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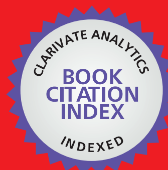

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Extract of *Achillea fragrantissima* Downregulates ROS Production and Protects Astrocytes from Oxidative-Stress-Induced Cell Death

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1. Introduction

Oxidative damage plays a pivotal role in the initiation and progress of many human diseases and also in the development of acute and chronic pathological conditions in brain tissue (Halliwell, 2006; Hyslop et al., 1995; Ischiropoulos & Beckman, 2003; Minghetti, 2005). Compared with other tissues, the brain is particularly vulnerable to oxidative damage due to its high rate of oxygen utilization and high contents of oxidizable polyunsaturated fatty acids (Floyd, 1999; Sastry, 1985). In addition, certain regions of the brain are highly enriched in iron, a metal that is catalytically involved in the production of damaging reactive oxygen species (ROS) (Hallgren & Sourander, 1958). Although ROS are critical intracellular signaling messengers (Schrecka & Baeuerlea, 1991), excess of free radicals may lead to peroxidative impairment of membrane lipids and, consequently, to disruption of neuronal functions, and apoptosis. Among the ROS that are responsible for oxidative stress, H_2O_2 is thought to be the major precursor of highly reactive free radicals, and is regarded as a key factor in both neuronal (Vaudry et al., 2002) and astroglial cell death (Ferrero-Gutierrez et al., 2008). H_2O_2 is normally produced in reactions predominantly catalyzed by superoxide dismutase (SOD) and monoaminoxidases (MAO) A and B in the brain (Almeida et al., 2006; Duarte et al., 2007). As with both Ca^{2+} and NO, H_2O_2 appears to play contradictory roles, in that it is potentially toxic at high concentrations, even though it is a central signaling compound at low concentrations (Miura et al., 2002). Brain cells have the capacity to produce peroxides, particularly H_2O_2, in large amounts (Dringen et al., 2005). Excess of H_2O_2 accumulates during brain injuries and neurodegenerative diseases, and can cross cell membranes to elicit its biological effects intracellularly (Bienert et al., 2006). Although H_2O_2 is generally poorly reactive, it forms highly toxic hydroxyl radicals, which may damage all

the major classes of biological macromolecules in the cell, through iron- or copper ion-mediated oxidation of lipids, proteins, and nucleic acids. This capability can partly account for H_2O_2-mediated neuronal and glial cell death. H_2O_2 also induces differential protein activation, which indicates varied biological effects of this molecule. In the mammalian central nervous system (CNS), the transition metal zinc is an endogenous molecule that is localized exclusively to the synaptic vesicles of glutamatergic neurons and that has a special role in modulating synaptic transmission. Chelatable zinc is released into the synaptic cleft with the neurotransmitter during neuronal execution (Assaf & Chung, 1984), and under normal circumstances the robust release of zinc is transient and is efficiently cleared from the synaptic cleft to ensure the performance of successive stimuli. However, in pathological conditions, zinc dyshomeostasis, with consequently elevated levels of extracellular zinc has been recognized as an important factor in the resulting neuropathology (Choi & Koh, 1998; Cote et al., 2005; Li et al., 2009). In neurotransmission, the amount of zinc in the synaptic cleft is in the 10- to 30-μM range, but in pathological conditions that involve sustained neuronal depolarization, e.g., ischemia, stroke, or traumatic brain injury, the levels of extracellular zinc can increase to 100- to 400-μM, at which it can contribute to the resulting neuropathology (Frederickson et al., 2005; Li et al., 2001). *In vivo* and *in vitro* studies showed that, at concentrations that can be reached in the mammalian CNS during excitotoxic episodes, injuries or diseases, zinc is toxic to both neurons and astrocytes (Bishop et al., 2007; Hwang et al., 2008; Kim et al., 1999a; Kim et al., 1999b; Koh et al., 1996; Ryu et al., 2002; Sheline et al., 2000; Stork & Li, 2009). Zinc induces oxidative stress and ROS production, which contribute to both glial cell death (Ryu et al., 2002) and neuronal cell death (Kim et al. 1999a; Kim et al. 1999b). Zinc decreased the GSH content of primary cultures of astrocytes (Kim et al., 2003; Ryu et al., 2002), increased their GSSG content (Kim et al., 2003) and inhibited glutathione reductase activity in these cells (Bishop et al., 2007); furthermore, it slowed the clearance of exogenous H_2O_2 by astrocytes, and promoted intracellular production of ROS (Bishop et al., 2007). Thus, ROS generation, glutathione depletion and mitochondrial dysfunction may be key factors in $ZnCl_2$–induced glial toxicity (Ryu et al., 2002). Astrocytes are the most abundant glial cell type in the brain. They play important roles in maintenance of homeostasis, in provision of metabolic substrates for neurons, and also in coupling cerebral blood flow to neuronal activity. They are prominent in protecting neurons against oxidative stress and cell death, and in providing trophic supports such as the glial cell-line-derived neurotrophic factor (GDNF) (Sandhu et al., 2009). There is evidence that dysfunctional astrocytes can enhance neuronal degeneration by diminishing secretion of trophic factors (Takuma et al., 2004). The study of astrocytes is particularly important, in light of the co- existence of apoptotic death of neurons and astrocytes in damaged brains affected by ischemia and neurodegenerative diseases. Despite their high antioxidative activities, astrocytes exhibit a high degree of vulnerability, and are not resistant to the effects of ROS. They respond to substantial or sustained oxidative stress with increased intracellular Ca^{2+}, loss of mitochondrial potential, and decreased oxidative phosphorylation (Robb et al., 1999). Since astrocytes determine the brain's vulnerability to oxidative injury, and form a tight functional unit with neurons, once astrocyte energy metabolism and antioxidant capacity are impaired, astrocytic death may critically impair neuronal survival (Feeney et al., 2008; Lu et al., 2008). Thus, protection of astrocytes from oxidative insult appears essential to brain function maintenance. Many herb and plant extracts are used as folk medicines for various kinds of diseases and organ dysfunctions. *Achillea fragrantissima* (*Af;* Asteraceae) is a desert plant that for many years has been used as a hypoglycemic medicinal plant in traditional medicine in the Arabian region (Yaniv et al.,

1987), and for the treatment of gastrointestinal disturbances (Segal et al., 1987). The ingredient responsible for the anti-spasmolytic activity was found to be a flavone aglycone named cirsiliol (5,3',4'-trihydroxy-6,7-dimethoxyflavone) that was shown to antagonize the spasmodic effects, inhibit Ca^{2+} influx and stimulate Ca^{2+} release from intracellular stores (Mustafa et al., 1992). In addition, the hydro-alcoholic extract of *Af* was shown to have a remarkable antiviral activity against poliomyelitis-1 virus (Soltan & Zaki, 2009). However, the effects of *Af* in the context of brain injuries and neurodegenerative diseases, have not been studied to date. In a recent study we have found that the ethanolic extract of *Achillea fragrantissima* inhibited lipopolysaccharide (LPS) –induced nitric oxide (NO) production by activated primary microglial cells. This extract also inhibited LPS - elicited expression of the pro-inflammatory cytokines interleukin1β (IL-1β) and tumor necrosis factor-α (TNFα), as well as expression of the proinflammatory enzymes, cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) by these cells (in preparation). Since oxidative stress has become accepted as a suitable target for early therapeutic intervention in brain injuries and neurodegenerative diseases, the present study addressed the astroprotective and antioxidant activities of this plant extract.

2. Materials and methods

2.1 Reagents

Dulbecco's modified Eagle's medium (DMEM), Leibovitz-15 medium, glutamine, antibiotics (10,000 IU/ml penicillin and 10,000 µg/ml streptomycin), soybean trypsin inhibitor, fetal bovine serum (FBS) and Dulbecco’s phosphate buffered saline (PBS) (without calcium and magnesium) were purchased from Biological Industries (Beit Haemek, Israel); dimethyl sulfoxide (DMSO) was obtained from Applichem (Darmstadt, Germany); Hydrogen peroxide was obtained from MP Biomedicals (Ohio, USA); 2,2'-Azobis(amidinopropane) (ABAP) was obtained from Wako chemicals (Richmond, VA), and other chemicals including $ZnCl_2$ and 2'7'-dichlorofluorescein diacetate (DCF) were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2 Preparation of *Af* Extracts

The plant was collected in the Arava Valley and authenticated. The voucher specimens have been kept in as part of the Arava Rift Valley Plant Collection; VPC (Dead Sea & Arava Science Center, Central Arava Branch, Israel, http://www.deadseaarava-rd.co.il/) under the accession code AVPC0040. Freshly collected plants were dried at 40 °C for three days and extracted in ethanol (96%). The liquid phase was then evaporated off, and the dry material was dissolved in DMSO to a concentration of 100 mg/ml to produce the *Af* extract.

2.3 High performance liquid chromatography (HPLC) conditions

The ethanolic extract of *Af* was subjected to HPLC chromatography. Separation was made using reverse phase column (Betasil C-18, 5 µm, 250 × 0.46 mm; Thermo-Hypersil, UK) by gradient elution with water-acetic acid (97 : 3 V/V) and methanol as described previously (Chen et al., 2010), and detection at 360 nm (Blue line) and 280 nm (Red line) (Fig. 1).

2.4 Liquid chromatography–mass spectrometry (LC–MS) conditions

The ethanolic extract of *Af* was subjected to MS/MS (Fig. 2). The mass spectra were performed on a liquid chromatography–mass spectrometry (LC–MS) Agilent 1100LC series

(Wald- bronn, Germany) and Bruker Esquire 3000plus MS (Bremen, Germany) instrument, operated in the electrospray ionization (ESI) in a positive ion mode. A reverse phase column (BetasilC-18,5 mm, 250 mm x 0.46 mm, Thermo-Hypersil,UK) was used. The MS conditions

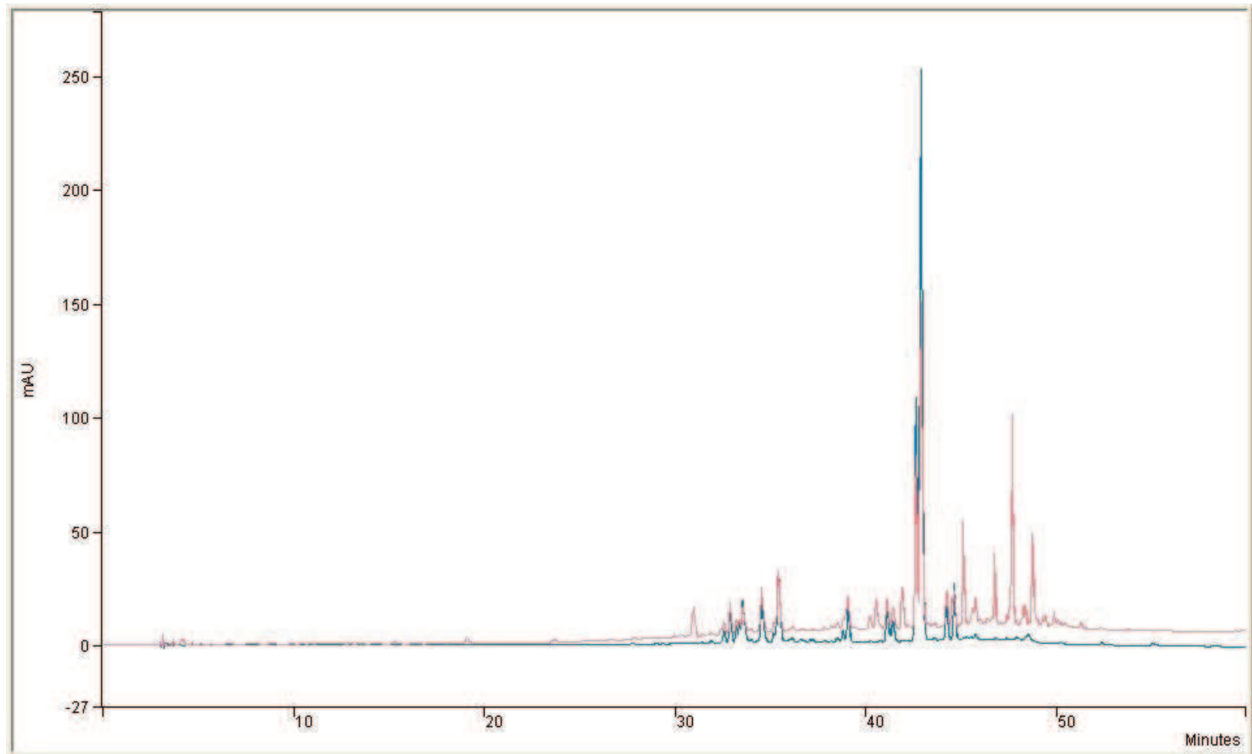


Fig. 1. HPLC analysis of the ethanolic extract of *Af*

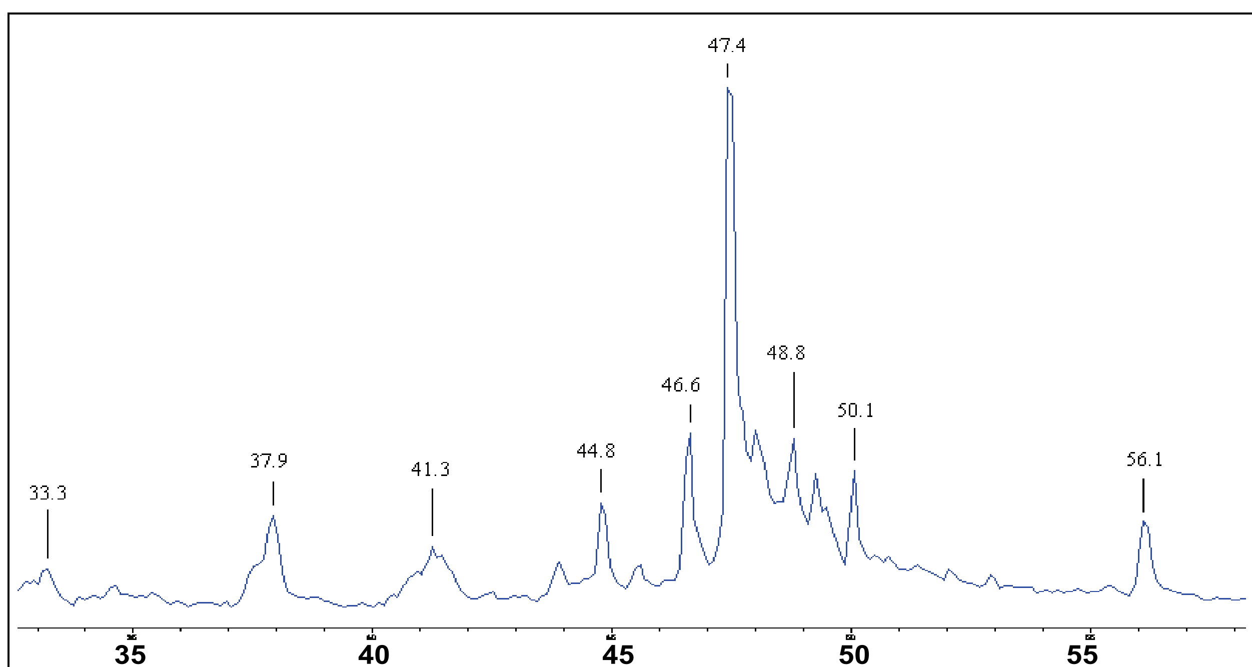


Fig. 2. Liquid chromatography–mass spectrometry (LC–MS) analysis of the ethanolic extract of *Af*

were optimized as follows: API electron spray interface, positive mode polarity, a drying gas flow of 10L/min, an nebulizer gas pressure of 60psi,a drying gas temperature of 300°C, a fragmentor voltage of 0.4V and capillary voltage of 4.5kV.
Four main peaks were identified by ESI-MS: Compound 1, ($C_{27}H_{34}O_{14}Na$), r.t. 50.1, m/z 605 [M+Na+146+146], m/z 582 [M+H+146+146]+, ; suggested as epicatechin-rhamnoside. Compound 2, ($C_{28}H_{33}O_{13}$), r.t. 48.8- m/z 577 [M+H+146+146]+; suggested as Acacetin rhamnoside. Compound 3, ($C_{22}H_{22}O_{10}Na$), r.t. 47.4- m/z 469 [M+Na+162]+, , m/z 447 [M+H+162]+,; suggested as Acacetin-glucoside, m/z 285 [M+H]+ aglycon. Compound 4, ($C_{24}H_{25}O_{12}$), r.t. 46.6- m/z 465 [M+H+162]+, suggested as Quercetin-glucoside, m/z 303 [M+H]+ aglycon.

2.5 Preparation of primary glial cell cultures

Cultures of primary rat glial cells were prepared from cerebral cortices of 1- to 2-day-old neonatal Wistar rats. Briefly, meninges and blood vessels were carefully removed from cerebral cortices kept in Leibovitz-15 medium; brain tissues were dissociated by trypsinization with 0.5% trypsin (10 min, 37 °C, 5% CO_2); and cells were washed first with DMEM containing soybean trypsin inhibitor (100 µg/ml) and 10% FBS and then with DMEM containing 10% FBS. Cells were seeded in tissue culture flasks pre-coated with poly-D-lysine (20 µg/ml in 0.1 M borate buffer pH 8.4) and incubated at 37 °C in humidified air with 5% CO_2. The medium was changed on the second day and every second day thereafter. At the time of primary cell confluence (day 10), microglial and progenitor cells were discarded by shaking (180 RPM, 37 °C) the flasks on a horizontal shaking platform. Astrocytes were then replated on 24-well poly-D-lysine-coated plastic plates, at a density of $1×10^5$/well, in DMEM (without phenol red) containing 2% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.
The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care, as found in the US guidelines, and was approved by the Institutional Animal Care and Use Committee of The Volcani Center, Agricultural Research Organization.

2.6 Treatment of astrocytes

Twenty four hours after plating, the original medium in which the cells were grown was aspirated off, and fresh medium was added to the cells. Dilutions of plant extracts first in DMSO and then in the growth medium were made freshly from stock solution just prior to each experiment and were used immediately. The final concentration of DMSO in the medium was 0.2%. Dilutions of H_2O_2 in the growth medium were made freshly from a 30% stock solution immediately prior to each experiment and were used immediately.

2.7 Determination of cell viability

Cell viability was determined using a commercial colorimetric assay (Roche Applied Science, Germany) according to the manufacturer's instructions. This assay is based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the incubation medium.

2.8 Evaluation of intracellular ROS production

Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2'7'-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by

intracellular esterases and then oxidized by ROS to a fluorescent compound 2'-7'-DCF. Astrocytes were plated onto 24 wells plates (300,000 cells/well) and treated with DCF-DA (20 μM) for 30 min at 37^0C. Following incubation with DCF, cultures were rinsed twice with PBS and then re-suspended (1) For measurement of H_2O_2-induced ROS: in DMEM containing 10% FBS, 8 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (2) For measurement of $ZnCl_2$ - induced ROS: in a defined buffer containing 116 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.4 mM KCl, 1 mM NaH_2PO_4, 14.7 mM $NaHCO_3$, and 10 mM HEPES, pH, 7.4. The fluorescence was measured in a plate reader with excitation at 485 nm and emission at 520 nm.

2.9 Cellular antioxidant activity of *Af* extract

Peroxyl radicals are generated by thermolysis of 2,2'-Azobis(amidinopropane) (ABAP) at physiological temperature. ABAP decomposes at approximately $1.36x10^{-6}s^{-1}$ at 37°C, producing at most $1x10^{12}$ radicals/ml/s (Bowry & Stocker, 1993; Niki et al., 1986; Thomas et al., 1997). Astrocytes were plated onto 24 wells plates (300,000 cells/well) and were incubated for 1 hr with *Af* extract. Then astrocytes were preloaded with DCF-DA for 30 min, washed, and ABAP (0.6 mM final concentration) was then added. The fluorescence, which indicates ROS levels, was measured in a plate reader with excitation at 485 nm and emission at 520 nm.

2.10 Differential pulse voltammetry analysis

Ethanolic extracts were obtained by dissolving 1 g of dry plant powder in 10 ml of ethanol overnight at room temperature. Before performing the differential pulse voltammetry (DPV) analysis, tetrabutylammonium perchlorate was added to the ethanolic extract to final concentration of 1% and the total reducing capacity of the *Af* extracts was analyzed, as described before (Butera et al., 2002). Briefly, the plant extract was placed in a cyclic voltammeter cell equipped with a working electrode (3.2 mm in diameters, glassy carbon), a reference electrode (Ag/AgCl), and an auxiliary electrode (platinum wire). The DPV potential was conducted at a scan rate of 40 mV/s, pulse amplitude 50 mV, sample width 17 ms, pulse width 50 ms, pulse period 200 ms. An electrochemical working station (CH Instruments Inc., 610B, Austin, TX, USA) was used. The output of the DPV experiments was a potential-current curve (Kohen et al., 1999).

2.11 Data analysis

Statistical analyses were performed with one-way ANOVA followed by Tukey-Kramer multiple comparison tests using Graph Pad InStat 3 for windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Protection by the *Af* extract of astrocytes from H_2O_2 -induced cell death

H_2O_2 exposure is used as a model of ischemia reperfusion. The concentration of H_2O_2 used in our experiments (175-200 microM) resembles the concentration reported by Hyslop *et al* to be the concentration of H_2O_2 that appears in the rat striatum under ischemic conditions (Hyslop et al., 1995). In order to characterize the astroprotective potential of the *Af* extract against H_2O_2 -induced oxidative stress, we have assessed changes in intracellular ROS production and in cell viability, using a model in which oxidative stress was induced by the

addition of this compound to cultured primary astrocytes. Exposure of normal primary astrocytes with H_2O_2 resulted in a time and concentration dependent astrocytic cell death 20 h later (data not shown). To find out whether the *Af* extract has a protective effect and to determine the optimal concentration of the extract needed for such an effect, astrocytes were pre-incubated with different concentrations of *Af* extract. H_2O_2 was then added, and cytotoxicity was determined after 20 h. Our results showed that the *Af* extract exerted a protective action against H_2O_2 -induced cell death in a dose-dependent manner (Fig. 3). No significant changes were observed in the viability of cells treated with similar concentrations of the *Af* extract in the absence of H_2O_2 (Fig. 3).

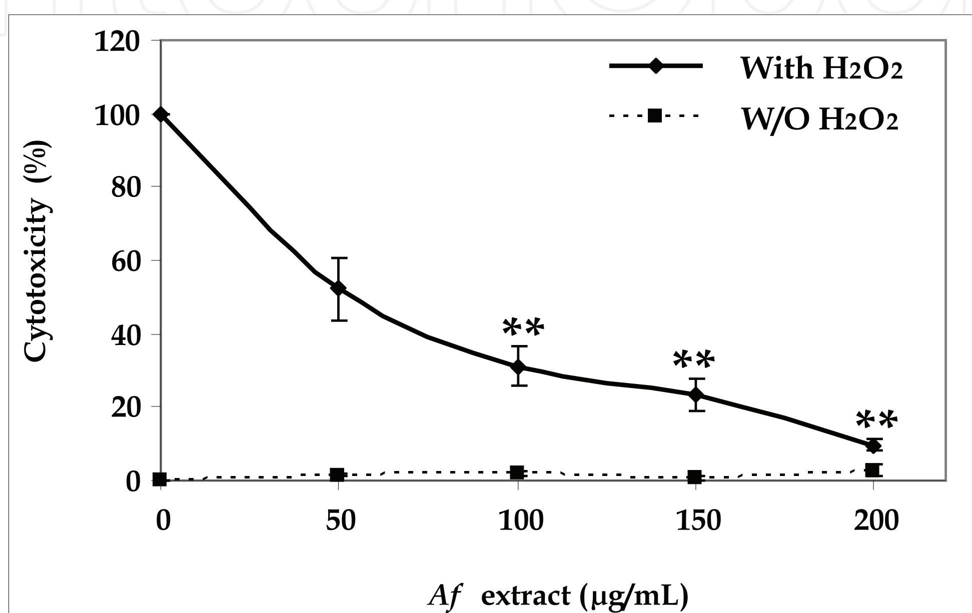


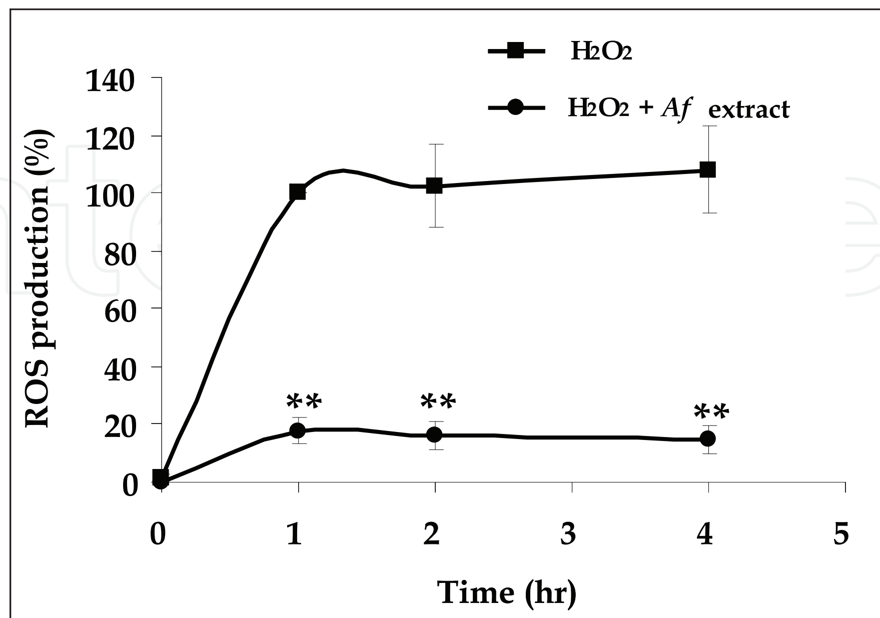
Fig. 3. Protection from H_2O_2-induced astrocytic cell death by different concentrations of the extract of *Af*

Astrocytes were treated with different concentrations of *Af* extract. H_2O_2 (200 µM) was added 2 h after the addition of *Af* extract. Cell death was determined 20 h later. Each point represents the means ± SEM of five experiments (*n* = *20*). ***p*<0.001 compared to cells treated with H_2O_2 alone.

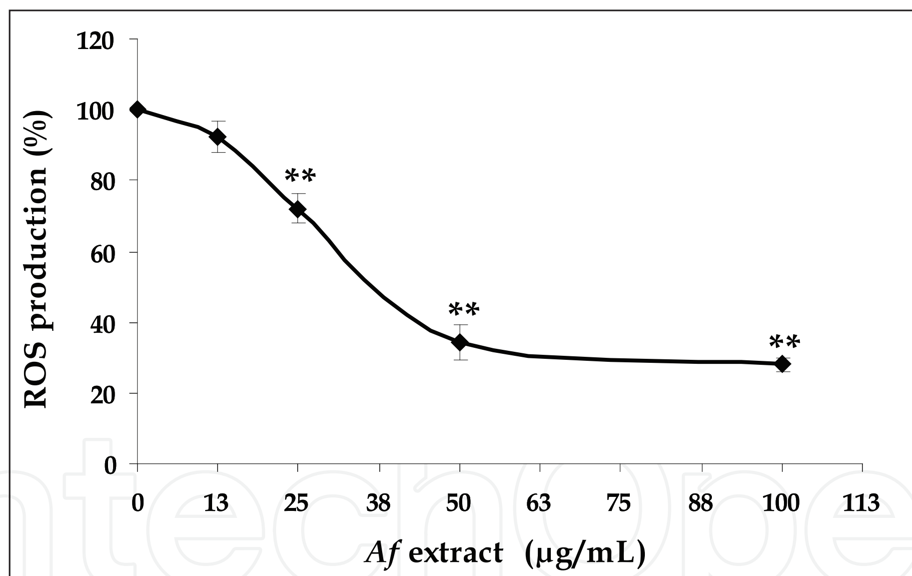
3.2 *Af* extract inhibits H_2O_2- and $ZnCl_2$-induced ROS generation

In order to gain more insight into the mechanisms by which the *Af* extract might exert its protective effects, and to determine whether this extract could inhibit ROS production induced by H_2O_2 and $ZnCl_2$, we assessed the intracellular generation of ROS by these toxic molecules, and tested whether treatment of astrocytes with the *Af* extract affected intracellular ROS levels. For the study of preventive effects against intracellular ROS formation the cells were preloaded with the ROS indicator DCF-DA, and were pretreated with various concentrations of *Af* extract before the application of H_2O_2 or $ZnCl_2$ stress, and ROS formation was determined by reading fluorescence every hour for 4 h. As can be seen in Fig. 4A, H_2O_2 induced ROS production in astrocytes, with the maximum levels produced after 1 h. Pretreatment of astrocytes with the *Af* extract inhibited the H_2O_2-induced elevation of the levels of intracellular ROS in a dose-dependent manner (Fig. 4B). We also found that treatment with $ZnCl_2$ increased ROS generation in astrocytes, and that, similarly to the effect

of the *Af* extract on H_2O_2-induced ROS, this extract greatly attenuated $ZnCl_2$-induced ROS generation (Fig. 5).



(A)



(B)

Fig. 4. The *Af* extract attenuates H_2O_2-induced ROS production in astrocytes

Astrocytes were preloaded with the redox - sensitive DCF-DA for 30 min and washed with PBS. Preloaded astrocytes were then pre-incubated for 2 h with various concentrations of *Af* extract. H_2O_2 (175 μM) was added to the culture and the fluorescence intensity representing ROS production was measured. (A) Pre-incubation with 100 μg/ml *Af* extract and measurements at the indicated time points (B) Pre-incubation with various concentrations of *Af* extract and measurements after 1 h. Each point represents the mean ± SEM of two experiments (n=7). **p<0.001 when ROS production following treatment with H_2O_2+*Af* extract was compared to cells treated with H_2O_2 alone at each of the equivalent time points.

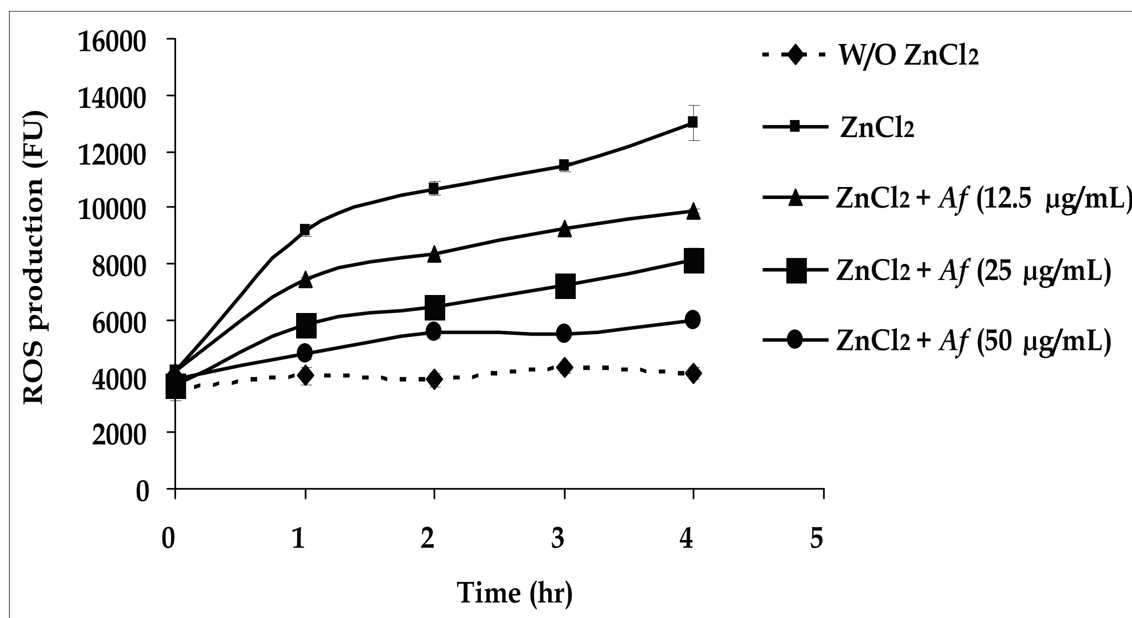


Fig. 5. Zinc induces ROS generation, and the *Af* extract attenuates ROS production following treatment of astrocytes with zinc

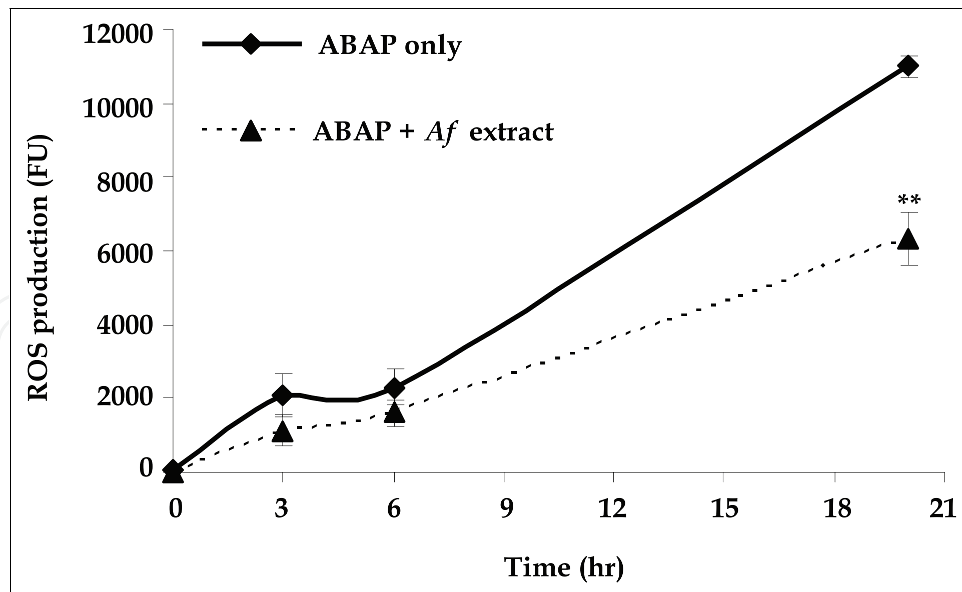
Astrocytes were preloaded with DCF-DA for 30 min and washed with PBS. They were then pre-incubated for 2 h with various concentrations of *Af* extract, after which, $ZnCl_2$ (50 µM) was added and the resulting fluorescence signal was measured at the indicated time points. Each point represents the mean ± SEM (n = 7). $p<0.01$ when ROS production following treatment with $ZnCl_2$+*Af* extract was compared to cells treated with $ZnCl_2$ alone at each of the equivalent time points

3.3 *Af* extract reduces 2,2'-azobis(amidinopropane) (ABAP)-mediated peroxyl radicals levels in astrocytes

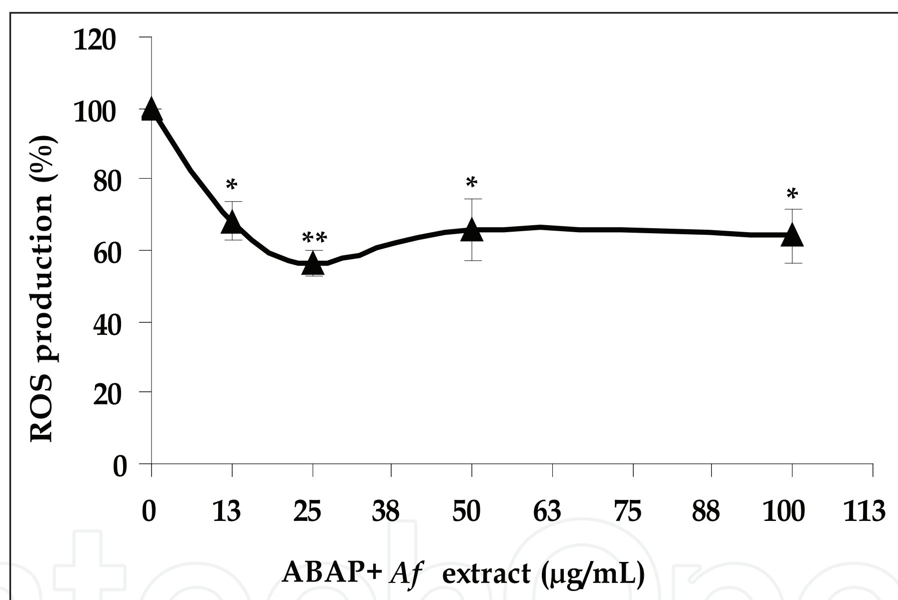
In addition to H_2O_2, various other species, such as peroxynitrite (ONOO-), nitric oxide (NO·) and peroxyl radicals have been found to oxidize DCFH to DCF in cell culture (Wang & Joseph, 1999), therefore we have used the cellular antioxidant activity assay to measure the ability of compounds present in the *Af* extract to prevent formation of DCF by ABAP-generated peroxyl radicals (Wolfe & Liu, 2007). The kinetics of DCFH oxidation in astrocytes by peroxyl radicals generated from ABAP is shown in Fig. 6A, where it can be seen that ABAP generated radicals in a time-dependent manner, and that treatment of cells with *Af* extract moderated this induction. Fig. 6B shows that the increase in ROS–induced fluorescence was inhibited by *Af* extract in a dose-dependent manner. This indicates that compounds present in the *Af* extract entered the cells and acted as efficient intracellular hydroperoxyl radical scavengers.

3.4 Differential pulse voltammetry (DPV) analysis of *the* antioxidant capacity of *Af* extract

Extract antioxidant capacity was evaluated by differential pulse voltammetry approach (DPV). Voltammetric techniques of analysis are increasingly being used for the determination of many substances of pharmaceutical importance (Zapata-Urzua et al., 2010) as well as of fruit extracts (Butera et al., 2002). These techniques are based on the measurement of current that results from oxidation or reduction at an electrode surface following an applied potential



(A)



(B)

Fig. 6. Peroxyl radical - induced oxidation of DCFH to DCF in primary astrocytes, and the inhibition of oxidation by *Af* extract

Astrocytes were incubated for 1 h with *Af* extract. They were then preloaded with DCF-DA for 30 min and washed with PBS, after which, 0.6 mM ABAP was added and ROS levels were measured at the indicated time points. Each point represents mean ± SEM of two experiments (n = 7). **A**. *Af* extract at 25 µg/ml. **B**. ROS production was measured 20 h after the addition of ABAP *p<0.01, **p<0.001 compared to cells treated with ABAP only at the equivalent time points.

difference. The DPV technique has excellent resolving power, and is able to differentiate between peaks due to different electroactive species in the same solution which are no more

that 50 mV apart (Smyth & Woolfson, 1987). In the present study we have used the DPV approach to analyze the total reducing capacity of the ethanolic *Af* extract. On the potential-current curve generated by DPV, the values of the potential are a characteristic of the antioxidant material and the values of the current are proportional to the amounts of the corresponding antioxidant. Analysis of the *Af* extract by DPV revealed two anodic waves that are caused by two major reducing groups of low-molecular-weight antioxidants, representing the total antioxidants in the extract (Fig. 7). The anodic wave potentials and their corresponding anodic currents, representing the amount of each antioxidant, are presented at Table 1.

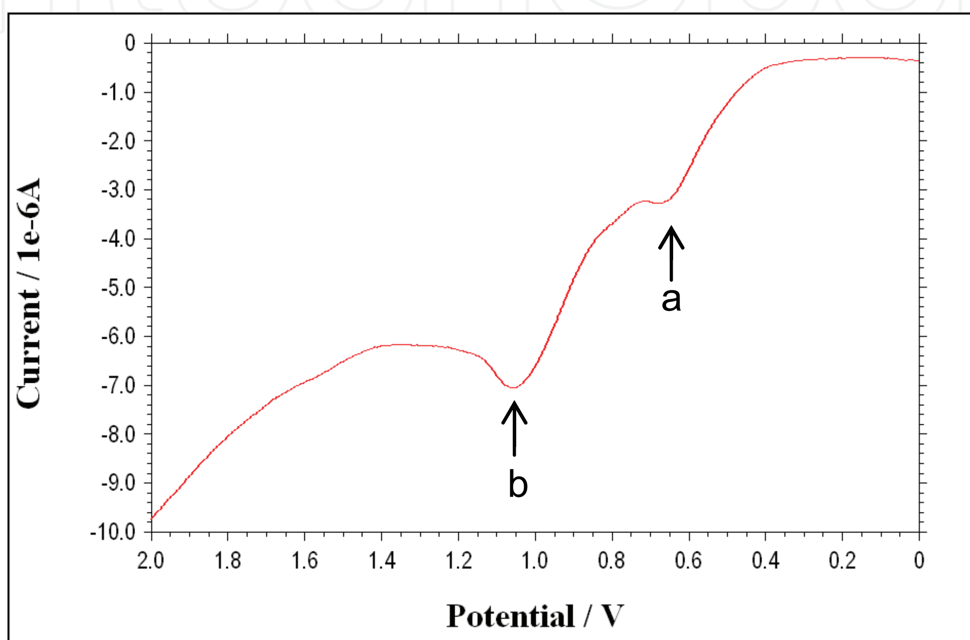


Fig. 7. Representative differential pulse voltammogram of the *Af* extract

Differential pulse voltammetry (DPV) was conducted from E = 0.0 V to final E = 2.0 V at a scan rate of 40 mV/s, pulse amplitude 50 mV, sample width 17 ms, pulse width 50 ms, pulse period 200 ms. Extracts were prepared in duplicate, and each sample was traced three times. **a** - first anodic wave; **b** - second anodic wave.

	Anodic wave a	**Anodic wave b**
Potential (V ± SD)	0.625±0.003	1.039±0.024
Current (μA ± SD)	3.233±0.251	7.027±0.063

Table 1. Anodic potentials and currents of the ethanolic extracts of *Af*

4. Discussion

The main findings of the present study were that an ethanolic extract of the desert plant *Af* could protect primary cultures of rat brain astrocytes from H_2O_2 -induced cell death, and reduced the levels of intracellular ROS produced after treatment with H_2O_2, $ZnCl_2$ or ABAP. This protective effect of *Af* and the reduction in ROS levels might be mediated by its antioxidant activities (as was demonstrated by the DPV experiments) or by modulation of

signals and processes induced by H_2O_2 and $ZnCl_2$. For example, it has been found, that H_2O_2 induced the phosphorylation of ERK1/2, AKT/protein kinase B and ATF-2 in C6 glioma cells (Altiok et al., 2006). It also has been demonstrated that cell death caused by zinc was accompanied by membrane translocation of protein kinase C-alpha (PKC-α), phosphorylation of extracellular signal-regulated kinase (ERK), and activation of group IV calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$) (Chang et al., 2010; Liao et al., 2011). It was also reported that Zn^{2+} bound to and inhibited glutathione reductase and peroxidase, the major enzymes responsible for glutathione (GSH) metabolism and cellular antioxidative defense mechanisms (Mize & Langdon, 1962; Splittgerber & Tappel, 1979).

Hydrogen peroxide also decreased astrocyte membrane fluidity, induced cytoskeletal reorganization, decreased the activities of the antioxidant enzymes catalase and superoxide dismutase (SOD) (Naval et al., 2007), and increased formation of cytonemes and cell-to-cell tunneling nanotube (TNT)-like connections (Zhu et al., 2005). Thus, the *Af* extract might interfere with any or all of the described processes, and enhance the resistance of astrocytes to $ZnCl_2$ and H_2O_2 toxicity, and to oxidative stress. Moreover, defense of glial cells against oxidative damage would be essential for maintaining brain functions.

There are two opportunities for compounds present in *Af* extract to elicit their antioxidant effects in our model: they can act at the cell membrane and break peroxyl radical chain reactions at the cell surface; or they can be taken up by the cell and react intracellularly with ROS. Therefore, the efficiency of cellular uptake and/or membrane binding, combined with the radical-scavenging activity dictates the efficacy of the tested compounds. In order to discriminate between these possibilities, astrocytes were pre-incubated with ABAP, which generates ROS intracellularly. According to our results, which show that *Af* extract inhibited intracellular ROS levels, in addition to other possible activities, compounds present in *Af* extract could enter the cells and react with ROS intracellularly.

Because many low-molecular-weight antioxidants might contribute to the cellular antioxidant defense properties, we analyzed the total antioxidant content of the *Af* extract by the DPV method, which enabled us to demonstrate the presence of two reducing equivalents in the *Af* extract. The advantages of DPV over other voltammetric techniques include excellent sensitivity with a very wide useful linear concentration range for organic species (10^{-6} to 10^{-3} M), short analysis times, simultaneous determination of several analytes, and ease of generating a variety of potential waveforms and measuring small currents.

Our LC-MS analysis identified quercetin-glucoside as one of the major peaks in the *Af* extract. Quercetin glycosides are widely consumed flavonoids that are found in many fruits and vegetables, e.g., onion, and, like other flavonoids, offer a wide range of potential health benefits, including prevention of atherosclerosis and cardiovascular diseases (Peluso, 2006; Terao et al., 2008). In recent years, intestinal absorption and metabolism of quercetin glucosides have been extensively investigated with regard to their bioavailability (Spencer et al., 2004; Walle, 2004). Quercetin glucosides are well absorbed by the small intestine because the presence of a glucose moiety significantly enhances absorption (Arts et al. 2004; Boyer et al., 2005; Hollman & Arts, 2000). In the process of intestinal absorption quercetin-glucosides are subjected to hydrolysis and subsequent conversion into conjugated glucuronides and/or sulfates (Murota & Terao, 2003). A variety of metabolites circulating in the blood-stream were identified (Day et al., 2001; Mullen et al., 2002), and some of them were found to possess a substantial antioxidant activity (da Silva et al., 1998; Manach et al., 1998). It was suggested that metabolites of quercetin glucosides accumulate in the aorta - a target site for its anti-atherosclerotic effect, and attenuate lipid peroxidation that occur in the

aorta, along with the attenuation of hyperlipidemia (Kamada et al., 2005; Terao, 1999; Terao et al., 2008).

Two other compounds in the *Af* extract were also identified by LC-MS: acacetin 7-o-rhamnoside, which was also identified in the aerial parts of several plants (El-Wakil, 2007; Sharaf et al., 1997), and acacetin 7-o-glucoside, which was also found in the anti-inflammatory extract of Mcfadyena unguis-cati L. (Aboutabl et al., 2008). All four compounds identified by LC-MS analysis as major peaks in *Af* extract, namely epicatechin-rhamnoside, Acacetin rhamnoside, Acacetin-glucoside, and Quercetin-glucoside, are stable compounds, that under our experimental conditions (ethanol extraction, resolubilization in DMSO, and tissue culture experiments at 37°C and neutral pH) would not react chemically with each other. Chemical interactions between these compounds might occur under high temperatures and extreme pH values.

Several studies have revealed that some herbal medications and antioxidants show promise in prevention of neurodegenerative diseases (Iriti et al., 2010). Substances that can restrict and/or protect brain cells from oxidative stress show promise as potential tools in the therapy of various brain injuries and neurodegenerative diseases. Desert plants survive various stress conditions, including oxidative stress., therefore it is reasonable to suppose that various endogenous molecules present in these plants might also assist animal cells to cope with stresses that develop during pathological conditions.

5. Conclusions

In light of their antioxidant and astroprotective properties, we suggest that *Af* extracts might serve as a new source of beneficial phytochemicals, and should be further evaluated for nutraceutical development as polyvalent cocktails for prevention or treatment of various brain injuries and neurodegenerative diseases, in which oxidative stress and astrocytic cell death form part of the pathophysiology.

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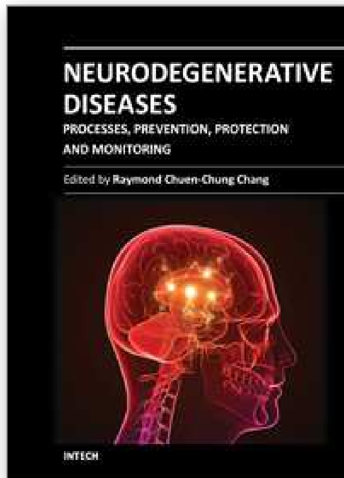

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Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring focuses on biological mechanisms, prevention, neuroprotection and even monitoring of disease progression. This book emphasizes the general biological processes of neurodegeneration in different neurodegenerative diseases. Although the primary etiology for different neurodegenerative diseases is different, there is a high level of similarity in the disease processes. The first three sections introduce how toxic proteins, intracellular calcium and oxidative stress affect different biological signaling pathways or molecular machineries to inform neurons to undergo degeneration. A section discusses how neighboring glial cells modulate or promote neurodegeneration. In the next section an evaluation is given of how hormonal and metabolic control modulate disease progression, which is followed by a section exploring some preventive methods using natural products and new pharmacological targets. We also explore how medical devices facilitate patient monitoring. This book is suitable for different readers: college students can use it as a textbook; researchers in academic institutions and pharmaceutical companies can take it as updated research information; health care professionals can take it as a reference book, even patients' families, relatives and friends can take it as a good basis to understand neurodegenerative diseases.

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