caspase antibody and reprobed with anti- β -actin antibody (one representative western blot is shown; $n = 3$). **b** The densities of the corresponding bands in the presence of *S. choloroleuca*, *S. mirzayanii*, *S. santolinifolia*, were measured and the ratio was calculated. The mean of three independent experiments is shown. *Significantly different from untreated cells. #Significantly different from H_2O_2 -treated cells (* or # $P < 0.05$, ** or ## $P < 0.01$)

Besides, the phytochemical analysis of these species of *Salvia* revealed that these extracts possess high levels of phenolic and flavonoid compounds including luteolin, catechine, rosmarinic acid and rutin. HPLC analysis also showed that *S. choloroleuca* has higher levels of these compounds (Asadi et al. 2011). It has been well documented that intake of polyphenols through diets reduces incidence of certain age related

neurological disorders including dementia (Vauzour et al. 2010). On the other hand, free radical scavenging activity of phenolic compounds and antioxidant effects of flavonoids was demonstrated (Rice-Evans et al. 1996). As was expected, these *Salvia* species had a remarkable antioxidant and free radical scavenging activity that was higher in *S. choloroleuca* because of its extreme phenolic and flavonoid content. To

Fig. 9 Western blot analysis to measure the effects of *Salvia* species on nuclear levels of PARP-1 in PC12 cells. The cells were pretreated with (10, 25, 50 and 100 μg/mL) of *Salvia* species for 24 h and then exposed to H₂O₂ (150 μM) for 24 h. **a** Twenty μg of nuclear extract was separated on SDS-PAGE, Western blotted, probed with anti-PARP antibody and reprobed with anti-β-actin antibody (one representative Western blot is

shown; $n = 3$). The densities of PARP bands in the presence of **b** *S. choloroleuca*, **c** *S. mirzayanii*, **d** *S. santolinifolia*, were measured and the ratio was calculated. The mean of three independent experiments is shown. *Significantly different from untreated cells. #Significantly different from H₂O₂-treated cells (* or # $P < 0.05$, ** or ## $P < 0.01$)

evaluate the detailed mechanism of this cytoprotection, we previously examined antioxidant and antiglycating activities of these plants. The overexpression of antioxidant enzymes such as catalase and super oxide dismutase, as well as the reduction of lipid

peroxidation and protein glycation, are the primary mechanisms of cell defense in oxidative stress conditions (Tusi et al. 2010). In our previous study we confirmed that all of these mechanisms are triggered by *Salvia* species in cells exposed to H₂O₂ (Asadi et al.

Table 1 Effects of *S. cholerolesuca*, *S. mirzayanii* and *S. santolinifolia* on glutathione level in H₂O₂-treated PC12 cells

Treatment	GSH (μmol/mg protein)
Control	6.51 ± 0.58
H ₂ O ₂	2.91 ± 0.15*
<i>S. cholerolesuca</i> (100 μg/mL)	9.43 ± 0.28 [#]
<i>S. cholerolesuca</i> (10 μg/mL) + H ₂ O ₂	7.63 ± 0.13 [#]
<i>S. cholerolesuca</i> (25 μg/mL) + H ₂ O ₂	8.71 ± 0.53 [#]
<i>S. cholerolesuca</i> (50 μg/mL) + H ₂ O ₂	10.81 ± 0.45 ^{##}
<i>S. cholerolesuca</i> (100 μg/mL) + H ₂ O ₂	10.95 ± 0.32 ^{##}
<i>S. mirzayanii</i> (100 μg/mL)	6.93 ± 0.19 [#]
<i>S. mirzayanii</i> (10 μg/mL) + H ₂ O ₂	6.8 ± 0.38 [#]
<i>S. mirzayanii</i> (25 μg/mL) + H ₂ O ₂	7.04 ± 0.14 [#]
<i>S. mirzayanii</i> (50 μg/mL) + H ₂ O ₂	7.72 ± 0.32 [#]
<i>S. mirzayanii</i> (100 μg/mL) + H ₂ O ₂	7.83 ± 0.33 [#]
<i>S. santolinifolia</i> (100 μg/mL)	8.97 ± 0.34 [#]
<i>S. santolinifolia</i> (10 μg/mL) + H ₂ O ₂	7.98 ± 0.32 [#]
<i>S. santolinifolia</i> (25 μg/mL) + H ₂ O ₂	8.21 ± 0.38 [#]
<i>S. santolinifolia</i> (50 μg/mL) + H ₂ O ₂	9.32 ± 0.50 [#]
<i>S. santolinifolia</i> (100 μg/mL) + H ₂ O ₂	9.88 ± 0.32 [#]

The data are presented as means ± SD ($n = 6$), * $P < 0.05$ versus control; [#] $P < 0.05$ versus H₂O₂ group

2010). Here, we attempted to investigate the mechanism of cell death that may be triggered by *Salvia* species.

PC12 cells issued from a pheochromocytoma of rat adrenal medulla. These cells can display neurite-like and differentiated processes when they are exposed to NGF. NGF is a necessary protein for the survival and development of the sympathetic and certain of the sensory neurons (Greene and Tischler 1976; Lee et al. 1977). PC12 cell line is a useful model for neuronal signaling pathways and differentiation, and in the presence of NGF, neurons begin to extend neurites. In this study, we found that H₂O₂ decreased neurite length and complexity, two markers of normal and functional neurons, while *Salvia* species could restore these criteria of differentiation. *Salvia* species could also protect PC12 cell against H₂O₂-induced apoptosis, as was morphologically evident by the decrease of chromatin condensation using Hoechst staining and the decrease of membrane interruption in AO/EB staining.

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 (Vento et al. 1998). 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 endoplasmic reticulum and the outer mitochondrial membrane that regulates the antioxidant pathway at the site of free radical generation (Shimohama et al. 1998). 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 (Kitamura et al. 1998). 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 (Gogvadze et al. 2006; Kagan et al. 2009).

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 (Brown and Borutaite 2008; Gogvadze et al. 2006). In addition, the released cytochrome *c* comes into equilibrium with the cytosolic redox state, i.e. the system of oxidants and antioxidants which are broadly in equilibrium with reduced oxidized glutathione. The oxidized form of cytochrome *c* can be reduced by various reductants including GSH. Based on our results, increased level of GSH was concomitant with increased level of reduced cytochrome *c*. In the present study, we found that pretreatment with *Salvia* species decreased the cytosolic levels of cytochrome *c*,

which was significantly elevated in H₂O₂-treated cells. Thus, it seems that part of neuroprotective effect of *Salvia* species 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 a significantly increased level of cleaved caspase-3 in H₂O₂-treated cells, compared to the control, which can be attributed to the activation of mitochondrial-mediated apoptotic pathway via cytochrome *c* releasing following H₂O₂ exposure. However, we cannot rule out the possibility of the ER-mediated apoptotic pathway, as the expression of cleaved caspase-3 can be also induced following the activation of this pathway (Tacconi et al. 2004; Tusi et al. 2011). To further confirm caspase-3 activity, we measured PARP-1 level, as a protein downstream to caspase-3, which is cleaved by caspases including caspase-3 into characteristic 89 and 24 KDa fragments during apoptotic cell death (Virág et al. 1998; Virág 2005). Interestingly, it has been reported that an interaction of PARP-1 with *bcl2* and ribosomal S38 results in the inhibition of PARP-1 activity. Thus, *bcl2* anti-apoptotic properties may be in part due to the inhibition of PARP-1, supporting the role of PARP-1 in the initiation of apoptosis (Sodhi et al. 2010). Because of the rapid activation of PARP-1, it seems to be a key factor that regulates whether cells die or survive following DNA damage (Bouchard et al. 2003). Moreover, Prabhakaran et al. (2004) have reported that inhibition of the caspase cascade merely could switch the apoptotic response of cell to other modes of cell death, such as necrosis. This change of cell death mode is accompanied by a marked increase in PARP-1 activity, ROS generation and reduction of MMP. Interestingly, they reported that in these conditions, inhibition of PARP-1 preserves cellular ATP levels and in turn blocks execution of the necrotic death pathway. Plant pretreated groups of our study, showed a significant decline of cleaved caspase-3, as well as both cleaved fragments of PARP-1, indicating the ability of these *Salvia* species to inhibit caspase dependent apoptotic pathway and their potential for inhibiting other switched modes of cell death.

One possible strategy to counter oxidative damage is regulating redox state. However, to guard against oxidative stress, mammalian cells have evolved

multifaceted, highly regulated defense systems. One of the most important systems is regulated by antioxidant response element that is present in the promoters of genes encoding phase II detoxification enzymes such as hemeoxygenase-1 and γ -glutamylcysteine synthetase (Venugopal and Jaiswal 1996). It has been shown that induction of these enzymes is a strategy to combat chemical toxicity. Based on our previous study, *Salvia* species could upregulate these enzymes significantly after exposure to H₂O₂ (Asadi et al. 2011). Besides, these enzymes confer cellular protection against inflammation by reducing deleterious production of pro-inflammatory mediators (Jeong et al. 2011), although the present study did not address it.

In summary, we provided the documentation of anti-apoptotic effects of three *Salvia* species from Iran, *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia*. It seems that these antioxidant species interfere with the intrinsic pathway of apoptosis that involves mitochondria. We found that they decreased Bax/Bcl-2 ratio and thereby decreased outer mitochondrial membrane break and cytochrome *c* release into the cytoplasm. As a consequence, the level of cleaved form of caspase-3 was decreased. We also found that PARP-1 which is a key factor in apoptosis was decreased in cells treated with *Salvia*. It should be noted that *S. choloroleuca* exerts its neuroprotective effect more efficiency than the two other species. Using these plants in animal models of AD is in progress in our laboratory that might be considered as an adjunct therapeutic strategy to combat neural demise in AD and other oxidative stress-related diseases.

Acknowledgments This work was supported by Shahid Beheshti University of Medical Sciences Research Fund.

Conflict of interest The authors have no conflict of interest.

References

- Ansari N, Khodaghali F, Amini M (2011) 2-Ethoxy-4,5-diphenyl-1,3-oxazine-6-one activates the Nrf2/HO-1 axis and protects against oxidative stress-induced neuronal death. *Eur J Pharmacol* 658:84–90
- Asadi S, Ahmadiani A, Esmaili MA, Sonboli A, Ansari N, Khodaghali F (2010) In vitro antioxidant activities and an investigation of neuroprotection by six *Salvia* species from Iran: a comparative study. *Food Chem Toxicol* 48:1341–1349

- Asadi S, Khodagholi F, Esmaeili MA, Tusi SK, Ansari N, Shaerzadeh F, Sonboli A, Ghahremanzamaneh M (2011) Chemical composition analysis, antioxidant, antiglycating activities and neuroprotective effects of *S. choloroleuca*, *S. mirzayanii* and *S. santolinifolia* from Iran. *Am J Chin Med* 39:615–638
- Beal MF (1992) Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Ann Neurol* 31:119–130
- Boesch-Saadatmandi C, Loboda A, Jozkowicz A, Huebbe P, Blank R, Wolfram S, Dulak J, Rimbach G (2008) Effect of ochratoxin A on redox-regulated transcription factors, antioxidant enzymes and glutathione-S-transferase in cultured kidney tubulus cells. *Food Chem Toxicol* 46:2665–2671
- Bouchard VJ, Rouleau M, Poirier GG (2003) PARP-1, a determinant of cell survival in response to DNA damage. *Exp Hematol* 31:446–454
- Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Brown GC, Borutaite V (2008) Regulation of apoptosis by the redox state of cytochrome *c*. *Biochim Biophys Acta* 1777:877–881
- Cai J, Yang J, Jones DP (1998) Mitochondrial control of apoptosis: the role of cytochrome *c*. *Biochim Biophys Acta* 1366:139–149
- Caroppi P, Sinibaldi F, Fiorucci L, Santucci R (2009) Apoptosis and human diseases: mitochondrion damage and lethal role of released cytochrome *c* as proapoptotic protein. *Curr Med Chem* 16:4058–4065
- Chen RH, Chang TY (1997) Involvement of caspase family proteases in transforming growth factor-beta-induced apoptosis. *Cell Growth Differ* 8:821–827
- Ellman GL (1959) Tissue sulfhydryl groups. *Arch Biochem Biophys* 82:70–77
- Fadeel B, Orrenius S, Zhivotovsky B (1999) Apoptosis in human disease: a new skin for the old ceremony? *Biochem Biophys Res Commun* 266:699–717
- Farrow SN, Brown R (1996) New members of the Bcl-2 family and their protein partners. *Curr Opin Genet Dev* 6:45–49
- Gaumer S, Guénal I, Brun S, Théodore L, Mignotte B (2000) Bcl-2 and Bax mammalian regulators of apoptosis are functional in *Drosophila*. *Cell Death Differ* 7:804–814
- Gogvadze V, Orrenius S, Zhivotovsky B (2006) Multiple pathways of cytochrome *c* release from mitochondria in apoptosis. *Biochim Biophys Acta* 1757:639–647
- Götz ME, Kunig G, Riederer P (1994) Oxidative stress: free radical production in neural degeneration. *Pharmacol Ther* 63:37–122
- Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* 73:2424–2428
- Halliwel B, Aruoma OI (1991) DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. *FEBS Lett* 281:9–19
- Hohmann J, Zupkó I, Rédei D, Csányi M, Falkay G, Máthé I, Janicsák G (1999) Protective effects of aerial parts of *Salvia officinalis*, *Melissa officinalis*, and *Lavandula angustifolia* and their constituents against enzyme-dependent and enzyme independent lipid peroxidation. *Planta Med* 65:576–578
- Jeong GS, Lee DS, Kim DC, Jahng Y, Son JK, Lee SH, Kim YC (2011) Neuroprotective and anti-inflammatory effects of mollugin via up-regulation of heme oxygenase-1 in mouse hippocampal and microglial cells. *Eur J Pharmacol* 654:226–234
- Kagan VE, Bayir HA, Belikova NA, Kapralov O, Tyurina YY, Tyurin VA, Jiang J, Stoyanovsky DA, Wipf P, Kochanek PM, Greenberger JS, Pitt B, Shvedova AA, Borisenko G (2009) Cytochrome *c*/cardiolipin relations in mitochondria: a kiss of death. *Free Radic Biol Med* 46:1439–1453
- Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG (1993) Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res* 53:3976–3985
- Kitamura Y, Shimohama S, Kamoshima W, Ota T, Matsuoka Y, Nomura Y, Smith MA, Perry G, Whitehouse PJ, Taniguchi T (1998) Alteration of proteins regulating apoptosis, Bcl-2, Bcl-x, Bax, Bak, Bad, ICH-1 and CPP32, in Alzheimer's disease. *Brain Res* 780:260–269
- Lee V, Shelanski ML, Greene LA (1977) Specific neural and adrenal medullary antigens detected by antisera to clonal PC12 pheochromocytoma cells. *Proc Natl Acad Sci USA* 74:5021–5025
- Lu Y, Foo LY (2002) Polyphenolics of *Salvia*—a review. *Phytochemistry* 59:114–140
- Mihara M, Moll UM (2003) Detection of mitochondrial localization of p53. *Methods Mol Biol* 234:203–209
- Mozafarian V (1996) A dictionary of Iranian plant names (Latin English Persian). Farhang Mosafer Publication, Tehran
- Poppe M, Reimertz C, Düssmann H, Krohn AJ, Luetjens CM, Böckelmann D, Nieminen AL, Kögel D, Prehn JH (2001) Dissipation of potassium and proton gradients inhibits mitochondrial hyperpolarization and cytochrome *c* release during neural apoptosis. *J Neurosci* 21:4551–4563
- Prabhakaran K, Li L, Borowitz JL, Isom GE (2004) Caspase inhibition switches the mode of cell death induced by cyanide by enhancing reactive oxygen species generation and PARP-1 activation. *Toxicol Appl Pharmacol* 195:194–202
- Raha S, Robinson BH (2001) Mitochondria, oxygen free radicals, and apoptosis. *Am J Med Genet* 106:62–70
- Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* 20:933–956
- Savelev SU, Okello EJ, Perry EK (2004) Butyryl- and acetylcholinesterase inhibitory activities in essential oils of *Salvia* species and their constituents. *Phytother Res* 18:315–324
- Schapira AH (1996) Oxidative stress and mitochondrial dysfunction in neurodegeneration. *Curr Opin Neurol* 9:260–264
- Shaerzadeh F, Ahmadiani A, Esmaeili MA, Ansari N, Asadi S, Tusi SK, Sonboli A, Ghahremanzamaneh M, Khodagholi F (2011) Antioxidant and antiglycating activities of *Salvia sahendica* and its protective effect against oxidative stress in neuron-like PC12 cells. *J Nat Med* 65:455–465
- Shimohama S, Fujimoto S, Sumida Y, Tanino H (1998) Differential expression of rat brain bcl-2 family proteins in development and aging. *Biochem Biophys Res Commun* 252:92–96
- Sodhi RK, Singh N, Jaggi AS (2010) Poly(ADP-ribose) polymerase-1 (PARP-1) and its therapeutic implications. *Vascul Pharmacol* 53:77–87

- Suto D, Sato K, Ohba Y, Yoshimura T, Fujii J (2005) Suppression of the pro-apoptotic function of cytochrome *c* by singlet oxygen via a haem redox state-independent mechanism. *Biochem J* 392:399–406
- Tacconi S, Perri R, Balestrieri E, Grelli S, Bernardini S, Annichiarico R, Mastino A, Caltagirone C, Macchi B (2004) Increased caspase activation in peripheral blood mononuclear cells of patients with Alzheimer's disease. *Exp Neurol* 190:254–262
- Touyz RM, Schiffrin EL (2004) Reactive oxygen species in vascular biology: implications in hypertension. *Histochem Cell Biol* 122:339–352
- Tusi SK, Ansari N, Amini M, Amirabad AD, Shafiee A, Khodagholi F (2010) Attenuation of NF-kappaB and activation of Nrf2 signaling by 1,2,4-triazine derivatives, protects neuron-like PC12 cells against apoptosis. *Apoptosis* 15:738–751
- Tusi SK, Khalaj L, Ashabi G, Kiaei M, Khodagholi F (2011) Alginate oligosaccharide protects against endoplasmic reticulum- and mitochondrial-mediated apoptotic cell death and oxidative stress. *Biomaterials* 32: 5438–5458
- Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39:44–84
- Vauzour D, Rodriguez-Mateos A, Corona G, Oruna-Concha MJ, Spencer JPE (2010) Polyphenols and human health: prevention of disease and mechanisms of action. *Nutrients* 2:1106–1131
- Vento R, Giuliano M, Lauricella M, Carabillò M, Di Liberto D, Tesoriere G (1998) Induction of programmed cell death in human retinoblastoma Y79 cells by C2-ceramide. *Mol Cell Biochem* 185:7–15
- Venugopal R, Jaiswal AK (1996) Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H: quinone oxidoreductase1 gene. *Proc Natl Acad Sci USA* 93:14960–14965
- Virág L (2005) Structure and function of poly(ADP-ribose) polymerase-1: role in oxidative stress-related pathologies. *Curr Vasc Pharmacol* 3:209–214
- Virág L, Salzman AL, Szabó C (1998) Poly(ADP-ribose) synthetase activation mediates mitochondrial injury during oxidant-induced cell death. *J Immunol* 161:3753–3759
- Wallace DC, Shoffner JM, Watts RL, Juncos JL, Torroni A (1992) Mitochondrial oxidative phosphorylation defects in Parkinson's disease. *Ann Neurol* 32:113–114
- Zawia NH, Lahiri DK, Cardozo-Pelaez F (2009) Epigenetics, oxidative stress, and Alzheimer disease. *Free Radic Bio Med* 46:1241–1249
- Zupkó I, Hohmann J, Rédei D, Falkay G, Janicsák G, Máthé I (2001) Antioxidant activities of leaves of *Salvia* species in enzyme-dependent and enzyme independent systems of lipid peroxidation and their phenolic constituents. *Planta Med* 67:366–368