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ARTICLE

Protective Effects of the Essential Oil of *Salvia fruticosa* and Its Constituents on Astrocytic Susceptibility to Hydrogen Peroxide-Induced Cell Death

Anat Elmann,*,† Sharon Mordechay,† Miriam Rindner,† Olga Larkov,‡ Meital Elkabetz,‡ and Uzi Ravid‡

†Department of Food Science, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel and ‡Department of Aromatic Plants, Agricultural Research Organization, Ramat Yishay, Israel

Oxidative stress has been implicated in pathologic processes associated with neurodegenerative diseases. Astrocytes, the most abundant glial cell type in the brain, protect neurons from reactive oxygen species (ROS), and any damage to them will affect neuronal survival. This study compares the ability of essential oils prepared from different herbs and spices to protect cultured primary brain astrocytes from H_2O_2-induced death. The results show that the essential oil of *Salvia fruticosa* (*Sf*) among the tested essential oils demonstrated remarkable protective activity. The protective effect of *Sf* could be attributed to α-humulene and α-pinene. Following incubation, α-humulene and *trans*-β-caryophyllene could be found in the cytosol of astrocytes. It is proposed that *Sf*, by attenuating H_2O_2-induced cell death, might be used as a functional food or may be offered as a means of therapy in the treatment of neurodegenerative diseases.

KEYWORDS: *Salvia fruticosa*; oxidative stress; astrocytes; neurodegenerative diseases; essential oils

INTRODUCTION

Salvia fruticosa Mill. (*Sf*), formerly known as *Salvia triloba* L. (Lamiaceae) and commonly known as Greek *Sf*, is a native species of the Eastern Mediterranean basin. It has a long history of use as a culinary herb as well as in the treatment of various disorders. This herb (especially its leaves) has a folk reputation in the Eastern Mediterranean region for the treatment of various skin, blood, and infectious ailments as well as ailments of the digestive, circulatory, respiratory, and osteomuscular systems (*1*,*2*). It is also used as a hypoglycemic herb (*3*) and against inflammations, hepatitis, and tuberculosis (*4*, *5*). However, there is no evidence for the activity of *Sf* or its essential oil in the context of neurodegenerative diseases.

Oxidative stress has long been associated with the development of various pathological conditions in the brain and with neurodegenerative disorders, including ischemia, schizophrenia, Alzheimer′s disease, Parkinson′s disease, and Huntington′s disease (*6*−*8*). Oxidative stress is a major factor leading to neuronal death by necrosis or apoptosis (*9*), and both are observed under conditions of severe oxidative stress in vivo (*10*). The reactive oxygen species (ROS) known to be responsible for neurotoxicity are H_2O_2, superoxide anions (O_2^-), and hydroxyl radicals ($OH^•$). Of these, H_2O_2 is thought to be the major precursor of highly reactive free radicals, and it has been reported to be produced in excess in the pathogenesis of brain injuries and neurodegenerative diseases. For example, amyloid β-peptide was reported to produce H_2O_2 through metal ion reduction in Alzheimer's disease (*11*, *12*). H_2O_2 may damage all of the major classes of biological macromolecules in the cells through direct oxidation of lipids, proteins, and nucleic acids. Specifically, it has been demonstrated that oxidative stress induced by H_2O_2 decreases astrocyte membrane fluidity, induces cytoskeletal reorganization, and increases the formation of cytonemes and cell to cell tunneling nanotube (TNT)-like connections (*13*). H_2O_2 was also shown to induce apoptosis in cultured cells of the central nervous system (CNS), for example, neurons and glial cells (*14*, *15*).

Astrocytes, which are the major cell type in the CNS, form an intimately connected network, with neurons providing mechanical and metabolic support (*16*, *17*) and playing an important role in the defense system of the brain against ROS. The brain is particularly vulnerable to oxidative damage because of the high rate of oxygen utilization and the high contents of oxidizable polyunsaturated fatty acid and redox-active transition metal ions. Oxidative stress causes cell death when intracellular levels of metabolic and antioxidant enzymes (especially glutathione related enzymes) and substrates (glutathione, glucose, and ATP) are exhausted. However, astrocytes contain more vitamin E and GSH, more of the enzymes involved in GSH metabolism, and more superoxide dismutase (SOD) than neurons, making these cells neuroprotective and resistant to oxidative stress relative to oligodendrocytes and neurons (*18*). Thus, astrocytes appear to play a key role in the defense system of the brain against ROS, determining the brain′s vulnerability to oxidative injury. Indeed, it has been demonstrated that cultured astrocytes protect oligodendrocytes and neurons in culture against H_2O_2 toxicity (*19*).

*Address correspondence to this author at the Food Science Department, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan, 50250 Israel (telephone +972-3-9683516; fax +972-3-9683692; e-mail aelmann@volcani.agri.gov.il).

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Table 1. Protective Effect of Essential Oils Prepared from Different Herbs and Spices against H_2O_2-Induced Astrocytic Cell Death[a]

plant family	plant species	protection (%)
Asteraceae	*Achillea fragrantissima* (Forssk.) Sch. Bip.	0 ± 5
	Artemisia arborescens L.	20 ± 4[b]
	Artemisia dracunculus L.	0 ± 7
	Artemisia judaica L.	7 ± 6
	Artemisia herba alba L.	0 ± 7
	Matricaria chamomilla L.	9 ± 5
Geraniaceae	*Pelargonium graveolens* L′Her.	10 ± 6
Lamiaceae	*Ocimum canum* Sims.	21 ± 2[b]
	Origanum majorana L.	30 ± 2[b]
	Origanum dayi Post	0 ± 5
	Rosmarinus officinalis L.	15 ± 2[b]
	Salvia dominica L.	13 ± 5
	Salvia sclarea L.	20 ± 1[b]
	Salvia fruticosa Mill.	51 ± 3[b]
	Thymus vulgaris L.	20 ± 2[b]
Myrtaceae	*Myrtus communis* L.	0 ± 8
Apiaceae	*Apium graveolens* L.	0 ± 6
	Carum carvi L.	7 ± 7
	Foeniculum vulgare Mill.	21 ± 6[b]
	Petroselinum crispum Mill.	21 ± 8[b]

[a] Results represent two experiments performed in quadruplicates. [b] Statistically significant difference ($p < 0.05$) between the tested compound and the corresponding control.

Epidemiological studies have shown that nutritional antioxidants may forestall the onset of dementia (*20*, *21*). Several studies have also shown that some herbal medications and antioxidants show promise toward preventing Alzheimer′s disease (*22*). Thus, because of the critical role of astrocytes in neuronal survival (*23*), it is of interest to assess the protective activity of essential oils derived from different herbs and spices on astrocytic susceptibility to H_2O_2 insult. The present study describes the protective effect of the essential oil from *Sf* and its constituents from H_2O_2-induced cell death of cortical astrocytes.

MATERIALS AND METHODS

Materials. Dulbecco′s Modified Eagle′s Medium (DMEM), Leibovitz-15 medium, glutamine, antibiotics (10000 IU/mL penicillin and 10,000 μg/mL streptomycin), soybean trypsin inhibitor, and fetal bovine serum (FBS) were purchased from Biological Industries (Beit Haemek, Israel); dimethyl sulfoxide (DMSO) was obtained from Applichem; hydrogen peroxide (H_2O_2) was obtained from MP Biomedicals; 1,8-cineole was purchased from Frutarom (Israel); (−)-camphor was purchased from BDH; (+)-camphene and (−)-β-pinene were purchased from Aldrich; and (−)-α-thujone, (±)-α-pinene, α-humulene, and (−)-*trans*-β-caryophyllene were purchased from Fluka.

Experimental Animals. Newborn Wistar rats (0−2 days old) were obtained from Harlan Laboratories. The experiments were performed in compliance with the appropriate laws and institutional guidelines and were approved by the Institutional Animal Care and Use Committee (no. 148/08).

Isolation of Essential Oils. All herbs and spices (**Table 1**) of the Asteraceae, Geraniaceae, Lamiaceae, and Myrtaceae were taken from a living material collection existing at Newe Ya′ar Research Center, Agricultural Research Organization, Israel, under cultivated conditions. The Israeli native plants were identified by A. Danin of the Hebrew University of Jerusalem and A. Dafni of Haifa University. The plants were cultivated in local clayey soil under drip irrigation, as described previously (*24*, *25*). Seeds of the Apiaceae family were taken from cooled seed storage and were sown at Newe Ya′ar. Plant samples of at least 5 kg were steam-distilled in a 130 L of direct steam pilot plant apparatus for 1 h. Yields of essential oils varied (0.1−2.0% v/w) between the different plants (see the Supporting Information). Essential oils were diluted with petroleum ether and were injected to the GC-MS.

GC-MS Analyses. GC-MS analyses were performed on MSD (Agilent Technologies, Palo Alto, CA) with a Combi Pal (CTC Analytic, Switzerland) autosampler equipped with an Rtx-5SIL MS (30 m×0.25 mm i.d. × 0.25 μm) fused-silica capillary column (Restek, Bellefonte, PA). Helium, at a constant pressure of 14.14 psi and a linear velocity of 47 cms^{-1}, was used as carrier gas. The injector was kept at 250 °C and set for the 1:50 split mode. The transfer line was kept at 280 °C. The column was maintained at 50 °C for 1 min and then programmed to 190 at 5 °C min^{-1} and then to 260 at 10 °C min^{-1}. The MSD was operated in the electron ionization mode at 70 eV, in the m/z range of 42−350. Identification of compounds was performed by comparing their relative retention indices and mass spectra with those of authentic samples, supplemented with the NIST 98 and HPCH 2205 GC-MS libraries.

Preparation of Primary Cultures of Astrocytes. Cultures of primary rat astrocytes were prepared from cerebral cortices of 1−2-day-old neonatal Wistar rats. Briefly, while in Leibovitz-15 medium, meninges and blood vessels were carefully removed, 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 precoated with poly-D-lysine (PDL, 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 in vitro 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 for 5−6 h on a horizontal shaking platform. Astrocytes were replated at 24-well PDL-coated plastic plates at a density of 1×10^5/well, in DMEM (w/o Phenol Red) containing 2% FBS, 2 mM glutamine, 100 U/mL penicilin, and 100 μg/mL streptomycin.

Treatment of Cells. Twenty-four hours after plating, the original medium of the cells was aspirated off and fresh medium was added to the cells. Dilutions of the essential oils first in DMSO and then in the growth medium and dilutions of H_2O_2 in the growth medium were made fresh from stock solution just prior to each experiment and were used immediately. The final concentration of DMSO in the medium was 0.2% (v/v). Each treatment was performed in quadruplicates.

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 cehydrogenase (LDH) activity released from the cytosol of damaged cells into the incubation medium.

Determination of the Uptake of *Sf* Oil Constituents by Astrocytes. Astrocytes (5×10^6/ sample) were incubated with *Sf* oil or its constituents, *trans*-β-caryophyllene and α-humulene, for different time periods. At the end of the incubation the treated astrocytes were washed six times for 2 min with astrocytic growth medium and were harvested on ice into 0.5 mL of 20% NaCl in water. In cases when cytosol and membranes were separated, the cells were flash frozen (in liquid nitrogen) and subsequently thawed three times and centrifuged (18620*g*, 20 min, 4 °C). The cytosol was transferred into 2 mL glass headspace vials containing 140 mg of NaCl, and the membrane fraction was discarded. The vials were stored at −20 °C until analysis. The content of the vials was then extracted with 1 mL of methyl *tert*-butyl ether (MTBE) containing 0.5 ppm of isobutylbenzene (IBB) as internal standard. The samples were shaken for 2 h at room temperature for phase separation. The organic (upper) phase was transferred to clean vials with sodium sulfate (anhydrous Na_2SO_4). After the sodium sulfate had precipitated, the dried organic phase was transferred into a clean vial and was evaporated to 100 μL using nitrogen. One microliter of each sample was injected to a GC-MS.

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

RESULTS

Screening the Effect of Essential Oils of Various Herbs and Spices on Astrocytic Susceptibility to Hydrogen Peroxide. To determine the astroprotective potential of the various essential

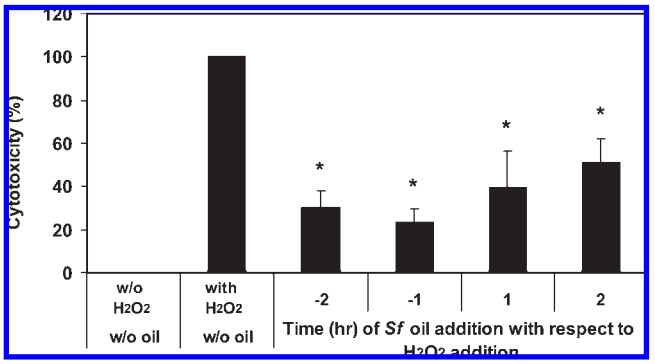

Figure 1. Pre-incubation of astrocytes with the essential oil of *Sf* is needed to exert its protective effect from H_2O_2 cytotoxicity. The essential oil of *Sf* (40 μg/mL) was added to astrocytes before (−2 h, −1 h) or after (1 h, 2 h) the addition of H_2O_2 (150 μM). Cytotoxicity was measured 20 h later. The results are the mean ± SD of a representative experiment of four experiments, each performed in quadruplicates. An asterisk (∗) indicates statistically significant difference (p< 0.01) between each time point tested and corresponding control.

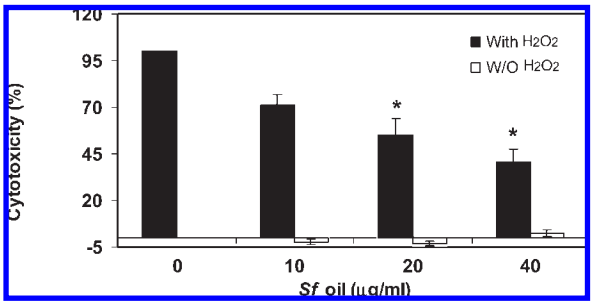

Figure 2. Protection from H_2O_2-induced astrocytic cell death by different concentrations of the essential oil of *Sf*. Astrocytes were pre-incubated for 2 h with different concentrations of the essential oil of *Sf* and were treated with H_2O_2 (150 μM). Cell death was determined 20 h later. The results are the mean ± SEM of two experiments, each performed in quadruplicates. An asterisk (∗) indicates statistically significant difference (p < 0.01) between the tested oil and corresponding control.

oils, we used a model in which oxidative stress is caused by the in vitro addition of H_2O_2 to primary astrocytes. Treatment of normal primary astrocytes with hydrogen peroxide resulted in astrocytic cell death and concentration-dependent release of LDH when assessed 20 h later (data not shown). The concentration of H_2O_2 (150 μM) used in our experiments was reported by Hyslop et al. to be the concentration of H_2O_2 that appears in the rat striatum under ischemic conditions (*26*). To conduct a first selection for prospective astroprotective activity, we compared the effect of essential oils prepared from 20 different herbs and spices belonging to 5 different plant families (**Table 1**) in minimizing the cytotoxic damage induced by H_2O_2. These essential oils differ in the identity of their main constituents (see the Supporting Information). Astrocytes were pretreated with the tested oil 2 h before their exposure to H_2O_2. Cell viability was assessed 20 h after H_2O_2 addition using the LDH assay. The distribution of the essential oils according to their extent of astroprotection is presented in **Table 1**. Under these experimental conditions, most of the oils (15 plants, 75% of the tested plants) could provide only 0−20% protection. Four plants (20% of the tested plants) were more potent and provided 21−30% protection, and the oil extracted from *Sf* exhibited the highest protective activity (51 ± 3%) against the H_2O_2 insult.

Effects of *Sf* Oil on Astrocytic Susceptibility to Hydrogen Peroxide. We further examined the optimal conditions, in terms of time and dose, for *Sf* oil to exert its protective effect. To elucidate the optimal time point for the addition of the *Sf* essential oil with respect to H_2O_2 insult, the cells were either pre-incubated in the presence of the oil for 1 or 2 h before the addition of H_2O_2 or treated 1 or 2 h after the addition of H_2O_2. The results, presented in **Figure 1**, demonstrate that *Sf* oil acts more efficiently when added 1−2 h before the H_2O_2 insult. To find the optimal concentration of the oil needed for its protective effect, astrocytes were pre-incubated with different concentrations of *Sf* essential oil. H_2O_2 was then added, and cytotoxicity was determined after 20 h. Our results show (**Figure 2**) that *Sf* oil protects against H_2O_2-induced cell death in a dose-dependent manner. No significant changes were observed in the viability of cells treated with similar concentrations of *Sf* essential oil in the absence of H_2O_2.

Protective Effect of the Pure Constituents of *Sf* Oil. The essential oil of *Sf* is composed of various compounds at different concentrations (*27*, *28*, and Supporting Information). Thus, to identify

Table 2. Protective Effect of the Main Constituents of *Salvia fruticosa* against H_2O_2-Induced Astrocytic Cell Death[a]

compd	LRI [b]	% of compd in the natural oil	concn of compd in the expts[c] (μg/mL)	protection (%)
1,8-cineole	1031	26.4	10	0
camphor	1149	18.9	8	0
camphene	949	9.5	4	22 ± 4
α-thujone	1108	9.1	4	0
trans-β-caryophyllene	1418	5	2	24 ± 5
α-pinene	931	4.4	2	69 ± 7[d]
β-pinene	976	4.7	2	0
α-humulene	1456	3.9	1.5	50 ± 9[d]

[a] Results represent the mean ± SEM of three experiments, each performed in quadruplicates. [b] Linear retention index on an Rtx-5SIL MS column. [c] Concentrations were chosen on the basis of the calculation as if the cells were treated with 40 μg/mL *Sf* oil. [d] Statistically significant difference (p<0.05) between the tested compounds and the corresponding control.

the active compound(s), which may be responsible for the protective effect of *Sf* oil, we further examined the protective effect of eight main constituents, which comprise 82% of the oil. The results, presented in **Table 2**, show that when tested at their relative concentrations in the oil (as determined by GC-MS analysis), a significant protective activity was exerted by α-humulene and α-pinene (50−69% protection), which are minor constituents of the oil (3.9 and 4.4%, respectively), and not by 1,8-cineole and camphor, which are the major constituents of the oil (26.4 and 18.9%, respectively). α-Thujone and β-pinene had no protective activity at all, and camphene and *trans*-β-caryophyllene had no significant protective activity (22 and 24%, respectively) as determined by statistical analysis. **Figure 3** shows the dose dependency of the protective effect of α-humulene and α-pinene.

Incorporation of *Sf* Oil Components into Astrocytes. To gain more insight into the mechanism by which the *Sf* oil and its constituents exert their protective effect, we tested whether *Sf* oil components are incorporated into astrocytes. For that purpose, we incubated the cells with *Sf* oil for 2 h, extracted the essential oil constituents from the cells, and analyzed their composition by GC-MS. Interestingly, α-pinene, α-thujone, and camphene were found in astrocyte homogenate in similar proportions as in the original *Sf* oil, whereas the proportions of 1,8-cineole and camphor were significantly lower in astrocytes than in the original oil, and the proportions of *trans*-β-caryophylene and α-humulene were significantly higher (∼7-fold) in astrocytes than in the

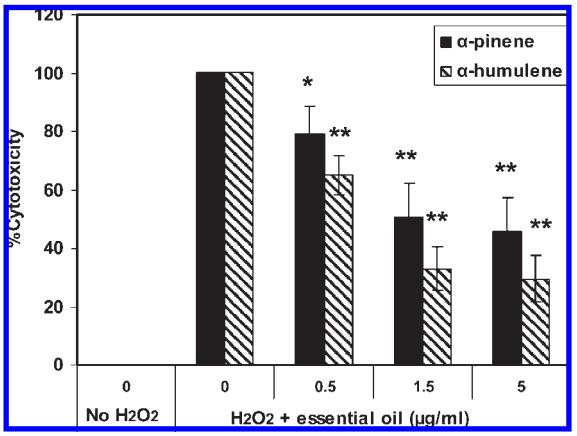

Figure 3. Protective effect of different concentrations of α-humulene and α-pinene against H_2O_2-induced astrocytic cell death. Astrocytes were pre-incubated for 2 h with different concentrations of α-humulene and α-pinene and were then treated with H_2O_2 (150 μM). Cytotoxicity was determined 20 h later. The results are the mean ± SD of a representative experiment of three similar experiments, each performed in quadruplicates. Asterisks (*) and (**) indicate statistically significant difference ($p < 0.05$) and $p < 0.01$, respectively) between the tested compound and corresponding control.

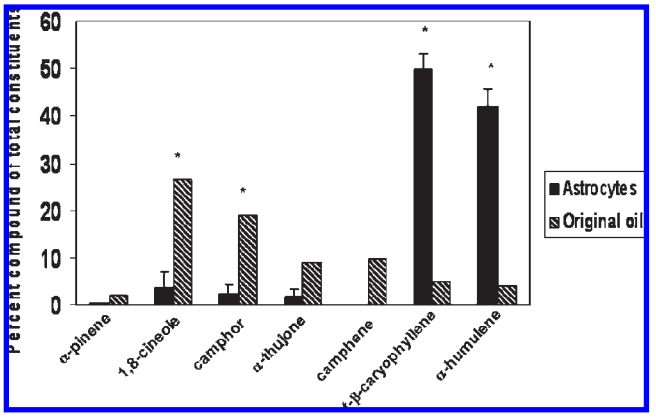

Figure 4. Incorporation of *Sf* oil constituents by astrocytes. Astrocytes were incubated with 30 μg/mL *Sf* oil for 2 h. Cells were then washed, harvested, and extracted, and the amount of each constituent in the homogenate and in the original oil was determined by GC-MS. The results are the mean ± SEM of five different experiments. An asterisk (*) indicates statistically significant difference ($p < 0.001$) between the percentage of the compound in the original oil and its percentage in astrocyte homogenate.

original oil (**Figure 4**). These data can be explained either by adherence/incorporation of these lipophilic molecules into the cell membranes or by their active transport into the cell cytosol. To test these possibilities, cells were incubated for different time periods with α-humulene or with *trans*-β-caryophyllene, washed, harvested, and separated into cytosolic and membrane fractions. **Figure 5** show that both α-humulene and *trans*-β-caryophyllene enter the cytosol of the cells in a time-dependent manner as was determined by GC-MS.

DISCUSSION

Essential oils are used as folk medicines against various kinds of inflammatory diseases, organ dysfunction, or systemic disorders. Extensive research has demonstrated, in experimental systems in vitro and in vivo, that essential oils and their components possess a wide spectrum of biological activities (*29*).

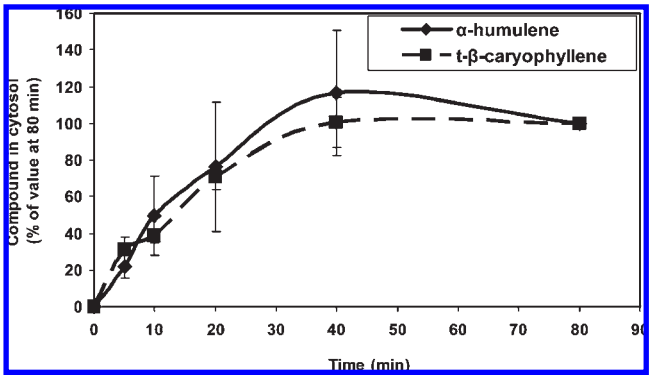

Figure 5. Incorporation of *trans*-β-caryophyllene and α-humulene by astrocytes. Astrocytes (5×10^6) were incubated with 20 μg/mL α-humulene or *trans*-β-caryophyllene for the indicated time points. Cells were then washed, harvested, and separated into cytosolic and membrane fractions. After extraction, the amounts of α-humulene and *trans*-β-caryophyllene in the cytosol were determined by GC-MS. The results are the mean ± SD of two or three similar experiments for *trans*-β-caryophyllene and α-humulene, respectively. One hundred percent indicates the amount of *trans*-β-caryophyllene (218 ng/sample) or α-humulene (205 ng/sample) found in the cytosol at the latest time point tested (80 min).

In the present study, the effectiveness of essential oils isolated from 20 different herbs and spices in counteracting oxidative damage has been evaluated in cultured astrocytes that were stressed by the addition of hydrogen peroxide. The main findings of this study are that of the 20 essential oils tested, the essential oil of *Sf* exhibited the highest protective activity against oxidative stress-induced death. The active protective constituents of the oil were α-humulene and α-pinene. Interestingly, none of the essential oils screened in this study, except *Sf* oil, contained α-humulene, which was found to be one of the astroprotective components in *Sf* oil. α-Humulene and *trans*-β-caryophyllene, which are lipophilic sesquiterpene hydrocarbons, penetrate the cells and could be found in the cytosol of astrocytes. In contrast, although the monoterpene hydrocarbons camphene, α-pinene, and β-pinene are also lipophilic molecules, they were not found in the cells in a higher proportion than in the *Sf* oil. The oxygenated monoterpenes, 1,8-cineole, camphor, and α-thujone, are more polar, and this might be the reason that they were not found in association with the astrocytes. Conclusive evidence to understand whether these compounds enter the cells in an active or passive manner awaits further research.

Most of the studies regarding the effects of phytochemicals on neurodegenerative diseases concentrate on various aspects related to neurons which their death is the final step in the degenerative process. However, very little research has been done regarding their effects on astrocytes, which play a critical role in neuroprotection and their response is involved in the early stages of these diseases. Increased oxidative stress and excess of H_2O_2 have been implicated in the pathology of various neurodegenerative disorders; thus, reducing oxidative stress is considered to be a promising approach to neuroprotection. Although experimental data are consistent in demonstrating neuroprotective effects of antioxidants in vitro and in animal models, the clinical evidence that antioxidants may prevent or delay the course of these diseases is still relatively unsatisfactory and insufficient to strongly modify clinical practices. Thus, substances that can restrict and/or protect brain cells from oxidative stress, not just by their chemical ability to serve as antioxidants, are more promising potential tools in the therapy of various neurodegenerative diseases. Although there are studies regarding the in vitro

antioxidant activity of various essential oils and their constituents, only a few studies have described the ability of essential oils to exert protective activity from oxidative stress in cellular or animal models. For example, camphene, which comprises 9.5% of the total constituents of the *Sf* oil, was also shown to protect rat alveolar macrophages against *tert*-butyl hydroperoxide (t-BHP) induced oxidative stress (*30*). This protective effect was evident from the decrease in lipid peroxidation, nitric oxide release, and ROS production, as well as from the increase in SOD activity along with glutathione content and the restoration of mitochondrial membrane potential. Another essential oil component, although not of *Sf* oil, is carvacrol, which was shown to protect human leukemic K562 cells from DNA damage induced by H_2O_2 treatment (*31*). Moreover, when given to rats in drinking water, carvacrol reduced the level of DNA lesions induced in freshly isolated hepatocytes and testicular cells by H_2O_2 (*32*).

Interestingly, the arithmetic sum of the percentages of astroprotection by all of the tested constituents is much higher than that of the protective effect of the *Sf* oil itself. This might be explained either by a masking effect exerted by the other constituents of the oil or, alternatively, by a similar mechanisms of action (i.e., redundancy) shared by the different astroprotective compounds. The components of *Sf* oil might exert their astroprotective effects by different mechanisms and might interfere with signals and processes induced by H_2O_2, either directly or through receptor-mediated signaling. For example, *trans*-β-caryophyllene, which is one of the components that penetrated astrocytes, was shown to be a functional agonist of the cannabinoid CB2 receptor, to inhibit adenylate cyclase activity, and to attenuate the LPS-stimulated Erk1/2 and JNK1/2 phosphorylation (*33*). Various constituents of essential oils were shown to possess different biological activities in the CNS, which indicates their entry into the brain. For example, α-humulene was found in the brain 0.5 h after oral administration (*34*), and 1,8-cineole and β-pinene were found to possess antinociceptive effects in rodents (*35*). In addition, our results show that α-humulene can cross the cell membrane and enter the cell. Thus, according to the above, and due to their small molecular size and lipophilicity, the volatile constituents of the essential oil of *Sf* are likely to readily cross the blood−brain barrier and exert their protective effects. Because neurodegenerative diseases are multifactorial, treatment strategies for these diseases have to include a variety of interventions directed at multiple targets. The various components of *Sf* oil have various complementary activities that might be beneficial for the treatment of such diseases. For example, α-humulene and *trans*-β-caryophyllene, which were shown by us to penetrate the cells, were shown to display topical and systemic anti-inflammatory effects in different experimental models (*33*, *36*, *37*). These compounds inhibit the LPS-induced NF-kB activation and neutrophil migration, preventing the production of pro-inflammatory cytokines by neutrophils and in peripheral blood (*33*, *38*). Furthermore, peroral *trans*-β-caryophyllene strongly reduces the carrageenan-induced inflammatory response in mice (*33*). Another example is 1,8-cineole, which is a main constituent (26.4%) of the *Sf* oil but had no apparent neuroprotective activity. This compound has been reported to possess anti-inflammatory activities both in vivo (*39*, *40*) and in vitro (*41*). Because neuroinflammation plays a key role in the initiation and progression of neurodegenerative diseases, these anti-inflammatory effects might be beneficial in the treatment of such diseases. An additional example of a beneficial effect of the components of *Sf* oil is the inhibitory activity of 1,8-cineole and α-pinene on acetyl cholinesterase activity (*42*), which is a therapeutic target in the treatment of Alzheimer's disease. Because of the broad range of beneficial bioactivities of the different constituents of this oil (e.g., anti-inflammatory, antioxidant, acetyl cholinesterase inhibitor, astroprotective), it might serve as a polyvalent "cocktail" for nutraceutical development.

In summary, in the present study, we have demonstrated that *Sf* essential oil is a source for different bioactive phytochemicals that can protect primary cultures of astrocytes from H_2O_2-induced oxidative damage. To the best of our knowledge, this is the first paper comparing the astroprotective effects of essential oils isolated from different herbs and spices. Moreover, no study has discussed the effect of *Sf* oil in the context of neurodegenerative diseases or demonstrated the incorporation of its constituents into astrocytes. Thus, our data, as well as others, may be useful for the consideration of *Sf* or its active constituents as a possible functional food ingredient, food supplement, or nutraceutical for the prevention or amelioration of neurodegenerative diseases.

ABBREVIATIONS USED

CNS, central nervous system; GC-MS, gas chromatography−mass spectrometry; ROS, reactive oxygen species; *Sf*, *Salvia fruticosa* Mill.; MTBE, methyl *tert*-butyl ether.

SAFETY

MTBE is highly flammable and is harmful if inhaled or ingested or through skin contact. Safety glasses must be worn, and the vapor should not be breathed; the work area must be well-ventilated.

Supporting Information Available: Composition and yields of essential oils of the different plants. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Carmona, M. D.; Llorach, R.; Obon, C.; Rivera, D. "Zahraa", a Unani multicomponent herbal tea widely consumed in Syria: components of drug mixtures and alleged medicinal properties. *J. Ethnopharmacol.* **2005**, *102*, 344–350.

(2) Ali-Shtayeh, M. S.; Yaniv, Z.; Mahajna, J. Ethnobotanical survey in the Palestinian area: a classification of the healing potential of medicinal plants. *J. Ethnopharmacol.* **2000**, *73*, 221–232.

(3) Perfumi, M.; Arnold, N.; Tacconi, R. Hypoglycemic activity of *Salvia fruticosa Mill.* from Cyprus. *J. Ethnopharmacol.* **1991**, *34*, 135–140.

(4) Wichtl, M. *Herbal Drugs and Phytopharmaceuticals*; CRS Press: London, U.K., 1994.

(5) Kaileh, M.; Berghe, W. V.; Boone, E.; Essawi, T.; Haegeman, G. Screening of indigenous Palestinian medicinal plants for potential anti-inflammatory and cytotoxic activity. *J. Ethnopharmacol.* **2007**, *113*, 510–516.

(6) Simonian, N. A.; Coyle, J. T. Oxidative stress in neurodegenerative diseases. *Annu. Rev. Pharmacol. Toxicol.* **1996**, *36*, 83–106.

(7) Ischiropoulos, H.; Beckman, J. S. Oxidative stress and nitration in neurodegeneration: cause, effect, or association?. *J. Clin. Invest.* **2003**, *111*, 163–169.

(8) Halliwell, B. Oxidative stress and neurodegeneration: where are we now? *J. Neurochem.* **2006**, *97*, 1634–1658.

(9) Bonfoco, E.; Krainc, D.; Ankarcrona, M.; Nicotera, P.; Lipton, S. A. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7162–7166.

(10) Choi, D. W. Ischemia-induced neuronal apoptosis. *Curr. Opin. Neurobiol.* **1996**, *6*, 667–672.

(11) Brera, B.; Serrano, A.; de Ceballos, M. L. β-Amyloid peptides are cytotoxic to astrocytes in culture: a role for oxidative stress. *Neurobiol. Dis.* **2000**, *7*, 395–405.

(12) Huang, X.; Atwood, C. S.; Hartshorn, M. A.; Multhaup, G.; Goldstein, L. E.; Scarpa, R. C.; Cuajungco, M. P.; Gray, D. N.; Lim, J.; Moir, R. D.; Tanzi, R. E.; Bush, A. I. The A β peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *J. Biochem.* **1999**, *38*, 7609–7616.
(13) Zhu, D.; Tan, K.; Zhang, X.; Sun, A.; Sun, G.; Lee, J. Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes. *J. Cell Sci.* **2005**, *15*, 3695–3703.
(14) Mischel, R. E.; Kim, Y. S.; Sheldon, R. A.; Ferriero, D. M. Hydrogen peroxide is selectively toxic to immature murine neurons in vitro. *Neurosci. Lett.* **1997**, *231*, 17–20.
(15) Kitamura, Y.; Ota, T.; Matsuoka, Y.; Tooyama, I.; Kimura, H.; Shimohama, S.; Nomura, Y.; Gebicke-Haerter, P. J.; Taniguchi, T. Hydrogen peroxide-induced apoptosis mediated by p53 protein in glial cells. *Glia* **1999**, *25*, 154–164.
(16) Maragakis, N. J.; Rothstein, J. D. Mechanisms of disease: astrocytes in neurodegenerative disease. *Nat. Clin. Pract. Neurol.* **2006**, *2*, 679–689.
(17) Cotrina, M.; Nedergaard, M. Astrocytes in the aging brain. *J. Neurosci. Res.* **2002**, *67*, 1–10.
(18) Wilson, J. X. Antioxidant defense of the brain: a role for astrocytes. *Can. J. Physiol. Pharmacol.* **1997**, *75*, 1149–1163.
(19) Desagher, S.; Glowinski, J.; Premont, J. Astrocytes protect neurons from hydrogen peroxide toxicity. *J. Neurol. Sci.* **1996**, *16*, 2553–2562.
(20) Solfrizzi, V.; Panza, F.; Capurso, A. The role of diet in cognitive decline. *J. Neural Transm.* **2003**, *110*, 95–110.
(21) Joseph, J. A.; Shukitt-Hale, B.; Casadesus, G. Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. *Am. J. Clin. Nutr.* **2005**, *81*, 313S–316S.
(22) Frank, B.; Gupta, S. A review of antioxidants and Alzheimer's disease. *Ann. Clin. Psychiatry* **2005**, *17*, 269–286.
(23) Drukarch, B.; Schepens, E.; Stoof, J. C.; Langeveld, C. H.; Van Muiswinkel, F. L. Astrocyte-enhanced neuronal survival is mediated by scavenging of extracellular reactive oxygen species. *Free Radical Biol. Med.* **1998**, *25*, 217–220.
(24) Putievsky, E.; Ravid, U.; Dudai, N. The influence of season and harvest frequency on essential oil and herbal yields from a pure clone of sage (*Salvia officinalis*) grown under cultivated conditions. *J. Nat. Prod.* **1986**, *49*, 1015–1017.
(25) Putievsky, E.; Dudai, N.; Ravid, U. Cultivation, selection and conservation of oregano species in Israel. *Proc. IPGRI Inter. Workshop on Oregano*, Valenzano (Bari), Italy, 1996; pp 103−110.
(26) Hyslop, P. A.; Zhang, Z.; Pearson, D. V.; Phebus, L. A. Measurement of striatal H_2O_2 by microdialysis following global forebrain ischemia and reperfusion in the rat: correlation with the cytotoxic potential of H_2O_2 in vitro. *Brain Res.* **1995**, *671*, 181–186.
(27) Ravid, U.; Putievsky, E. Essential oils of Israeli wild species of Labiatae. In *Essential Oils and Aromatic Plants*, 1st ed.; Baerheim Svendsen, A., Scheffer, J. J. C., Eds.; Martinus Nijhoff/Dr. W. Junk Publishers: Dordrecht, The Netherlands, 1985; pp 155−161.
(28) Putievsky, E.; Ravid, U.; Diwan-Rinzler, N.; Zohary, D. Genetic affinities and essential oil composition of *Salvia officinalis* L., *S. fruticosa* Mill., *S. tomentosa* Mill. and their hybrids. *Flavour Fragrance J.* **1990**, *5*, 121–123.
(29) Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M. Biological effects of essential oils—a review. *Food Chem. Toxicol.* **2008**, *46*, 446–475.
(30) Tiwari, M.; Kakkar, P. Plant derived antioxidants—geraniol and camphene protect rat alveolar macrophages against t-BHP induced oxidative stress. *Toxicol. In Vitro* **2009**, *23*, 295–301.
(31) Horvathova, E.; Turcaniova, V.; Slamenova, D. Comparative study of DNA-damaging and DNA-protective effects of selected components of essential plant oils in human leukemic cells K562. *Neoplasma* **2007**, *54*, 478–483.
(32) Slamenova, D.; Horvathova, E.; Marsalkova, L.; Wsolova, L. Carvacrol given to rats in drinking water reduces the level of DNA lesions induced in freshly isolated hepatocytes and testicular cells by H_2O_2. *Neoplasma* **2008**, *55*, 394–399.
(33) Gertsch, J.; Leonti, M.; Raduner, S.; Racz, I.; Chen, J. Z.; Xie, X. Q.; Altmann, K. H.; Karsak, M.; Zimmer, A. β-Caryophyllene is a dietary cannabinoid. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 9099–9104.
(34) Chaves, J. S.; Leal, P. C.; Pianowisky, L.; Calixto, J. B. Pharmacokinetics and tissue distribution of the sesquiterpene α-humulene in mice. *Planta Med.* **2008**, *74*, 1678–1683.
(35) Liapi, C.; Anifandis, G.; Chinou, I.; Kourounakis, A. P.; Theodosopoulos, S.; Galanopoulou, P. Antinociceptive properties of 1,8-cineole and β-pinene, from the essential oil of *Eucalyptus camaldulensis* leaves, in rodents. *Planta Med.* **2007**, *73*, 1247–1254.
(36) Fernandes, E. S.; Passos, G. F.; Medeiros, R.; da Cunha, F. M.; Ferreira, J.; Campos, M. M.; Pianowski, L. F.; Calixto, J. B. Anti-inflammatory effects of compounds α-humulene and (−)-*trans*-caryophyllene isolated from the essential oil of *Cordia verbenacea*. *Eur. J. Pharmacol.* **2007**, *569*, 228–236.
(37) Passos, G. F.; Fernandes, E. S.; da Cunha, F. M.; Ferreira, J.; Pianowski, L. F.; Campos, M. M.; Calixto, J. B. Anti-inflammatory and anti-allergic properties of the essential oil and active compounds from *Cordia verbenacea*. *J. Ethnopharmacol.* **2007**, *110*, 323–333.
(38) Medeiros, R.; Passos, G. F.; Vitor, C. E.; Koepp, J.; Mazzuco, T. L.; Pianowski, L. F.; Campos, M. M.; Calixto, J. B. Effect of two active compounds obtained from the essential oil of *Cordia verbenacea* on the acute inflammatory responses elicited by LPS in the rat paw. *Br. J. Pharmacol.* **2007**, *151*, 618–627.
(39) Santos, F. A.; Silva, R. M.; Campos, A. R.; De Araujo, R. P.; Lima Junior, R. C.; Rao, V. S. 1,8-Cineole (eucalyptol), a monoterpene oxide attenuates the colonic damage in rats on acute TNBS-colitis. *Food Chem. Toxicol.* **2004**, *42*, 579–584.
(40) Juergens, U. R.; Dethlefsen, U.; Steinkamp, G.; Gillissen, A.; Repges, R.; Vetter, H. Anti-inflammatory activity of 1,8-cineole (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial. *Am. J. Respir. Med.* **2003**, *97*, 250–256.
(41) Juergens, U. R.; Engelen, T.; Racke, K.; Stober, M.; Gillissen, A.; Vetter, H. Inhibitory activity of 1,8-cineole (eucalyptol) on cytokine production in cultured human lymphocytes and monocytes. *Pulm. Pharmacol. Ther.* **2004**, *17*, 281–287.
(42) Perry, N. S. L.; Houghton, P. J.; Theobald, A.; Jenner, P.; Perry, E. K. In-vitro inhibition of human erythrocyte acetylcholinesterase by *Salvia lavandulaefolia* essential oil and constituent terpenes. *J. Pharm. Pharmacol.* **2000**, *52*, 895–902.

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