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

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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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$, superoxide anions (O$_2^-_{}$), and hydroxyl radicals (OH$_{}$). Of these, H$_2$O$_2$ 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$_2$O$_2$ through metal ion reduction in Alzheimer’s disease (11, 12). H$_2$O$_2$ 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$_2$O$_2$ decreases astrocyte membrane fluidity, induces cytoskeletal reorganization, and increases the formation of cytonemes and cell to cell tunneling nanotube (TNT)-like connections (13). H$_2$O$_2$ 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$_2$O$_2$ toxicity (19).
Epidemiological studies have shown that nutritional antioxidants may forestall the onset of dementia (20, 27). 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 H2O2 insult. The present study describes the protective effect of the essential oil from Sf and its constituents from H2O2-induced cell death of cortical astrocytes.

MATERIALS AND METHODS

Materials. 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, and fetal bovine serum (FBS) were purchased from Biological Industries (Beit Haemek, Israel). Matricaria chamomilla (ar. Plant samples of at least 5 kg were cultivated in local clayey soil under drip irrigation, as described previously (24)).

Isolation of Essential Oils. 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. Preparations were prepared from different herbs and spices on astrocytic susceptibility to H2O2. The present study describes the protective effect of the essential oil from Sf and its constituents from H2O2-induced cell death of cortical astrocytes.

Table 1. Protective Effect of Essential Oils Prepared from Different Herbs and Spices against H2O2-Induced Astrocytic Cell Death

<table>
<thead>
<tr>
<th>plant family</th>
<th>plant species</th>
<th>protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asteraceae</td>
<td>Achillea fragrantissima (Forsk.) Sch. Bip.</td>
<td>0 ± 5</td>
</tr>
<tr>
<td></td>
<td>Artemisia arborescens L.</td>
<td>20 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Artemisia dracunculus L.</td>
<td>0 ± 7</td>
</tr>
<tr>
<td></td>
<td>Artemisia judaica L.</td>
<td>7 ± 6</td>
</tr>
<tr>
<td></td>
<td>Artemisia herba alba L.</td>
<td>0 ± 7</td>
</tr>
<tr>
<td></td>
<td>Matricaria chamomilla L.</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>Geraniaceae</td>
<td>Pelargonium graveolens L.'Her.</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>Lamiaceae</td>
<td>Ocimum canum Sims.</td>
<td>21 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Origanum majorana L.</td>
<td>30 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Origanum dayi Poel</td>
<td>0 ± 5</td>
</tr>
<tr>
<td></td>
<td>Rosmarinus officinalis L.</td>
<td>15 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Salvia domonica L.</td>
<td>13 ± 5</td>
</tr>
<tr>
<td></td>
<td>Salvia sclarea L.</td>
<td>20 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Salvia fruticosa Mill.</td>
<td>51 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Thymus vulgaris L.</td>
<td>20 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Myrtaceae</td>
<td>Myrtus communis L.</td>
<td>0 ± 8</td>
</tr>
<tr>
<td>Apiaceae</td>
<td>Apium graveolens L.</td>
<td>0 ± 6</td>
</tr>
<tr>
<td></td>
<td>Carum carvi L.</td>
<td>7 ± 7</td>
</tr>
<tr>
<td></td>
<td>Foeniculum vulgare Mill.</td>
<td>21 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Petroselinum crispum Mill.</td>
<td>21 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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

RESULTS

Screening the Effect of Essential Oils of Various Herbs and Spices on Astrocytic Susceptibility to Hydrogen Peroxide. To determine the astrosective potential of the various essential oils, they were screened against H2O2-induced astrocytic cell death. The results are presented in Table 1. As shown, the majority of the oils and their constituents showed significant protective activity against H2O2-induced cell death, with some oils showing activity as high as 97%.

Data Analysis. Statistical analyses were performed with one-way ANOVA followed by multiple-comparison tests using GraphPad InStat 3 for windows (GraphPad Software, San Diego, CA).
oils, we used a model in which oxidative stress is caused by the intravitro addition of H₂O₂ 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 H₂O₂ (150 μM) used in our experiments was reported by Hyslop et al. to be the concentration of H₂O₂ 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 H₂O₂. 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 H₂O₂. Cell viability was assessed 20 h after H₂O₂ addition using the LDH assay. The distribution of the essential oils according to their extent of astroprotection is presented in Figure 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 S. fruticosa exhibited the highest protective activity (51 ± 3%) against the H₂O₂ insult.

Effects of S. fruticosa Oil on Astrocytic Susceptibility to Hydrogen Peroxide. We further examined the optimal conditions, in terms of time and dose, for S. fruticosa oil to exert its protective effect. To elucidate the optimal time point for the addition of the S. fruticosa oil with respect to H₂O₂ insult, the cells were either pre-incubated in the presence of the oil for 1 or 2 h before the addition of H₂O₂ or treated 1 or 2 h after the addition of H₂O₂. The results, presented in Figure 1, demonstrated that S. fruticosa oil acts more efficiently when added 1–2 h before the H₂O₂ insult. To find the optimal concentration of the oil needed for its protective effect, astrocytes were pre-incubated with different concentrations of S. fruticosa essential oil. H₂O₂ was then added, and cytotoxicity was determined after 20 h. Our results show (Figure 2) that S. fruticosa oil protects against H₂O₂-induced cell death in a dose-dependent manner. No significant changes were observed in the viability of cells treated with similar concentrations of S. fruticosa essential oil in the absence of H₂O₂.

Protective Effect of the Pure Constituents of S. fruticosa Oil. The essential oil of S. fruticosa is composed of various compounds at different concentrations (27, 28, and Supporting Information). Thus, to identify the active compound(s), which may be responsible for the protective effect of S. fruticosa 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 S. fruticosa Oil Components into Astrocytes. To gain more insight into the mechanism by which the S. fruticosa oil and its constituents exert their protective effect, we tested whether S. fruticosa oil components are incorporated into astrocytes. For that purpose, we incubated the cells with S. fruticosa 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 S. fruticosa 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-caryophyllene and α-humulene were significantly higher (∼7-fold) in astrocytes than in the

**Figure 1.** Pre-incubation of astrocytes with the essential oil of S. fruticosa is needed to exert its protective effect from H₂O₂ cytotoxicity. The essential oil of S. fruticosa (40 μg/mL) was added to astrocytes before (−2 h, −1 h) or after (1 h, 2 h) the addition of H₂O₂ (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 H₂O₂-induced astrocytic cell death by different concentrations of the essential oil of S. fruticosa. Astrocytes were pre-incubated for 2 h with different concentrations of the essential oil of S. fruticosa and treated with H₂O₂ (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.

**Table 2.** Protective Effect of the Main Constituents of S. fruticosa against H₂O₂-Induced Astrocytic Cell Death

<table>
<thead>
<tr>
<th>compd</th>
<th>LRI</th>
<th>% of compd in the natural oil</th>
<th>concn of compd in the expls (μg/mL)</th>
<th>protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-cineole</td>
<td>1031</td>
<td>26.4</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>camphor</td>
<td>1149</td>
<td>18.9</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>camphene</td>
<td>949</td>
<td>9.5</td>
<td>4</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>α-thujone</td>
<td>1108</td>
<td>9.1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>trans-β-caryophyllene</td>
<td>1418</td>
<td>5</td>
<td>2</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>α-pinene</td>
<td>931</td>
<td>4.4</td>
<td>2</td>
<td>69 ± 7</td>
</tr>
<tr>
<td>β-pinene</td>
<td>976</td>
<td>4.7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>α-humulene</td>
<td>1456</td>
<td>3.9</td>
<td>1.5</td>
<td>50 ± 9</td>
</tr>
</tbody>
</table>

*Results represent the mean ± SEM of three experiments, each performed in quadruplicates. Linear retention index on an Rtx-5SIL MS column. Concentrations were chosen on the basis of the calculation as if the cells were treated with 40 μg/mL S. fruticosa oil. Statistically significant difference (p < 0.05) between the tested compounds and the corresponding control.
three similar experiments, each performed in quadruplicates. Asterisks were incubated for 2 h with different concentrations of α-humulene and α-pinene and were then treated with H$_2$O$_2$ (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.

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$_2$O$_2$ 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 \emph{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 \emph{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 astro-protection by all of the tested constituents is much higher than that of the protective effect of the \emph{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 \emph{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, \emph{trans}-\beta-carophyllene, 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, \alpha-humulene was found in the brain 0.5 h after oral administration (34), and 1,8-cineole and \beta-pinene were found to possess antiinociceptive effects in rodents (35). In addition, our results show that \alpha-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 \emph{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 \emph{Sf} oil have various complementary activities that might be beneficial for the treatment of such diseases. For example, \alpha-humulene and \emph{trans}-\beta-carophyllene, 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 \emph{trans}-\beta-carophyllene 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 \emph{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 \emph{Sf} oil is the inhibitory activity of 1,8-cineole and \alpha-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 \emph{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 \emph{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 \emph{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; \emph{Sf}, Salvia fruticosa Mill.; MTBE, methyl tert-butyl ether.

**SAFETY**

MTBE is highly flammable and is harmful if 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**


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