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# Antioxidative Activity of Volatile Extracts Isolated from *Angelica tenuissimae* Roots, Peppermint Leaves, Pine Needles, and Sweet Flag Leaves

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Volatile extracts were isolated from dried medicinal plants [*Angelica tenuissimae* roots (AT, *Angelica tenuissima* Nakai), peppermint leaves (PL, *Mentha arvensis* L.), pine needles (PN, *Pinus sylvestris* L.), and sweet flag leaves (SF, *Acorus gramineus* Rhizoma)] using steam distillation under reduced pressure, followed by continuous liquid−liquid extraction (DRP−LLE). The extracts were then analyzed by gas chromatography (GC) and gas chromatography−mass spectrometry (GC−MS). The major volatile constituents of AT, PL, PN, and SF were 3-butylidene-4,5-dihydrophthalide (32 mg/g), menthol (18 mg/g), thunbergol (2.1 mg/g), and *cis*-asarone (37 mg/g), respectively. The inhibitory activity (%) of the extracts against hexanal oxidation ranged from 33 to 98% at a level of 50 $\mu$g/mL. Among the volatile extracts, PL and PN increased cell viabilities by 10 and 24%, respectively, at a dose of 1 $\mu$g/mL, compared to that of $H_2O_2$-treated brain neuroblastoma cells, SK-N-SH. However, a 20% reduction in the malonaldehyde level, an index for lipid peroxidation, was observed at only 1 $\mu$g/mL concentration of PN.

**KEYWORDS: Medicinal plants; antioxidant activity; volatile extracts; *Angelica tenuissima* Nakai; *Mentha arvensis* L.; *Pinus sylvestris* L.; *Acorus gramineus* Rhizoma**

## INTRODUCTION

Naturally occurring antioxidants in plants are classified as vitamins, phenolic compounds, and volatile compounds of herbs and spices. Among them, herbs and spices have received much attention as preventive medicines because they have beneficial health effects such as antioxidant and antibacterial activity. They are also used as flavor-boosting agents and food preservatives. *Angelica tenuissimae* roots (AT, *Angelica tenuissima* Nakai), peppermint leaves (PL, *Mentha arvensis* var. piperascens), pine needles (PN, *Pinus sylvestris* L.), and grassleaf sweet flag leaves (SF, *Acorus gramineus* Rhizoma) are traditional medicinal plants in East Asian countries such as Korea and China and are known to have a lot of volatile compounds (*1*). The essential oil of AT, in which 3-butylidenephthalide and 3-butylidene-4,5-dihydrophthalide were found to be major components, has been used as a medicine to stop pain and provide relief from female diseases (*2*). The volatile extract of PL is used as an ingredient in various foods, as an antibacterial agent and as a promoter of gas secretion (*3*). In East Asia, parts of the pine tree such as pine needles, cones, cortices, and pollen are widely consumed as an ingredient in folk medicines or as various dietary supplements (*4*). The essential oil of pine needle (*Pinus rigida* Mill or *Pinus densiflora*) is widely employed as an ingredient in several types of perfumes and deodorants (*5*). Red pine needles are especially used as a nourishing tonic drug and tea in Korea (*6*). SF leaves are listed in *Korean Pharmacopoeia* as a sedative, digestive, analgesic, diuretic, and antifungal agent (*7*). In addition, oriental medicine practitioners consider that sweet flag leaves have beneficial effects in the treatment of strokes and in the improvement of learning and memory (*8*, *9*).

Reactive oxygen species (ROS) may be the causative factor involved in many human degenerative diseases, and antioxidants have been found to have some degree of preventive and therapeutic effects on these disorders (*10*). Hydrogen peroxide ($H_2O_2$), one of the main ROS, causes lipid peroxidation and DNA oxidative damage in cells (*11*). Brain neurons are particularly vulnerable to $H_2O_2$ because of their relatively low levels of antioxidant enzymes and dependence on mitochondrial respiration (*12*). Although numerous studies have been carried out to investigate the health benefits and volatile component analysis of extracts isolated from medicinal plants (*13*−*15*), investigation of their antioxidant activities and cytoprotective effects have seldom been done.

In the present study, we isolated the volatile extracts from the four medicinal plants listed above and characterized their volatile compounds in terms of antioxidant activity. In addition, the cytoprotective characteristics of the volatile extracts against

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$H_2O_2$ exposure in brain neuroblastoma cells, SK-N-SH, were tested and measured.

## MATERIALS AND METHODS

**Materials.** Dried *Angelica tenuissimae* roots, peppermint leaves, pine needles, and sweet flag leaves were purchased at a local oriental herbal pharmacy (Kyung-Dong market) in Seoul, Korea. They were identified by Prof. Byung-Soo Koo, College of Oriental Medicine, Dongguk University, Kyeongju, Korea.

**Chemicals.** Hexanal (99%), α-tocopherol (vitamin E; 95%), 2,6-di-*tert*-butyl-4-methylphenol (BHT; 99%), 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and hydroxypropyl-β-cyclodextrin were purchased from Sigma Chemical Co. (St. Louis, MO). The protein assay kit was obtained from Pierce Chemical Co. (Rockford, IL). Fetal bovine serum, minimum essential media (MEM), and antibiotics were purchased from Life Technologies, Inc. (Grand Island, NY). Undecane (99%) and various reference chemicals used for identifying the constituents were bought from Aldrich Chemical Co. (Milwaukee, WI). Dichloromethane was obtained from Junsei Chemical Co., Ltd. (Tokyo, Japan). All other chemicals were analytical-grade.

**Isolation of Volatile Constituents by Steam Distillation under Reduced Pressure Followed by Continuous Liquid−Liquid Extraction (DRP−LLE).** Dried medicinal plants (20 g) were placed in a 3 L round-bottom flask with 1 L of deionized water. The solution was steam-distilled at 55 °C for 3 h under reduced pressure (95 mmHg). The distillate (900 mL) was subjected to continuous liquid−liquid extraction for 6 h using 100 mL of dichloromethane. After the extract was dried over anhydrous sodium sulfate, the solvent was removed in a rotary flash evaporator. The distillation was stopped when the volume of extract was reduced to approximately 1 mL. The extract was transferred to a vial; the distillation flask was washed with a minimum amount of dichloromethane; and the washings were added to the vial. The solvent was further removed under a purified nitrogen stream until the volume was reduced to 0.4 mL. These concentrated extracts were used to identify volatile constituents and to determine antioxidant activities in the aldehyde/carboxylic acid assays.

**Determination of Total Volatile Constituents in Extracts.** The mass of extract was determined according to a method developed by Lee and Shibamoto (*16*). The extract was then analyzed by GC with a mass selective detector (GC−MS) to determine the percentage of the total peak area of volatile components and solvent. The total mass of volatile components was calculated by multiplying the percentage representing the total peak area of components by the total mass of the extract. Each experiment was repeated twice. The detector response to the solvent was found to be linear over a range of 0.2−1.0 μL injected, with a $R^2$ value of 0.99.

**Identification and Quantification of Volatile Constituents Isolated from Extracts.** Volatile constituents obtained by DRP−LLE were identified by comparison of the compound's Kovats index, *I* (*17*), mass spectrum with that of a reference standard, and mass fragmentation pattern of authentic constituents. The volatile components were also matched by co-injection with authentic compounds. The mass of each volatile component was determined according to the previously reported article (*16*). The average concentration of each chemical was calculated using the following equation:

$$\text{concentration } (\mu\text{g/g}) = \frac{\text{weight of extract (without solvent)} \times \text{GC peak area \%/100 } (\mu\text{g})}{\text{weight of herbs (20 g)}}$$

**Aldehyde/Carboxylic Acid Assay.** The inhibitory effect of volatile extracts on the oxidation of aldehyde to carboxylic acid was determined according to previously published reports (*18*, *19*). Various amounts of volatile extracts were added to 2 mL of a dichloromethane solution of hexanal (3 mg/mL) containing 0.2 mg/mL of undecane as a GC internal standard. The oxidation of the sample solution was initiated by heating at 60 °C for 10 min in a sealed vial, which was then stored at room temperature. The headspace of each vial was purged with pure oxygen (1.5 L/min, 3 s) every 24 h for the first 10 days. The decrease in hexanal was monitored at 5-day intervals. Standards of BHT and α-tocopherol were also examined for their antioxidative activity using the same methodology. The quantitative analysis of hexanal was conducted according to an internal standard method (*20*). All analyses were performed in triplicate.

**Instrumental Analysis.** A HP model 6890 gas chromatograph coupled to a HP 5973 quadruple mass spectrometer (capillary direct interface) was used for determination of the Kovats index and identification of volatile constituents. A 30 m × 0.32 mm i.d. ($d_f$ = 0.25 μm) DB-WAX bonded-phase fused-silica capillary column (J and W Scientific, Folsom, CA) was used. The linear velocity of the helium carrier gas was 44 cm/s at a splitless mode. MS was operated at a scan range of *m*/*z* 50−550, an ionization voltage of 70 eV, an ion source temperature of 280 °C, and a scanning rate of 2.94 scans/s.

A HP model 5890 GC equipped with a 30 m × 0.32 mm i.d. ($d_f$ = 0.25 μm) HP-1 bonded-phase fused-silica capillary column and a FID was used for hexanal analysis. The column flow of the helium carrier gas was 1.5 mL/min at a split ratio of 1:20. The injector and the detector temperatures were 280 and 300 °C, respectively. The oven temperature was programmed from 40 (2 min isothermal) to 180 °C at 5 °C/min.

**Protective Potential of Volatile Extracts Against $H_2O_2$-Induced Cytotoxicity.** The human neuroblastoma cell line, SK-N-SH, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in MEM (minimal essential medium) with phenol red supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics. The culture medium was changed every 2−3 days, and cells were trypsinized and subcultured at near confluence every 5−7 days at a split ratio of ∼1:4−1:6. To evaluate the effects of volatile extracts against $H_2O_2$-induced cytotoxicity, cells were preincubated with varying concentrations of volatile extracts or dimethyl sulfoxide (DMSO) for 24 h and then were incubated for another 16 h together with volatile extracts and 200 μM $H_2O_2$ in a fresh medium under 5% $CO_2$ and saturated humidity at 37 °C. The cell viabilities were determined by the conversion capacity of the viable cells from MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) to its formazan (*21*). The protective potential of volatile extracts against $H_2O_2$-induced cytotoxicity was expressed as follows: protective potential (%) = [A/B] × 100, where A and B are viabilities of sample-treated and $H_2O_2$-exposed cells and of $H_2O_2$-exposed cells alone, respectively. All experiments were performed a minimum of 3 times.

**Lipid Peroxidation in SK-N-SH Cells.** After treatment with volatile extracts for 24 h and 100 μM $H_2O_2$ for 16 h, cells were washed twice with PBS (0.01 M phosphate-buffered saline solution at pH 7.4) and then collected in a 1.5 mL Eppendorf tube containing 0.25 mL of PBS. The collected cells were centrifuged (1000*g* for 30 min). The pellets were resuspended in 1 mL of PBS containing 0.002% butylated hydoxy toluene (BHT) and recentrifuged at 1000*g* for 3 min. The pellet was used to quantify lipid peroxides using the thiobarbituric-acid-reactive substance (TBARS) assay (*22*). Briefly, cell homogenates (prepared in 0.5 mL PBS with 1% SDS) were mixed with 1 mL of 0.67% thiobarbituric acid in 10% trichloroacetic acid and 0.04% BHT in glass test tubes, and the mixtures were incubated in a boiling water bath for 30 min. After the tubes were cooled in ice, 1.5 mL of *n*-butanol was added and the reaction mixture was centrifuged at 1000*g* for 10 min. The fluorescence of the supernatant was determined at an excitation length of 515 nm and at an emission length of 553 nm by the fluorometer (JASCO FP 550, Japan). The concentrations of TBARS were calculated using tetramethoxypropane as a reference standard and were normalized to the protein content (*22*). A total of 8 μg/mL of SAC (*S*-allyl-L-cystein) was used as a positive control.

## RESULTS AND DISCUSSION

**Measurement and Identification of Total Volatile Constituents in Extracts.** The yields of total volatile components for AT, PL, PN, and SF were 3.16, 3.48, 2.51, and 4.41% (w/w), respectively. The masses of total volatile components isolated from the 20 g of AT, PL, PN, and SF were 633, 695, 502, and 883 mg, respectively. The values are averages of values ($n$ = 2). **Table 1** shows the components identified and their concentrations. Over ∼31−60 peaks were observed in the GC

**Table 1.** Volatile Compounds Identified in Extracts from *Angelica*, Peppermint, Pine Needles, and Sweet Flag

| compound | I[b] | concentration (μg/g)[a] AT[c] | PL[d] | PN[e] | SF[f] | identification |
|---|---|---|---|---|---|---|
| alcohols | | | | | | |
| neomenthol | 1570 | | 717 | | | RI[g], MS[h] |
| terpinen-4-ol | 1592 | | | 61 | 11 | RI[g], MS[h], Co-GC[i] |
| β-terpineol | 1615 | | | 64 | | RI[g], MS[h] |
| menthol | 1636 | 50 | 18 379 | 76 | 32 | RI[g], MS[h], Co-GC[i] |
| pinocarveol | 1637 | | | 472 | | MS[h] |
| furfuryl alcohol | 1657 | | | | 4 | RI[g], MS[h], Co-GC[i] |
| trans-verbenol | 1665 | | | 692 | | MS[h] |
| borneol | 1690 | | | 428 | 109 | RI[g], MS[h] |
| *p*-mentha-1,5-diene-8-ol | 1714 | | | 71 | | RI[g], MS[h] |
| myrtenol | 1776 | | | 157 | | RI[g], MS[h] |
| *trans*-carvenol | 1820 | | | 95 | | RI[g], MS[h], Co-GC[i] |
| *p*-cymene-8-ol | 1832 | | | 670 | | RI[g], MS[h] |
| benzyl alcohol | 1862 | | | | 36 | RI[g], MS[h], Co-GC[i] |
| 2-phenylethyl alcohol | 1891 | | 138 | 139 | | RI[g], MS[h], Co-GC[i] |
| β-eudesmol | 2021 | | | | 50 | RI[g], MS[h] |
| 1,6-germacradien-5-ol | 2030 | | 53 | | | MS[h] |
| elemol | 2055 | 8 | | | | RI[g], MS[h] |
| *p*-cymene-7-ol | 2076 | | | 115 | | RI[g], MS[h] |
| spathulenol | 2088 | 625 | | 1158 | | RI[g], MS[h] |
| eugenol | 2144 | | 145 | | | RI[g], MS[h], Co-GC[i] |
| α-cadinol | 2149 | | 71 | 158 | | RI[g], MS[h] |
| thymol | 2175 | | 126 | | | RI[g], MS[h], Co-GC[i] |
| *iso*-spathulenol | 2186 | 118 | | | | RI[g], MS[h] |
| muurolol | 2208 | | 208 | 503 | | RI[g], MS[h] |
| caryophylla-3,8-dien-5-ol | 2353 | | | 309 | | MS[h] |
| 4-vinyl phenol | 2358 | | | 138 | | RI[g], MS[h], Co-GC[i] |
| thunbergol | 2498 | | | 2129 | | RI[g], MS[h] |
| aldehydes | | | | | | RI[g], MS[h] |
| furfural | 1454 | 67 | | | 83 | RI[g], MS[h], Co-GC[i] |
| campholene aldehyde | 1472 | | | 53 | | MS[h] |
| 5-methyl furfural | 1559 | | | 91 | 29 | RI[g], MS[h] |
| myrtenal | 1597 | | | 118 | | RI[g], MS[h] |
| cuminaldehyde | 1751 | | | 30 | | RI[g], MS[h] |
| 5-hydroxymethyl furfural | 2469 | | | | 33 | RI[g], MS[h] |
| esters | | | | | | RI[g], MS[h] |
| menthyl acetate | 1539 | | 2439 | | | RI[g], MS[h] |
| linalyl acetate | 1552 | 28 | | | | RI[g], MS[h] |
| bornyl acetate | 1564 | | | 2069 | | RI[g], MS[h] |
| methyl eugenol | 1992 | | | 505 | | RI[g], MS[h], Co-GC[i] |
| menthyl jasmonate | 2303 | | 38 | | | MS[h] |
| allyl phenoxyacetate | 2556 | 850 | | | | MS[h] |
| benzyl benzoate | 2581 | | | | 66 | RI[g], MS[h] |
| methyl dehydroabietate | 2654 | | | 161 | | MS[h] |
| ketones | | | | | | RI[g], MS[h] |
| menthone | 1448 | 59 | 2323 | 70 | | RI[g], MS[h], Co-GC[i] |
| pinocarvone | 1543 | | | 115 | | RI[g], MS[h] |
| pulegone | 1622 | 36 | | | | RI[g], MS[h] |
| verbenone | 1676 | | | 338 | | RI[g], MS[h] |
| *cis*-piperitone oxide | 1680 | | 282 | | | RI[g], MS[h] |
| piperitone | 1705 | | 4656 | | | RI[g], MS[h] |
| 2-hydroxypiperitone | 1784 | | 40 | | | RI[g], MS[h] |
| *cis*-jasmone | 1915 | | 65 | | | RI[g], MS[h] |
| 2-acetylpyrrole | 1943 | | | | 30 | RI[g], MS[h], Co-GC[i] |
| 5-methoxy-2,8,8-trimethyl-dipyran-4-one | 2329 | | | 583 | | RI[g], MS[h] |
| 3-butylidene phthalide | 2450 | 1603 | | | | RI[g], MS[h] |
| 3-butylidene-4,5-dihydrophthalide (ligustilide) | 2521 | 32 673 | | 241 | | RI[g], MS[h] |
| terpenoids | | | | | | RI[g], MS[h] |
| bicycloelemene | 1471 | 120 | | | | RI[g], MS[h] |
| β-elemene | 1575 | 166 | | | | RI[g], MS[h], Co-GC[i] |
| β-caryophyllene | 1577 | | | 617 | 62 | RI[g], MS[h] |
| α-humulene | 1647 | | | 193 | 15 | RI[g], MS[h], Co-GC[i] |
| *trans*-β-fernesene | 1660 | 53 | | | | RI[g], MS[h] |
| germacrene D | 1683 | 55 | 61 | | | RI[g], MS[h] |
| β-selinene | 1693 | | | 165 | | RI[g], MS[h] |
| α-muurolene | 1706 | | | 58 | 35 | RI[g], MS[h] |
| zingiberene | 1708 | 180 | | | | RI[g], MS[h] |
| bicyclogermacrene | 1711 | 619 | | | | RI[g], MS[h] |
| β-bisabolene | 1714 | 53 | | | | RI[g], MS[h] |
| δ-cadinene | 1739 | 76 | | | | RI[g], MS[h] |
| β-sesquiphellandrene | 1752 | 188 | | | | RI[g], MS[h] |
| curcumene | 1756 | 25 | | | | RI[g], MS[h] |
| calamenene | 1806 | | 40 | | | RI[g], MS[h] |
| caryophyllene oxide | 1949 | | | 2133 | | RI[g], MS[h] |
| tetramethyl bicyclo(2.2.2.)oct-2-ene | 1964 | | | 126 | | RI[g], MS[h] |
| cadina-1,4-diene | 2037 | | | 64 | | RI[g], MS[h] |
| dehydroabietan | 2446 | | | 14 | | RI[g], MS[h] |

**Table 1 (Continued)**

| compound | I[b] | concentration (μg/g)[a] AT[c] | PL[d] | PN[e] | SF[f] | identification |
|---|---|---|---|---|---|---|
| miscellaneous compounds | | | | | | RI[g], MS[h] |
| 1-isopropyl-2-methoxy-4-methyl benzene | 1579 | | | 186 | | RI[g], MS[h] |
| euasarone | 2207 | | | | 1674 | RI[g], MS[h], Co-GC[i] |
| dihydroactinidiolide | 2280 | | 66 | 153 | | RI[g], MS[h] |
| *cis*-asarone | 2318 | 36 | 32 | | 37 307 | RI[g], MS[h], Co-GC[i] |
| decanoic acid | 2367 | | | 160 | | RI[g], MS[h] |
| *trans*-asarone | 2445 | | | | 2380 | RI[g], MS[h], Co-GC[i] |
| dodecanoic acid | 2465 | | | 238 | | RI[g], MS[h], Co-GC[i] |
| tridecanoic acid | 2676 | | | 604 | | RI[g], MS[h], Co-GC[i] |

[a] Solvent peak excluded. [b] *I* means Kovats retention index on DB-WAX. [c] AT = *Angelica tenuissimae* roots. [d] PL = peppermint leaves. [e] PN = pine needles. [f] SF = sweet flag leaves. [g] Identification based in retention index. [h] Identification based on comparison of mass spectra. [i] Identification based on co-injection with authentic compounds.

chromatograms of the extracts. Among them, 21, 21, 46, and 21 constituents were identified and quantified in the extracts of AT, PL, PN, and SF, respectively. The identified volatiles included 27 alcohols, 6 aldehydes, 10 esters, 1 heterocyclic compound, 15 ketones, 23 terpene hydrocarbons, and 8 miscellaneous compounds.

The most abundant keton of AT was 3-butylidene-4,5-dihydrophthalide (ligustilide, 33 mg/g). Other identified ketones were 3-butylidene phthalide (1.6 mg/g), *l*-menthone (60 μg/g), and pulegone (36 μg/g). According to a previous report, ligustilide and phthalides are key components in the methanol extract of AT grown in both Korea and Japan (*2*). Two esters, allyl phenoxyacetate (0.85 mg/g) and linanyl acetate (28 μg/g), were also identified. A total of 10 terpene hydrocarbons were found. Four alcohols were identified: spathulenol (0.63 mg/g), *iso*-spathulenol (0.12 mg/g), menthol (50 μg/g), and elemol (8.7 μg/g).

The most abundant alcohol of PL was menthol (18 mg/g). Menthol was identified in all four plants used in this study. Other alcohols identified in the extract were neomenthol (0.72 mg/g), muurolol (0.21 mg/g), eugenol (0.15 mg/g), and thymol (0.13 mg/g). Seven ketones were also found in PL. Two esters, menthyl acetate (2.4 mg/g) and menthyl jasmonate (39 μg/g), were identified. In addition, three terpene hydrocarbons were identified: dihydroactinidiolide (66 μg/g), germacrene D (62 μg/g), and calamenene (40 μg/g). In a previous report, menthol, 1,8-cineol, and myrcene were identified as key volatile components in peppermint (*Mentha piperita*) (*23*).

The most abundant terpene hydrocarbon of PN was caryophyllene oxide (2.1 mg/g). Other terpene hydrocarbons were β-caryophyllene (0.6 mg/g), δ-cadinene (0.59 mg/g), and α-humulene (0.19 mg/g). Thunbergol (2.1 mg/g) and spathulenol (1.2 mg/g) were the dominant alcohols among 18 alcohols identified. Three esters were identified: bornyl acetate (2.1 mg/g), methyl eugenol (0.51 mg/g), and methyl dehydroabietate (0.16 mg/g). Five ketones were identified: 5-methoxy-2,8,8-trimethyl-dipyran-4-one (0.58 mg/g), verbenone (0.34 mg/g), ligustilide (0.24 mg/g), pinocarvone (0.12 mg/g), and *l*-menthone (70 μg/g). Four aldehydes were also identified: myrtenal (0.12 mg/g), 5-methylfurfural (91 μg/g), campholene aldehyde (53 μg/g), and cuminaldehyde (31 μg/g). Furthermore, four miscellaneous compounds, tridecanoic acid (0.60 mg/g), dodecanoic acid (0.24 mg/g), decanoic acid (0.16 mg/g), and octanoic acid (73 mg/g), were identified. According to previously reported studies (*4*, *24*), α-pinene and limonene were identified in pine needles, but they were not identified in the present study. In my opinion, these most volatile components were lost during the distillation−extraction−concentration procedure because of their low molecular weight. However, other volatile chemicals identified in PN, such as caryophyllene, terpinene-4-ol, terpineol, and cadinene, were also found in this study. Concentrations of phenolic compounds such as thunbergol and spathulenol were high in PN, which accounts for its potent antioxidant activity.

**Table 2.** Inhibitory Effects (%) of Different Amounts of Volatile Extracts, α-Tocopherol, and BHT on the Oxidation of Hexanal throughout a Storage Period of 30 Days at Room Temperature

| concentration (μg/mL) | inhibitory effect[a] (%) AT[b] | PL[b] | PN[b] | SF[b] | α-tocopherol | BHT |
|---|---|---|---|---|---|---|
| 0 | 11 ± 0.7 | 11 ± 3.2 | 12 ± 2.3 | 7 ± 6.2 | 10 ± 4.2 | 10 ± 4.2 |
| 50 | 33 ± 12 | 90 ± 2.9 | 93 ± 3.6 | 86 ± 5.4 | 91 ± 8.8 | 89 ± 5.8 |
| 100 | 33 ± 6.1 | 84 ± 4.3 | 95 ± 5.3 | 91 ± 9.0 | 98 ± 0.2 | 100 ± 0.1 |
| 200 | 88 ± 12 | 87 ± 7.4 | 99 ± 2.8 | 91 ± 7.7 | 99 ± 1.3 | 100 ± 0.1 |
| 500 | 88 ± 9.7 | 85 ± 3.6 | 100 ± 0.3 | 92 ± 2.8 | 100 ± 1.5 | 100 ± 0.1 |

[a] The values are mean ± standard deviation ($n = 3$). [b] AT, PL, PN, and SF are abbreviations of *Angelica tenuissimae* roots, peppermint leaves, pine needles, and sweet flag leaves, respectively.

The most abundant miscellaneous compound in SF was *cis*-asarone (37 mg/g). Other miscellaneous compounds were *trans*-asarone (2.4 mg/g), euasarone (1.6 mg/g), and 1-(2-hydroxy-4-methoxyphenyl) ethanone (72 μg/g). Alcohols such as borneol (0.11 mg/g) and β-eudesmol (50 μg/g) were identified, as well as four terpene hydrocarbons, three aldehydes, and three esters. The volatiles found in SF, such as β-asarone, α-asarone, and caryophyllene, were consistent with the findings of previous reports (*25*, *26*).

**Antioxidant Activity of Volatile Extracts in the Aldehyde/Carboxylic Acid Assay.** The aldehyde/carboxylic acid assay is a simple and robust assay for measuring the antioxidant activity of volatile chemicals or extracts. This method is based on the autoxidation of aldehydes to carboxylic acids with active oxygen species such as a hydroxyl radical (*27*, *28*). In this assay, volatile extracts dissolved in organic solvent, such as dichloromethane, can easily be measured for antioxidant potential. This assay is also suitable for evaluating the long-term antioxidant potential of natural antioxidants because either aldehyde oxidation or carboxylic acid formation is monitored for 30 days. This method has been used to measure the antioxidant activity of natural volatile extracts isolated from beans, herbs, and spices (*18*). Moreover, the method has been validated with various volatile chemicals and with typical antioxidants such as BHT and α-tocopherol (*19*). The percentage of hexanal remaining in solutions treated with different amounts of volatile extract, α-tocopherol, and BHT is shown in **Table 2**. At concentrations up to 50 μg/mL, the inhibitory effect of the extracts on the formation of hexanoic acid was seen in the following descending order: PN > PL > SF > AT. After 30 days, the control samples exhibited over 89% oxidation of hexanal to hexanoic acid. All

**Table 3.** Effect of Several Volatile Extracts Against $H_2O_2$-Induced Cytotoxicity in SK-N-SH

| sample[a] | concentration ($\mu$g/mL) | absorbance | relative viability to $H_2O_2$-treated |
|---|---|---|---|
| control | | 0.78 ± 0.03[b] | |
| AT + $H_2O_2$ (200 $\mu$M) | 0 | 0.42 ± 0.01 | 100.0 ± 2.2 |
| | 0.1 | 0.41 ± 0.02 | 98.6 ± 3.9 |
| | 1 | 0.42 ± 0.02 | 101.3 ± 5.2 |
| PL + $H_2O_2$ (200 $\mu$M) | 0 | 0.44 ± 0.02 | 100.0 ± 5.3 |
| | 1 | 0.49 ± 0.02 | 110.4 ± 3.4 |
| | 10 | 0.47 ± 0.02 | 107.1 ± 5.1 |
| PN + $H_2O_2$ (200 $\mu$M) | 0 | 0.43 ± 0.01 | 100.0 ± 2.7 |
| | 0.1 | 0.48 ± 0.02 | 110.8 ± 3.9 |
| | 1 | 0.49 ± 0.01 | 123.8 ± 1.4 |
| SF + $H_2O_2$ (200 $\mu$M) | 0 | 0.45 ± 0.03 | 100.0 ± 6.5 |
| | 1 | 0.41 ± 0.02 | 89.9 ± 3.9 |
| | 10 | 0.33 ± 0.01 | 72.0 ± 3.0 |

[a] Samples: AT, PL, PN, and SF are abbreviations of *Angelica tenuissimae* roots, peppermint leaves, sweet flag leaves, and pine needles, respectively. [b] The value was representative (mean ± standard deviation, $n = 3$) of a minimum of five independent experiments.

volatile extracts from the medicinal plants inhibited hexanal oxidation by over 87% at concentrations up to 200 $\mu$g/mL. At all concentrations of the volatile extracts from PN, over 90% of hexanal remained in the samples, which means that the PN extract possessed potent antioxidant activity against hexanal oxidation. In the case of PL, phenolic compounds such as eugenol and thymol contribute to the antioxidant activity. However, it is hard to explain the potent antioxidant activity of PN with identified volatile components. At a concentration of 100 $\mu$g/mL, the antioxidant activities of PN and SF were almost equal to that of α-tocopherol. The inhibitory effect of AT was 33 and 88% inhibition at concentrations of 50 and 200 $\mu$g/mL, respectively.

**Protective Potential against $H_2O_2$-Induced Cytotoxicity and Lipid Peroxidation.** The protective effect of volatile extracts on the survival of $H_2O_2$-treated brain neuroblastoma cells, SK-N-SH, was measured. The relative cell survival of SK-N-SH cells decreased in response to treatment with various concentrations of $H_2O_2$ for 16 h, with an average $IC_{50}$ value of about 200 $\mu$M (data not shown). Treatments with PL and PN extract for 24 h prior to the addition of 200 $\mu$M of $H_2O_2$ induced a slight increase in cell survival (**Table 3**). At a dose of 1 $\mu$g/mL, PL increased cell viability by 10%, compared to control levels. The viabilities of cells pretreated with PN at 0.1 and 1 $\mu$g/mL were 111 and 124% of the control value, respectively. However, AT and SF, which inhibited hexanal oxidation, did not lead to any increase in cell survival. We also tested whether volatile extracts could inhibit the formation of malondialdehyde (MDA), as a convenient index for determining the extent of lipid peroxidation, in $H_2O_2$-treated SK-N-SH cells. As shown in **Figure 1**, PN and SAC (*S*-allyl-L-cystein) were active in the inhibition of lipid peroxidation. A 20 and 27% reduction in the MDA level were observed at a 1 $\mu$g/mL concentration of PN and an 8 $\mu$g/mL concentration of positive control, SAC, respectively. In contrast, volatile extracts of AT, PL, and SF, which demonstrated inhibitory activity against hexanal oxidation or $H_2O_2$-induced cytotoxicity, did not display any inhibition of the MDA level. This is similar to the report by Puertas-Mejia et al. (*29*), which suggests that the antioxidant activities may vary according to the assay used, because all methods are based on different chemical and physical principles of oxidation monitoring.

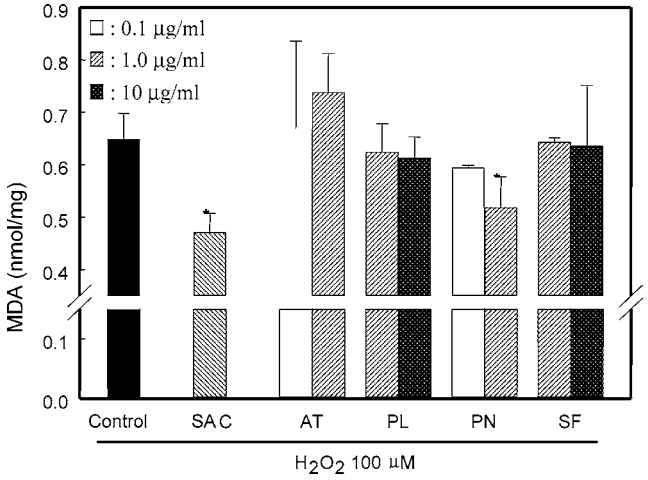


**Figure 1.** Effect of volatile extracts on $H_2O_2$-induced lipid peroxidation in SK-N-SH cells. Cells were preincubated with varying concentrations of volatile extract for 24 h and then were incubated for another 16 h together with volatile extracts and 100 $\mu$M $H_2O_2$. Each value was representative of a minimum of five independent experiments. SAC (8 $\mu$g/mL) was used as a positive control. Concentrations tested were 1 and 10 $\mu$g/mL for PL and SF and 0.1 and 1 $\mu$g/mL for AT and PN. (*) $p < 0.05$, compared with the control as analyzed by Student's *t* test.

In conclusion, the volatile constituents in AT, PL, PN, and SF were identified, and the volatile extracts exhibited varying degrees of antioxidant activity. Among the volatile extracts, PL and PN increased cell viabilities compared to that of $H_2O_2$-treated brain neuroblastoma cells, SK-N-SH. However, a 20% reduction in the malonaldehyde level, an index for lipid peroxidation, was observed at only 1 $\mu$g/mL concentration of PN. The aldehyde/carboxylic assay involves the abstraction of a hydroxyl radical (*28*). The results of the present study indicate that volatile extracts of medicinal plants contain various scavengers of radicals. It is difficult to pinpoint the specific components that give rise to the antioxidant activities of volatile extracts isolated from medicinal plants because there are many volatile antioxidants present in these plants. However, in light of previous reports and the chemical structures of the identified components in this study, the antioxidant activity of volatile extracts of medicinal plants is probably due to the presence of several phenolic compounds. This study suggests that the antioxidant activities of the medicinal plants are due, in part, to the presence of volatile compounds. Further work will be required to fully characterize the potential of volatile extracts as antioxidants in food or biological systems.

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