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Evaluation of Antioxidant Activity of Australian Tea Tree (*Melaleuca alternifolia*) Oil and Its Components

HYUN-JIN KIM,[†] FENG CHEN,*[,†] CHANGQING WU,[†] XI WANG,[‡] HAU YIN CHUNG,[§] AND ZHENGYU JIN[||]

Departments of Food Science and Human Nutrition and Genetics and Biochemistry, Clemson University, Clemson, South Carolina 29634, Department of Biology, Food and Nutritional Sciences Program, and Food Science Laboratory, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China, and School of Food Science and Technology, Southern Yangtze University, Wuxi 214036, China

Antioxidant activity of Australian tea tree (*Melaleuca alternifolia*) oil (TTO) was determined using two different assays. In the 2,2-diphenyl-1-picrylhydrazyl assay, 10 μL/mL crude TTO in methanol had approximately 80% free radical scavenging activity, and in the hexanal/hexanoic acid assay, 200 μL/mL crude TTO exhibited 60% inhibitory activity against the oxidation of hexanal to hexanoic acid over 30 days. These results were equivalent to the antioxidant activities of 30 mM butylated hydroxytoluene in both tests at the same experimental conditions. This indicated that the TTO could be a good alternative antioxidant. Inherent antioxidants, i.e., α-terpinene, α-terpinolene, and γ-terpinene, in the crude TTO were separated and identified chromatographically using silica gel open chromatography, C_{18}-high-pressure liquid chromatography, and gas chromatography−mass spectrometry. Their antioxidant activities decreased in the following order in both assays: α-terpinene > α-terpinolene > γ-terpinene.

KEYWORDS: Tea tree oil; *Melaleuca alternifolia*; antioxidant activity; DPPH; terpenoids; terpinen-4-ol; HPLC

INTRODUCTION

Research on bioactive principles of essential oils extracted from various herbs and spices has become increasingly popular because essential oils have been discovered to have many functional properties such as antimicrobial, antioxidant, and anticancer activities (*1*−*9*). As a result, essential oils have been widely used as fumigants, cosmetics, and aromatherapeutic agents. Nowadays, many research groups are focusing their investigation in the pharmacological actions of essential oils from aromatic and medicinal plants (*10*−*15*). Among them, Australian tea tree (*Melaleuca alternifolia*) oil (TTO) is one of the most important studied subjects because of its demonstrated broad spectrum of activities. TTO is mainly extracted by steam distillation from *M. alternifolia* that is also commonly known as Australian TTO, which, however, bears no resemblance to the taste or odor of the real tea, *Camellia sinensis*, Camelliaceae. TTO is composed of approximately 100 compounds, mainly monoterpenes, sesquiterpenes, and their alcohol derivatives (*16*), among which major components include terpinen-4-ol, γ-terpinene, α-terpinene, α-terpineol, α-terpinolene, 1,8-cineole, etc. The international standard ISO 4730 requires commercial TTO to have a minimum terpinen-4-ol content of 30% and a maximum 1,8-cineole content of 15% (*17*). However, most customers want to buy TTO products containing the highest content of terpinen-4-ol and the lowest content of 1,8-cineole because it has been found that terpinen-4-ol has strong antimicrobial (*18*) and antiinflammatory effects (*19*), while 1,8-cineole, also known as eucalyptol, is probably an allergen that is considered undesirable in TTO products (*20*). Although the various functionalities, such as antibacterial, antifungal, anti-inflammatory, etc., of TTO have been investigated in recent years (*21*−*28*), the antioxidant activity of TTO and its bioactive components has not been reported. Therefore, it is our interest to further explore the relationship between terpenic compounds and antioxidant activity in TTO in order to provide a more complete characterization of its biofunctional benefits. Also, additional knowledge of the antioxidant activity of TTO may help to increase its market value.

Many studies have shown that natural antioxidants in aromatic and medicinal plants are closely related with their biofunctionalities, such as the reduction of chronic diseases (e.g., DNA damage, mutagenesis, carcinogenesis, etc.) (*9, 29*−*30*) and inhibition of growth of pathogenic bacteria (*31*), which are often associated with the termination of free radical propagation in biological systems. Thus, antioxidant capacity is widely used

* To whom correspondence should be addressed. Tel: 864-656-5702. Fax: 864-656-0331. E-mail: fchen@clemson.edu.
[†] Department of Food Science and Human Nutrition, Clemson University.
[‡] Department of Genetics and Biochemistry, Clemson University.
[§] The Chinese University of Hong Kong.
[||] Southern Yangtze University.

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as a parameter to characterize medicinal plants and their bioactive components. In this study, the antioxidant activity of TTO was investigated using two complimentary in vitro assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and the hexanal/hexanoic acid assay. Furthermore, some TTO components showing strong antioxidant activity were separated from the crude TTO by silica gel open column chromatography and C_{18}-high-pressure liquid chromatography (HPLC) and identified by gas chromatography−mass sprectrometry (GC-MS). The antioxidant activities of TTO and its bioactive components were compared with that of a commercial standard antioxidant, butylated hydroxytoluene (BHT).

MATERIALS AND METHODS

Materials and Chemicals. The crude TTO, α-pinene, γ-terpinene, hexanal, and dry silica gel (70−230 mesh, 60 Å) were purchased from Aldrich Chemical Co. (Milwaukee, WI). α-Terpinene, α-terpinolene, and terpinen-4-ol were obtained from Fluka Chemical Co. (Milwaukee, WI). DPPH and BHT were purchased from Sigma Chemical Co. (St. Louis, MO).

Fractionation and Identification of Antioxidants from TTO. *Silica Gel Column Chromatography.* The column (30 cm × 2.5 cm) packed with silica gel (70−230 mesh, 60 Å) was equilibrated with a mixed solvent of dichloromethane/methanol (4:1 v/v). Five milliliters of the crude TTO was loaded from the top of the column and then linearly eluted with gradually increasing methanol from 0 to 100% at 10 mL/min. Each collected fraction was a size of 10 mL.

HPLC Separation. The Pinnacle II C_{18} column (150 mm × 4.6 mm, 5 μm; Restek, PA) was connected with a Shimadzu LC-10AT HPLC system (Kyoto, Japan) and equilibrated with methanol. Fifty microliters of each fraction separated by silica gel open column chromatography was injected into the HPLC column and eluted with methanol at a flow rate of 1 mL/min. Each fraction of the eluant collected at the detector exit was a size of 1 mL. The absorbance of the eluant was scanned from 200 to 500 nm by Shimadzu SPD-M10V photodiode array detector.

GC-MS Identification. A Shimadzu GC-MS system consisting of a GC-17A with a QP5050 Mass Spectrometer was equipped with a DB-5 capillary column (60 m × 0.25 mm, thickness 0.25 μm; J&W Scientific, Folsom, CA) for all chemical quantitative and qualitative analyses in this experiment. The oven temperature was programmed from 60 to 240 °C at the ramp rate of 3 °C/min and held at 240 °C for 10 min. The injector and ion source temperatures were set at 200 and 250 °C, respectively. The detector voltage was 70 eV, and the scanning mass range was *m*/*z* 43−350. Helium was used as the carrier gas at a column flow rate of 1.1 mL/min. The sample injection volume was 3 μL with a split ratio of 10. Identification of compounds was based on comparison of their mass spectra and retention indices (RIs) with those of the authentic standards. RIs were calculated using series of *n*-alkanes (C_8−C_{30}). When standard compounds were not available, each unknown compound was tentatively identified by comparing the mass spectrum with that of the Wiley and NIST mass spectral databases and the previously published RIs (*1*, *6*).

Antioxidative Capacity. The antioxidative capacities of TTO and its antioxidative components were determined by two methods: the DPPH free radical scavenging assay and the hexanal/hexanoic acid assay. A standard antioxidant BHT was used as the control.

DPPH Free Radical Scavenging Assay. Scavenging activity on DPPH free radicals by the crude TTO and its components was measured according to the method of Yamaguchi et al. (*32*) with minor modification. A sample of 0.4 mL was mixed with 0.4 mL of 0.5 mM DPPH solution. After 30 min of incubation in the darkness at room temperature, the absorbance of the reaction mixture was spectrophotometrically measured at 517 nm. The scavenging activity of DPPH free radical was calculated by using the following formula:

$$\text{scavenging effect (\%)} = \left(1 - \frac{\text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}}\right) \times 100 \quad (1)$$

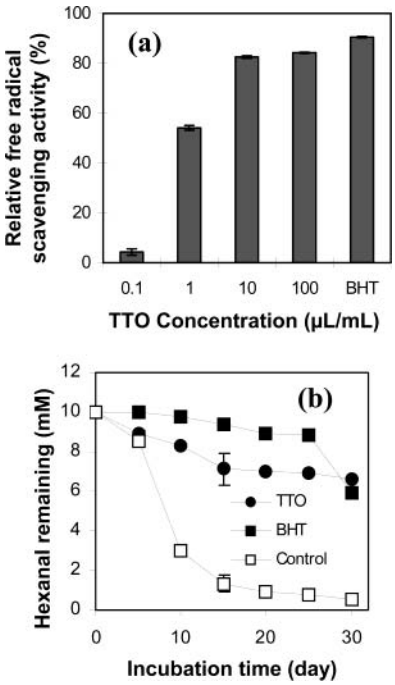

Figure 1. Comparison of antioxidative capacities of the crude TTO (*M. alternifolia*) and BHT as control using two different methods. (**a**) Free radical scavenging activity was spectrophotometrically measured at 517 nm using the DPPH assay, and (**b**) the remaining hexanal content was determined by GC-MS after oxidation of hexanal to hexanoic acid. BHT (30 mM) was used as a reference antioxidant in both methods, and 200 μL/mL of TTO was used for the hexanal/hexanoic acid assay.

Hexanal/Hexanoic Acid Assay. The inhibitory effect of the crude TTO and its components on the oxidation of aldehyde to carboxylic acid was determined using the hexanal/hexanoic acid assay (*33*, *34*). Sample (0.5 mL) was added into a 2 mL dichloromethane solution of hexanal (3 mg/mL) and undecane (0.2 mg/mL) as an internal standard. The reaction mixture was incubated at 60 °C for 10 min in a 20 mL sealed glass vial to initiate the oxidation of hexanal and then stored at room temperature for 30 days. The headspace of each vial was purged with pure air (1.5 L/min, 3 s) every 24 h only for the first 10 days. The remaining hexanal in the solution was determined by GC-MS every 5 days by taking 1 μL from the solution. For hexanal quantification, the GC oven temperature was programmed from 60 to 160 °C at 10 °C/min and then from 160 to 210 °C at 20 °C/min. The injector and ion source temperatures were 200 and 280 °C, respectively. The injection volume was 1 μL, and its split ratio was 10:1. Quantification of hexanal was conducted based on the standard hexanal calibration curve.

Statistical Analysis. The data on the antioxidant activities of the crude TTO and its components were subjected to the analysis of variance. The least significant difference was used to find the significant difference of antioxidant activities between various sample concentrations at $p < 0.05$.

RESULTS AND DISCUSSION

Antioxidative Capacity of Crude TTO. The antioxidative capacity of the crude TTO was determined with two different methods, the free radical scavenging test using DPPH solution and the inhibitory test of hexanal oxidation. Various concentrations (0.1, 1, 10, and 100 μL/mL) of the crude TTO dissolved in methanol were used for the free radical scavenging activity test. Results shown in **Figure 1a** indicated that the DPPH free radical scavenging activity of the crude TTO increased with increasing TTO concentration. Also, the free radical scavenging activity using 0.5 mM DPPH solution could be saturated by the crude TTO at the concentration of 10 μL/mL and above. In

this range, the DPPH free radical scavenging activity of the crude TTO approached 80%, which was comparable to the DPPH free radical scavenging activity of 30 mM BHT. Contrary to high concentrations, 1 μL/mL of the crude TTO had 54% of the free radical scavenging activity.

The percentage of remaining hexanal in solutions treated with 200 μL/mL of the crude TTO and 30 mM BHT over a period of 30 days was shown in **Figure 1b**. In the hexanal/hexanoic acid assay, results showed that the inhibitory activity of the crude TTO was marginally better than that of BHT over 30 days, although 200 μL/mL of the crude TTO exhibited weaker inhibitory activity than 30 mM BHT during the first 25 days. Besides, approximately 60% hexanal was inhibited to be oxidized to hexanoic acid in both reaction solutions, while hexanal in the control solution without any antioxidant was almost completely oxidized. These results shown in **Figure 1a**,**b** indicated that the antioxidative activity of the crude TTO was comparable with that of the well-known synthetic antioxidant such as BHT.

Separation of Antioxidants from Crude TTO. The antioxidants in the crude TTO were separated with silica gel open column chromatography and C_{18}-HPLC and identified by GC-MS. Twelve fractions were collected from the crude TTO through the silica gel column, but only fractions 5 and 6 exhibited higher antioxidant activity than any other fractions (data not shown). However, further chromatographic analysis by GC-MS showed that the chemical profiles of both fractions were not only similar to each other but also similar to the whole profile of the crude TTO. This meant that silica gel open column with a solvent mixture of dichloromethane and methanol as the eluant could not successfully separate the complex components in the crude TTO. Therefore, fraction 6 showing stronger antioxidant activity was further separated by the reverse phase C_{18} column connected to the HPLC system. **Figure 2a**,**b** showed the six fractions separated by the C_{18} column and their corresponding antioxidant activities measured by the aforementioned two methods. Among the six fractions collected, fraction 3 exhibited the highest free radical scavenging activity at 67%. Likewise, in the inhibition assay against the oxidation of hexanal to hexanoic acid, the least amount of hexanal oxidized in the reaction solutions was observed in the fraction 3 over a period of 15 days (**Figure 2c**). This indicated that fraction 3 had a relatively higher antioxidative activity than other fractions. Thus, fraction 3 within six fractions separated by the C_{18} column was selected for further chemical separation and identification.

Identification of Antioxidants in the Fraction 3 Separated by C_{18}-HPLC. Compounds in fraction 3 separated by C_{18}-HPLC were further separated and identified by GC-MS. As shown in **Figure 3a**, the crude TTO had over 90 compounds including major components such as terpinen-4-ol (43.2%), γ-terpinene (20.6%), and α-terpinene (9.6%). These three compounds accounted for about 73% of the total amount of the crude TTO, which was similar to the results of other research groups (*18*−*19*, *35*). Unlike silica gel open column chromatographic separation, C_{18}-HPLC was much more efficient in separating TTO components. The fraction 3 collected from C_{18}-HPLC had only 10 compounds, among which five compounds (α-pinene, α-terpinene, α-terpinolene, γ-terpinene, and terpinen-4-ol) were positively identified by comparison with authentic standard compounds. The other five compounds were tentatively identified by comparing their RIs and mass spectra with those in the previous publications and the Wiley and NIST mass spectral databases, respectively. These 10 identified compounds in fraction 3 are listed in the **Table 1**. γ-Terpinene, α-terpinene, and α-terpinolene were the three major compounds whose concentrations were quantified by means of the standard calibration curves. It was also determined that the crude TTO had 0.09 M α-pinene, 0.62 M α-terpinene, 0.19 M α-terpinolene, 1.25 M γ-terpinene, and 2.12 M terpinen-4-ol. However, the most plentiful compound in the crude TTO, terpinen-4-ol, was not the dominant chemical in fraction 3 (**Figure 3b**).

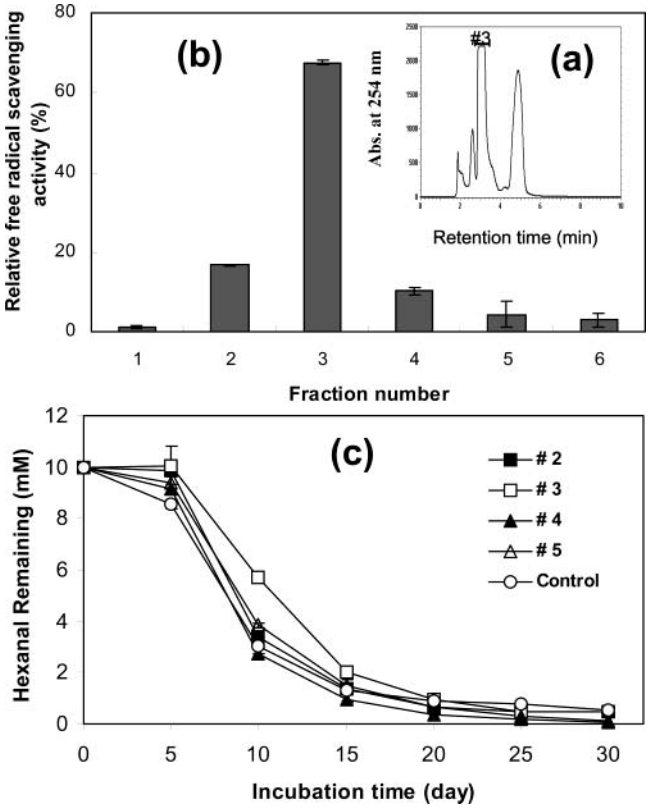

Figure 2. Antioxidative activities of fractions separated by C_{18}-HPLC. (**a**) C_{18}-HPLC chromatogram of fraction 6 of TTO from silica gel open column separation. The sample was eluted with methanol at a flow rate of 1 mL/min; each fraction was collected for 1 mL. (**b**) DPPH scavenging activity of fractions separated by C_{18}-HPLC. (**c**) Hexanal/hexanoic acid assay of the fractions separated by C_{18}-HPLC.

Antioxidant Activity of Major Compounds in Fraction 3 Separated By C_{18}-HPLC. The antioxidant activities of the identified major compounds in fraction 3 were further investigated by the DPPH assay and the hexanal/hexanoic acid assay, the results of which are shown in **Figures 4** and **5**, respectively. In the DPPH assay, DPPH free radical scavenging activity was measured for BHT and four compounds (α-pinene, α-terpinene, α-terpinolene, and γ-terpinene) at various concentrations. Although terpinen-4-ol is a major component of the TTO and a dominant component in fractions 1 and 2 separated by C_{18}-HPLC (data not shown), it was only in a minor amount in fraction 3. Also, it was found that fractions 1 and 2 showed much weaker antioxidant activity than fraction 3 (**Figure 2b**,**c**), which suggested that terpinen-4-ol was a weak antioxidant. This result was consistent with the other research findings of terpinen-4-ol when studying the antioxidant activities in citrus, clove, and nutmeg essential oils (*36*, *37*). Therefore, terpinen-4-ol was excluded from any further antioxidant assays. As shown in **Figure 4**, all samples in the DPPH test exhibited to different degrees dose-dependent relationships between concentrations (0−180 mM) of the chemical species and the DPPH free radical scavenging activity. The antioxidant activities of α-terpinene and α-terpinolene were rapidly saturated at 10 and 30 mM,

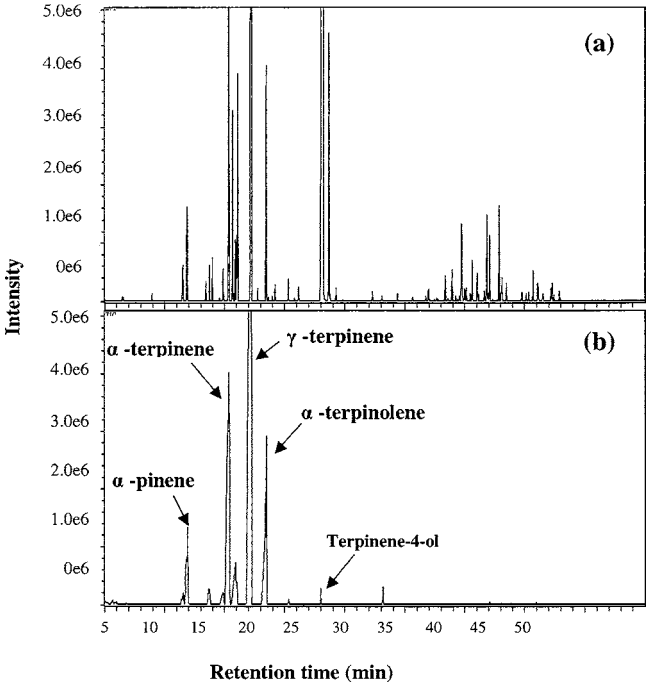

Figure 3. GC-MS chromatograms of the crude TTO and fraction 3 separated by HPLC C_{18} column. A Shimadzu GC 17A-QP5050 Mass Spectrometer system coupled with a DB-5 capillary column (60 m × 0.25 mm × 0.25 μm) was used to separate and identify the components in TTO. The oven temperature was programmed from 60 to 240 °C at 3 °C/min and held at 240 °C for 10 min. The injector and ion source temperatures were 200 and 250 °C, respectively.

Table 1. TTO Components in Fraction 3 Separated by C_{18}-HPLC, Identified, and Quantified by GC-MS

peak no.	identified compds	retention time (min)	RI[a]	area % (compd/TTO)	concentration (M/TTO)
1	α-thujene	11.547	927	0.8 ± 0.09	NC[b]
2	α-pinene	11.897	936	2.1 ± 0.23	0.09 ± 0.01
3	β-pinene	13.695	982	0.5 ± 0.05	NC
4	α-phellandrene	14.86	1009	0.4 ± 0.02	NC
5	α-terpinene	15.371	1020	9.6 ± 0.77	0.60 ± 0.00
6	limonene	15.914	1032	0.8 ± 0.05	NC
7	β-phellandrene	16.035	1034	0.8 ± 0.03	NC
8	γ-terpinene	17.242	1062	20.6 ± 0.43	1.16 ± 0.04
9	α-terpinolene	18.482	1088	3.3 ± 0.14	0.17 ± 0.01
10	terpinene-4-ol	23.008	1189	43.2 ± 0.14	2.12 ± 0.27

[a] RI was calculated using a series of *n*-alkanes (C_8–C_{30}). [b] Not calculated.

respectively, and their corresponding DPPH scavenging capacities were 77 and 74%, respectively. In addition, the DPPH scavenging activity of γ-terpinene nearly increased linearly with its increasing concentration within the range between 0 and 180 mM. Its activity achieved 68% at 180 mM, which was lower than that of both α-terpinene and α-terpinolene but higher than that of α-pinene. As compared with all other compounds, α-pinene did not exhibit potent antioxidant activity in the concentration range in the DPPH test. Nevertheless, α-terpinene had the highest DPPH scavenging activity at 10 mM except for that of BHT at over 80%. At 180 mM, the antioxidant activities of α-terpinene and α-terpinolene were close (82 and 86%, respectively). The activity of α-terpinene was also very close to that of 180 mM BHT (85%). Therefore, among the three compounds in fraction 3, i.e., α-terpinene, α-terpinolene, and γ-terpinene, α-terpinene had the strongest DPPH free radical scavenging activity, followed by α-terpinolene and γ-terpinene.

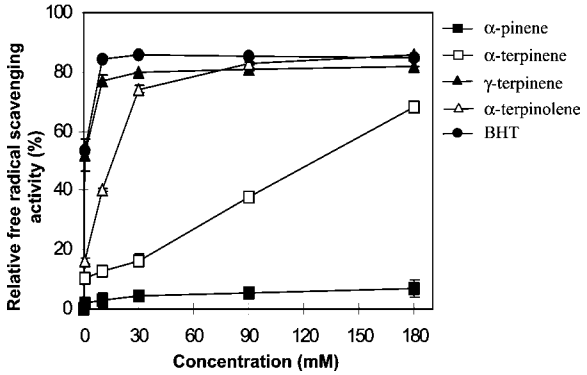

Figure 4. Relative DPPH free radical scavenging activity of TTO components spectrophotometrically measured at 517 nm.

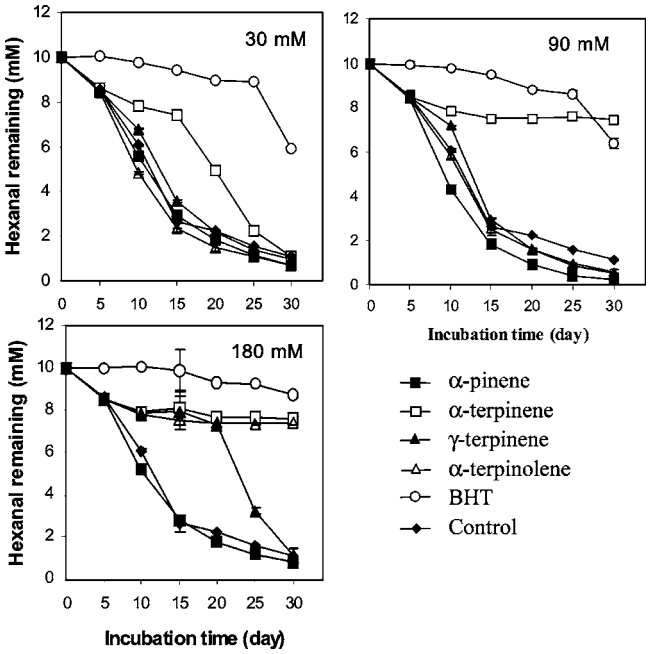

Figure 5. Antioxidant activity of TTO components at various concentrations (30, 90, and 180 mM). The remaining hexanal content was determined by GC-MS after oxidation of hexanal to hexanoic acid.

In addition, the DPPH free radical scavenging activities of α-terpinene, α-terpinolene, and γ-terpinene were correspondingly 29.8, 0.4, and 11.9%, respectively, when they were reconstituted using pure chemicals at the same concentration levels as their original concentrations in the 1 μL TTO/mL of methnol, i.e., α-terpinene, α-terpinolene, and γ-terpinene at 0.62, 0.19, and 1.25 mM, respectively. The sum of all of the DPPH free radical scavenging activities from the three compounds was 41%. However, the original crude TTO at 1 μL/mL in methanol had a DPPH free radical scavenging activity of 54% (**Figure 1**). Therefore, we hypothesized that there might be synergistic effects among these compounds in the assay or there might be other antioxidants unidentified in the TTO. Further investigation of the complimentary antioxidant activity among these compounds is shown in **Figure 5**. In the hexanal/hexanoic acid assay with the sample concentrations at 30 and 90 mM, only α-terpinene exhibited the inhibitory effect against the oxidation of hexanal to hexanoic acid. Although α-terpinene had stronger inhibitory activity than the other compounds at 30 mM, its inhibitory effect could not remain at a strong efficacy after 30 days but decreased drastically. At concentrations of 90 mM or above, α-terpinene exhibited strong inhibitory activity (about 65%) over 30 days in contrast with the lower concentrations.

Nevertheless, at 180 mM, α-terpinolene, γ-terpinene, and α-terpinene all showed strong inhibitory activity. α-Terpinolene had very similar inhibitory activity to α-terpinene (about 65%) over 30 days, but γ-terpinene could keep its strong inhibition activity (about 65%) for only 20 days and then rapidly decreased. After 30 days, the activity of 180 mM γ-terpinene was similar to that of the control solution without any antioxidant. The inhibition activity of BHT was stronger than that of any components isolated from the crude TTO except for the 90 mM α-terpinene for over 30 days. α-Pinene did not show any inhibition effect in this assay as in the DPPH assay. Results in **Figure 5** showed that α-terpinene was the strongest antioxidant in TTO in this assay, the same as that in the DPPH assay. α-Terpinolene only exhibited strong antioxidant activity as that of α-terpinene to inhibit the hexanal oxidation at high concentration, i.e., 180 mM. In addition, although γ-terpinene had the antioxidant activity at high concentration, its antioxidative efficacy in the hexanal oxidation test was weaker than that of α-terpinene and α-terpinolene.

In conclusion, many studies have addressed the benefits of using TTO (*M. alternifonia* L.) because it contains the major bioactive chemical terpinen-4-ol. However, our present study revealed that the majority antioxidant activity in TTO was attributed to the three terpenic compounds, i.e., α-terpinene, α-terpinolene, and γ-terpinene, rather than the chemical terpinen-4-ol. In addition, it was found that the potency of TTO antioxidant activity was comparable to that of the common synthetic antioxidant BHT. This suggests that TTO might become a useful antioxidant relevant to the maintenance of oxidative stability of food matrix.

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