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Enhancing Antioxidant, Antiproliferation, and Free Radical Scavenging Activities in Strawberries with Essential Oils

CHIEN Y. WANG,*,† SHIOW Y. WANG,‡ JUN-JIE YIN,§ JOHN PARRY,||,⊥ AND LIANGLI LUCY YU||

Produce Quality and Safety Laboratory and Fruit Laboratory, U. S. Department of Agriculture, ARS, Beltsville, Maryland 20705, Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland 20740, Department of Nutrition and Food Science, University of Maryland, College Park, Maryland 20742, and Agricultural Research Station, Virginia State University, Petersburg, Virginia 23806

Several natural antimicrobial compounds derived from essential oils of plants were investigated for their efficacies in inhibiting decay and extending the shelf life of strawberries (*Fragaria* x *ananassas* Duch.). The severity of decay in strawberries stored at 10 °C was significantly reduced by treatment with thymol. Treatments with menthol or eugenol also suppressed the fungal growth, but to a lesser extent. All of these three natural antimicrobial compounds extended shelf life of strawberries as compared to the control. Strawberries treated with thymol, menthol, or eugenol also maintained better fruit quality with higher levels of sugars, organic acids, phenolics, anthocyanins, flavonoids, and oxygen radical absorbance capacity than the untreated fruits. The free radical scavenging properties of strawberry fruit were evaluated against 2,2-diphenyl-1-picryhydrazyl (DPPH•), hydroxyl (HO•), and superoxide radicals ($O_2^{\bullet -}$) using electron spin resonance measurements. Higher radical scavenging capacities were found against DPPH• and HO• in all treated fruit, particularly in berries treated with thymol, compared to those in the control groups. In addition, strawberry extracts were evaluated for their antiproliferative activities using HT-29 colon cancer cells. Extracts from all treated fruit exhibited significantly stronger inhibition on HT-29 cell proliferation than those from the control fruit. These data provide evidence that, in addition to possessing antimicrobial activity, the essential oils also increase free radical scavenging capacity and antiproliferative activity in fruit and, in turn, enhance the resistance of fruit tissues to deterioration and spoilage.

KEYWORDS: Strawberries; eugenol; menthol; thymol; antioxidant; antiproliferation

INTRODUCTION

Investigations on the beneficial use of naturally occurring or GRAS (generally recognized as safe) substances and sustainable nonchemical techniques are needed to minimize our dependency on potentially hazardous chemicals to reduce decay and postharvest losses of fruits and vegetables. Several naturally occurring essential oils have been reported to have antimicrobial properties and have shown promise in reducing postharvest diseases and disorders in horticultural crops (*1*−*3*). The combination of modified atmosphere packaging with eugenol or thymol is beneficial in reducing the microbial spoilage and the losses of sensory quality, nutrition, and functional properties of grape during storage at 1 °C (*4*). Two other essential oils, carvacrol and cinnamic acid, were found to delay the spoilage of fresh-cut kiwifruit and honeydew melon at chilling temperatures without adversely affecting sensory quality (*5*).

Several essential oils including carvacrol, thymol, eugenol, and piperine have been shown to have inhibitory effects on lipid accumulation with some growth inhibition (*6*). However, little is known about the effects of these substances on fruit antioxidant properties, nor have these compounds been evaluated for their effects on antiproliferative components in fruit tissues. The relationship between antioxidant levels and antimicrobial resistance as well as antiproliferative activities in other crops by other treatments has received much attention recently (*7*−*9*).

In this study, we investigated the potential use of natural essential oils for their effect on the postharvest life of berries. The essential oils tested in this study included thymol, eugenol, and menthol, which are derived from plant sources. Thymol is the major constituent in the essential oils of thyme and *Origanum* (*10*, *11*). Eugenol is the main essential oil in clove oil and can also be obtained from other spices as well (*12*, *13*). Menthol occurs naturally in peppermint and other mint oils. The

* Author to whom correspondence should be addressed. Tel.: 301-504-5981. Fax: 301-504-5107. E-mail: ChienYi.Wang@ars.usda.gov.
† Produce Quality and Safety Laboratory, USDA.
‡ Fruit Laboratory, USDA.
§ Center for Food Safety and Applied Nutrition, FDA.
|| Department of Nutrition and Food Science, University of Maryland.
⊥ Agricultural Research Station, Virginia State University.

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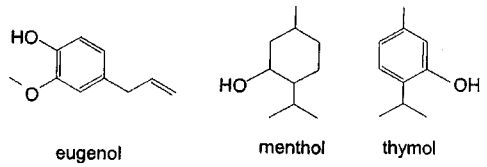

Figure 1. Chemical structures of the active compounds of the essential oils used in this study.

effects of these essential oils on fruit quality, phenolic content, anthocyanin content, antioxidant activity, and free radical scavenging activities of berry tissue were investigated to determine if these natural compounds affect the resistance of tissue to decay in addition to their inherent antimicrobial properties. The influence of these essential oils on levels of the antiproliferative components in strawberries was also studied using HT-29 human colon cancer cells.

MATERIALS AND METHODS

Plant Materials and Treatments. Strawberries (*Fragaria* x *ananassas* Duch.) used in this study were grown at a farm near Beltsville, MD; hand-harvested at a commercially mature stage; sorted to eliminate damaged, shriveled, and unripe fruit; and selected for uniform size and color. Selected berries were randomized and used for the experiments. Ten to 15 fruits were placed into 1 L polystyrene containers with snap-on lids. Two hundred milligrams of each essential oil, including thymol, eugenol and menthol, were put into a small beaker and placed in the sealed fruit container, which was then kept at 10 °C. The chemical structures of the active compounds of these essential oils used in this study are shown in **Figure 1**.

Analysis of Sugars and Organic Acids. The berry fruits were homogenized and centrifuged, and the supernatants were dried in vacuo in derivatizing vials. Procedures described by Li and Schuhmann (*14*) were modified for the derivatization of sugars and organic acids. A known amount of β-phenyl-D-glucopyranoside was included in all samples as an internal standard. A Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and a 25-m cross-linked methyl silicon gum capillary column (0.2-mm ID, 0.33-μm film thickness) was used for the analysis of sugars and organic acids.

Total Anthocyanin and Total Phenolic Content. Five grams of fresh berries were extracted with 25 mL 80% acetone containing 0.2% formic acid. Total anthocyanin content in fruit extracts was determined using the pH differential method (*15*). Total phenolic content was determined with Folin-Ciocalteu reagent by the method of Slinkard and Singleton (*16*) using gallic acid as a standard.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The automated sample preparation was performed using a Precision 2000 instrument. The ORAC assay was carried out using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system and a microplate fluorescence reader following the protocol previously described (*17*).

HPLC Analysis of Strawberry Flavonoids. High-performance liquid chromatography (HPLC) was used to separate and determine individual anthocyanin and phenolic compounds in strawberry tissue samples using procedures modified from a method published previously (*18*). Five grams of fruit samples was extracted twice with 15 mL of acetone using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) for 1 min. Combined extracts (30 mL) were concentrated to 1 mL using a Buchler Evapomix (Fort Lee, NJ) in a water bath at 35 °C. The concentrated sample was dissolved in 10 mL of acidified water (3% formic acid, v/v) and then passed through a C_{18} Sep-Pak cartridge (Waters), which was previously activated with methanol followed by water and then 3% aqueous formic acid. Anthocyanins and other phenolics were adsorbed onto the column while sugars, acids, and other high-polarity compounds were eluted with 10 mL of 3% aqueous formic acid. The anthocyanins and other phenolics were then recovered with 2.0 mL of acidified methanol containing 3% formic acid. The methanol eluate was passed through a 0.45 μm membrane filter (Millipore, MSI, Westboro, MA), and 20 μL was analyzed by HPLC. The samples were analyzed using a Waters (Waters Associates, Millipore, Milford, MA) HPLC system equipped with two pumps (600 E system controller) coupled with a photodiode array detector (Waters 990 series). Samples were injected at ambient temperature (20 °C) and components were separated using a reverse-phase Nova-Pak C_{18} column (150 × 3.9 mm, particle size 4 μm) with a guard column (Nova-Pak C_{18}, 20 × 3.9 mm, particle size 4 μm) (Sentry guard holder universal). The mobile phase consisted of acidified water containing 2.5% formic acid (A) and acetonitrile (B) in a linear gradient from 5% to 20% B in the first 15 min, followed by a linear gradient from 20 to 30% B for 5 min and then an isocratic mixture for 5 min, followed by a linear gradient from 30 to 90% B for 5 min, and then an isocratic mixture for 2 min before returning to the initial conditions. The flow rate was 1.0 mL/min, and the wavelengths of detection were set at 320, 350, and 510 nm. Scanning between 240 and 550 nm was performed, and data were collected by the Waters 990 3-D chromatography data system. Retention times and spectra were compared to those of pure standards.

Radical DPPH$^\bullet$ Scavenging Activity. Radical DPPH$^\bullet$ scavenging capacities of individual selected phenolic acids were determined by an electron spin resonance (ESR) spectrometry method, using the stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH$^\bullet$) (*19*). ESR analysis was conducted using a Varian E-109X-Band ESR spectrometer (Varian, Inc., Palo Alto, CA) at ambient temperature. Each aqueous strawberry extract was mixed with DPPH$^\bullet$ stock solution to initiate the antioxidant−radical reaction. The final concentration was 250 μM for DPPH$^\bullet$ in all reaction mixtures. The final amount of strawberry fruit used for measurement was 0.25 mg of fresh weight equivalent per milliliter of reaction mixture. The control reaction contained no antioxidant. The DPPH$^\bullet$ was prepared with 50% ethanol in water (v/v). ESR signals were recorded at 1, 5, 10, 20, and 30 min following the initiation of the reaction, with 20 mW incident microwave power and 100 kHz field modulation of 2 G (*20*). The ESR spectra recorded at 30 min of reaction are presented.

Hydroxyl Radical (HO$^\bullet$) Scavenging Activity. Hydroxyl radical (HO$^\bullet$) scavenging capacities of the strawberry extracts were examined by the ESR method (*21*). The ESR assay was based on the competition between the trapping agent and the antioxidative activity in the extract. HO$^\bullet$ was generated by Fenton reaction, while 5,5-dimethyl *N*-oxide pyrroline (DMPO) was used as the trapping agent. The reaction mixture contained 25 μL of freshly prepared 1 mM $FeSO_4$, 25 μL of 1 mM EDTA, 25 μL of 250 mM DMPO, 25 μL of 1 mM H_2O_2, and 25 μL of diluted fruit extract or H_2O for the blank. The final amount of strawberry antioxidants used for measurement was 4.15 mg of fresh weight equivalents per milliliter of reaction mixture. The ESR measurements were conducted at 4 min of each reaction at ambient temperature, with the following spectrometer settings: microwave power of 10 mW, field modulation frequency of 100 kHz, and a modulation amplitude of 1 G.

Superoxide Anion Radical ($O_2^{\bullet-}$) Scavenging Activity. $O_2^{\bullet-}$ scavenging activity was determined by the ESR method and the xanthine/xanthine oxidase system was used to generate the $O_2^{\bullet-}$ (*22*). The antioxidant−radical reaction was initiated by addition of xanthine oxidase solution (XOD), whereas 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO) was used as the trapping agent (*23*). The total volume of the reaction mixture was 125 μL and the concentrations were 5 mM for xanthine, 125 mM for BMPO, 0.5 mM for diethylenetriaminepentaacetic acid (DTPA), and 0.25 units/mL for XOD. The final amount of strawberry antioxidants used for measurement was 12.5 μg of fresh weight equivalents per milliliter of reaction mixtrure. The ESR spectra were recorded at 5 min of reaction at ambient temperature with the same spectrameter settings as those for hydroxyl radicals.

Inhibition of HT-29 Cancer Cell Proliferation. Cell proliferation inhibition study was carried out according to a method previously described by Parry et al. (*9*). The HT-29 human colorectal adenocarcinoma cell line characterized by Fogh and Trempe (*24*) was propagated in T-150 flasks in McCoy's 5A media containing 10% FBS and 1% antibiotic/antimycotic. Flasks were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 (*25*, *26*). Cell proliferation was examined using a Perkin-Elmer ATP1step lite luminescence kit (*27*,

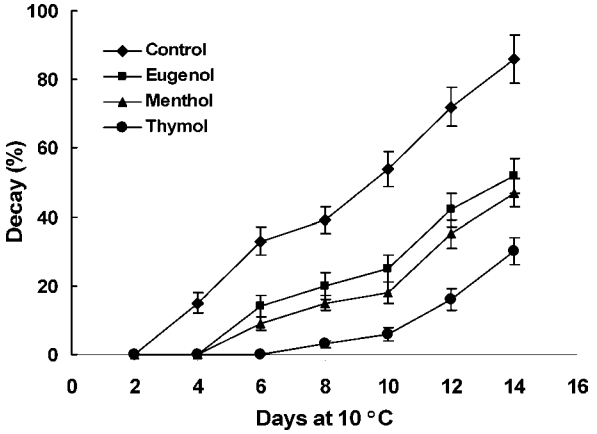

Figure 2. Effect of essential oils on decay of strawberries during storage at 10 °C. Error bars denote ±SD.

Table 1. Effect of Essential Oils on Sugar and Organic Acid Contents in Strawberries after 14 days of Storage at 10 °C[a]

essential oils	sugars (mg/g of fresh weight)			organic acids (mg/g of fresh weight)	
	fructose	glucose	sucrose	malic	citric
control	21.7 ± 1.6	18.6 ± 0.9	12.4 ± 1.1	0.85 ± 0.07	6.38 ± 1.5
eugenol	25.8 ± 0.8	20.2 ± 1.1	14.3 ± 0.9	0.97 ± 0.09	9.63 ± 0.4
menthol	28.2 ± 1.0	22.1 ± 1.2	12.9 ± 1.0	0.88 ± 0.06	8.96 ± 0.6
thymol	27.6 ± 0.7	25.8 ± 1.4	13.7 ± 1.2	0.93 ± 0.08	9.21 ± 0.7

[a] Data expressed as mean ± standard deviation, $n = 6$.

28) purchased from Perkin-Elmer Life and Analytical Sciences, Boston, MA. The initial cell density was 2.5×10^3 per well. After 24 h of incubation in the control media at 37 °C in 5% CO_2, cells in each well were treated with media containing the DMSO solution of the strawberry fruit extract (3 mg/mL), while the control cells were treated with same volume of DMSO. Media were changed daily and live cells were counted on day 1 through day 4 of treatments. The antiproliferative effects of strawberry fruit extracts from various essential oil treatments were estimated by the cell density, which was expressed as percent control cells after 1, 2, 3, or 4 days of treatment.

Statistical Analysis. Data were subjected to analysis of variance using NCSS (NCSS 2007, Kaysville, UT). The values of ORAC, total phenolics, total anthocyanin, and cell proliferation inhibition were evaluated by the Tukey−Kramer multiple-comparison test used in NCSS. Differences at $P \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

Treatments with eugenol, menthol, and thymol reduced decay of strawberries (**Figure 2**). The effectiveness of these and other essential oils against a wide range of microorganisms has been evaluated and demonstrated previously (*1*). A possible mechanism for the antimicrobial activity is that essential oils may interfere with the membranes of the microbes by changing their permeability for cations, and the dissipation of the ion gradients leads to impairment of vital processes in cells and eventually results in cell death (*29*). In addition to reducing decay, we found that treatment with eugenol, menthol, and thymol also maintained higher amounts of sugars and organic acids (**Table 1**); increased the contents of total phenolics, anthocyanins, and ORAC (**Figure 3**); and enhanced the amount of individual flavonoids, including *p*-coumaroyl glucose, ellagic acid, ellagic acid glucoside, quercetin 3-glucoside quercetin 3-glucuronide, kaempferol 3-glucoside, kaempferol 3-glucuronide, cyanidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3-glucoside succinate, and pelargonidin 3-glucoside succinate in berry fruits

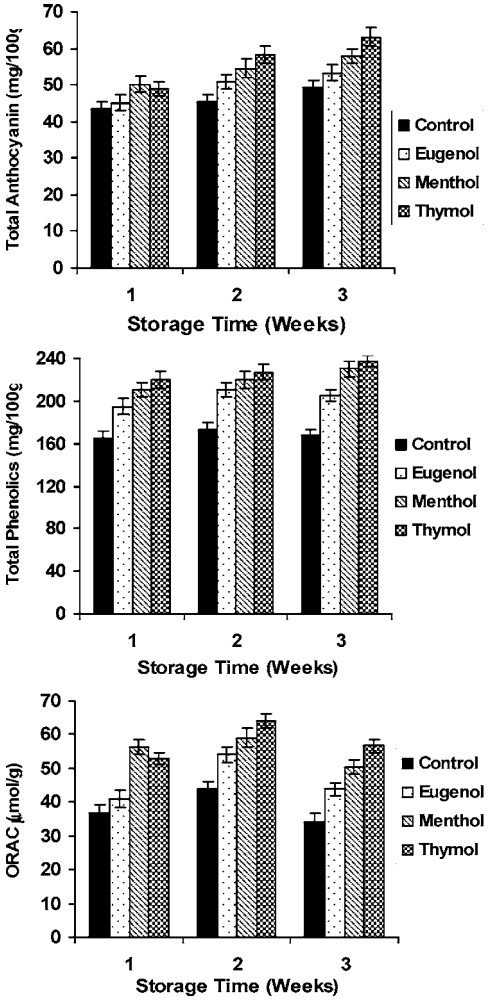

Figure 3. Effect of essential oils on total anthocyanin, phenolics, and oxygen radical absorbance capacity in strawberries during storage at 10 °C. Error bars denote ±SD.

(**Table 2**). The results indicate that these essential oils suppressed spoilage in berry fruits not only with their antimicrobial properties but also with their promotion of decay resistance in the fruit tissues through increasing the amounts of phenolic compounds, anthocyanins, flavonoids, and antioxidant capacities.

Among all the essential oils tested, thymol treatment was demonstrated to be the best at slowing berry decay (**Figure 2**) and resulted in the highest ORAC values in the fruit tissues (**Figure 3**). These results are consistent with that previously reported by Yanishlieva et al. (*30*). They found that thymol had the highest antioxidant effectiveness and activity during the oxidation of triacylglycerols in sunflower oil. Berries treated with other essential oils used in this study including eugenol and menthol also had higher ORAC values and lower percentage of decay compared to those in the control, although not as effective as those treated with thymol. Total phenolic content has been positively correlated to antioxidant capacity (*31*−*33*), and the antimicrobial activity in essential oils from spices is also thought to be associated with phenolic compounds (*34*). Thus, increased phenolic content in berry fruits treated with essential oils might have contributed to the increase in ORAC values and to the decrease in the percent of spoiled fruit (**Figure 3**).

In vitro studies have shown that essential oils exert their antibacterial activity at levels between 0.2 and 10 μL/mL (*35*). The minimum inhibitory concentration (MIC) of thymol to

Table 2. Effect of Essential Oils (Eugenol, Menthol, and Thymol) on Phenolic Content (Resveratrol, *p*-Coumaroyl Glucose, Ellagic Acid, Ellagic Acid Glucoside, Quercetin 3-Glucoside, and Quercetin 3-Glucuronide, Kaempferol 3-Glucoside and Kaempferol 3-Glucuronide) and Anthocyanin Content (Cyanidin 3-Glucoside, Pelargonidin 3-Glucoside, Cyanidin 3-Glucoside Succinate, and Pelargonidin 3-Glucoside Succinate) (μg/g fresh wt) in Strawberry ('Allstar') Fruit Extracts[a]

compound	control	eugenol	menthol	thymol
p-coumaric acid[b]	10.4 ± 0.5	8.6 ± 0.3	17.8 ± 1.4	15.3 ± 1.6
ellagic acid[c]	21.7 ± 1.3	19.7 ± 1.8	22.2 ± 1.9	29.9 ± 2.7
ellagic acid glucoside[c]	27.6 ± 2.8	25.3 ± 2.1	29.2 ± 2.6	29.6 ± 2.3
quercetin 3-glucoside and quercetin 3-glucuronide[d]	34.5 ± 3.1	36.2 ± 1.7	39.6 ± 3.5	59.7 ± 3.8
kaempferol 3-glucoside[e]	7.5 ± 0.3	7.7 ± 0.2	8.6 ± 0.3	9.5 ± 0.5
kaempferol 3-glucuronide[e]	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
cyanidin 3-glucoside[f]	15.9 ± 0.6	19.0 ± 1.4	36.4 ± 2.8	82.2 ± 6.4
pelargonidin 3-glucoside[f]	276.7 ± 9.8	226.2 ± 9.2	304.8 ± 13.4	331.4 ± 12.3
cyanidin 3-glucoside succinate[f]	12.9 ± 1.6	15.0 ± 1.8	21.9 ± 2.2	24.1 ± 2.1
pelargonidin 3-glucoside succinate[f]	42.2 ± 3.3	54.0 ± 4.6	55.6 ± 3.9	73.9 ± 4.8

[a] Data expressed as mean ± standard deviation, $n = 3$. [b] Data expressed as μg/g of fresh wt of *p*-coumaric acid per gram of fresh weight. [c] Data expressed as μg of ellagic acid equivalents per gram of fresh weight. [d] Data expressed as μg of quercetin equivalents per gram of fresh weight. [e] Data expressed as μg of kaempferol equivalents per gram of fresh weight. [f] Data of anthocyanidin expressed as μg of cyanidin 3-glucoside equivalents per gram of fresh weight.

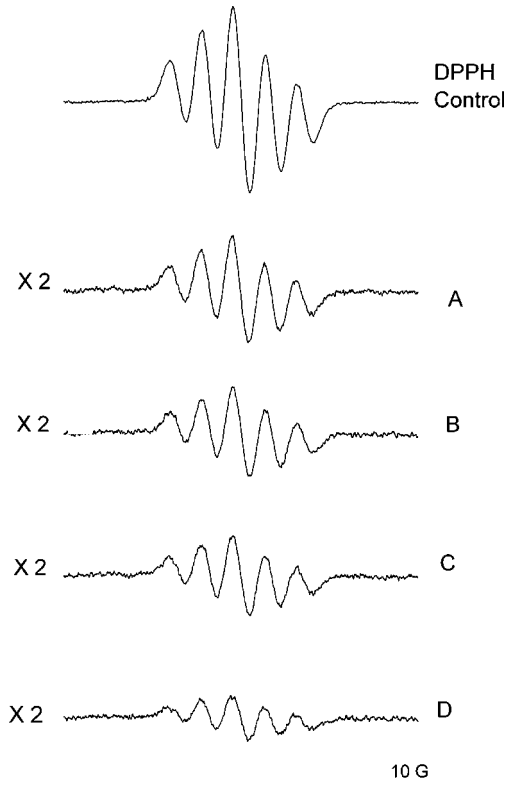

Figure 4. DPPH•-scavenging activities of strawberry extracts determined by ESR. Each aqueous strawberry extract was mixed with DPPH• stock solution to initiate the antioxidant–radical reaction. The final concentration was 250 μM for DPPH• in all reaction mixtures. ESR signals were recorded at 30 min following the start of the reaction, with 20 mW incident microwave power and 100 kHz field modulation of 2 G. The control reaction contained no antioxidant (**A**). Strawberry fruit were treated with (**B**) eugenol, (**C**) menthol, or (**D**) thymol. The final amount of strawberry fruit used for measurement was 0.25 mg of fresh weight.

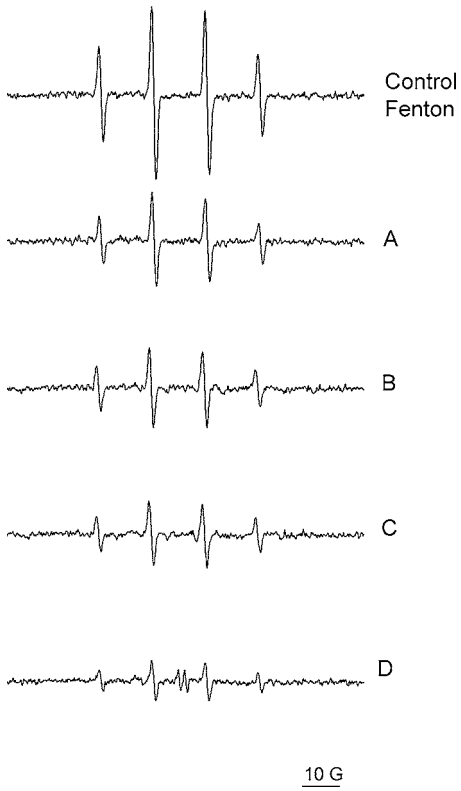

Figure 5. Hydroxyl radical (HO•) scavenging activities of strawberry extracts determined by ESR. The reaction mixture contained 25 μL of freshly prepared 1 mM $FeSO_4$, 25 μL of 1 mM EDTA, 25 μL of 250 mM DMPO, 25 μL of 1 mM H_2O_2, and 25 μL of diluted fruit extract or H_2O for the blank. The control reaction contained no antioxidant (**A**). Strawberry fruit were treated with (**B**) eugenol, (**C**) menthol, or (**D**) thymol. The final amount of strawberry fruit used for measurement was 4.15 mg of fresh weight. The ESR measurements were conducted at 4 min of each reaction at ambient temperature, using a Varian E-109X-Band ESR spectrometer.

enable a balance between sensory acceptability and antimicrobial efficacy was suggested as 50 mg/L (*36*). The concentration that we used in this study (200 mg/L) was within this range and above the MIC. Shelef et al. (*37*) found that similar doses of essential oils added to microbiological media exhibited greater inhibition against bacteria than when they were added to food system. This discrepancy in the effectiveness may pose a problem in the commercial use of essential oils as antimicrobial agents, because treatment with high concentrations of essential oils may cause chemical injury or affect the flavor of food products (*38*).

The scavenging activities of strawberry fruit against DPPH•, HO•, and $O_2^{\bullet-}$ are demonstrated in **Figures 4**, **5**, and **6**, respectively. Fruit treated with eugenol, menthol, or thymol had markedly lower radical signals for DPPH• and HO• than those of the control, indicating that the treated fruit had higher

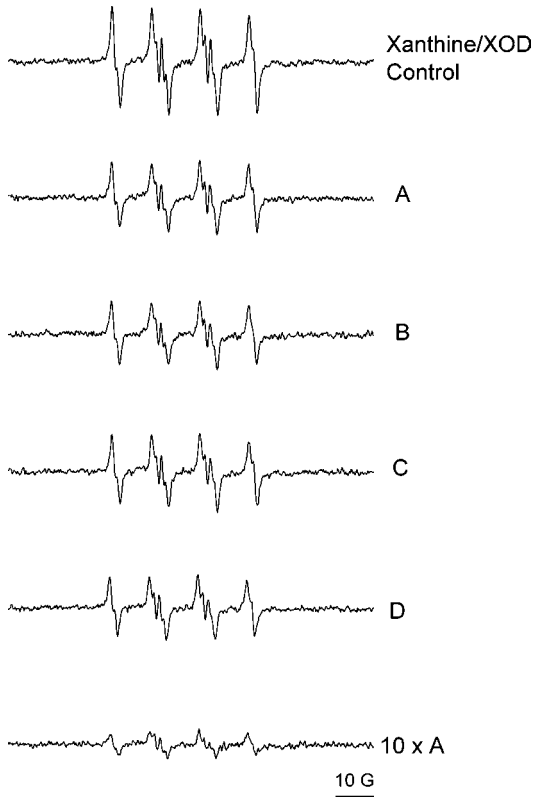

Figure 6. Superoxide radical ($O_2^{\bullet -}$) scavenging activities of strawberry extracts determined by ESR. The antioxidant–radical reaction was initiated by addition of xanthine oxidase solution (XOD), whereas 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO) was used as the trapping agent. The total volume of the reaction mixture was 125 μL, and the concentrations were 5 mM for xanthine, 125 mM for BMPO, 0.5 mM for diethylenetriaminepentaacetic acid (DTPA), and 0.25 units/mL for XOD. The control reaction contained no antioxidant (**A**). Strawberry fruit were treated with (**B**) eugenol, (**C**) menthol, or (**D**) thymol. The final amount of strawberry fruit used for measurement was 12.5 μg of fresh weight (for **B**, **C**, and **D**) or 125 μg of fresh weight for **10** $\times$ **A**. The ESR spectra were recorded at 5 min of reaction at ambient temperature with 10 mW incident microwave power and 100 kHz field modulation of 1 G.

scavenging activities for these radicals. Thymol-treated fruit in particular had the highest scavenging activities among all the treatments. These results are consistent with the data obtained for decay reduction, quality retention (sugars and organic acids), and antioxidant and antiproliferation activities. However, no discernible differences were detected for the $O_2^{\bullet -}$ radical signals among all treatments (**Figure 6**). Apparently, the scavenging capabilities of strawberry fruit themselves are so strong that only 12.5 μg of fresh weight equivalents per milliliter were needed to produce a distinct signal for superoxide radicals. This was equivalent to a 1000-fold dilution of the original fruit extract. It would be difficult for any treatment to alter (either increase or suppress) the scavenging capability of strawberry fruit for the superoxide radicals.

The strawberry extracts from day 14 samples were evaluated for their potential antiproliferative activities. These extracts were selected because that was when the treatments showed the most differences on their effects on decay. The extracts from all the treated samples showed inhibition of HT-29 cell proliferation compared with the control (**Figure 7**).

Besides antibacterial properties, essential oils or their components have been reported to exhibit antifungal, antiviral, antimycotic, antitoxigenic, antiparasitic, and insecticidal properties (*35*, *39*). Our study demonstrated that they also possess antioxidant and antiproliferative functions in addition to the above properties. The exact mechanisms for the treated strawberries to increase the contents of total phenol, individual flavonoids, and the antiproliferative effects are not clear. It is possible that treatment with eugenol, menthol, or thymol triggers a signal that resembles a mild stress to the fruit. As a defense response, fruit produces additional phenolic compounds and flavonoids and increases their antioxidant and antiproliferative activities. In our study, we have provided evidence that essential oils—eugenol, menthol, or thymol—could enhance the scavenging activities of strawberry fruit for DPPH$^{\bullet}$ and HO$^{\bullet}$ radicals. Eugenol, menthol, or thymol also increased the antiproliferative effect of fruit on human colorectal adenocarcinoma HT-29 cells.

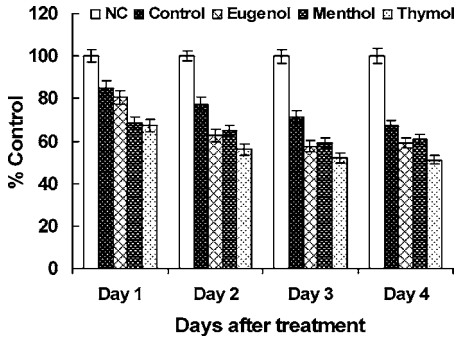

Figure 7. Proliferation rate of HT-29 colon cancer cells treated with strawberry extracts (3 mg/mL). Antiproliferative effects of extracts from various essential oil treatments were expressed as percent control cells after 1, 2, 3, or 4 days of treatment. Error bars denote ±SD.

Antioxidants are efficient free radical scavengers and are effective in suppressing ROS in plant tissue and reducing oxidative stress caused by decay organisms. Therefore, an increase in antioxidant capacity and free radical scavenging activity would reduce the physiological deterioration and enhance the resistance of tissue against microbial invasion and reduce the spoilage of fruit.

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