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# Unusual Antioxidant Behavior of α- and γ-Terpinene in Protecting Methyl Linoleate, DNA, and Erythrocyte

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The antioxidant effects of α-terpinene (α-TH) and γ-terpinene (γ-TH) on the oxidation of methyl linoleate (LH), DNA, and erythrocytes induced by 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) were investigated. The results from erythrocytes and DNA were treated by means of chemical kinetic equations. It was found that either α- or γ-TH was able to scavenge ∼0.4 radicals when they protected DNA. α-TH can trap ∼0.7 radicals when protecting erythrocytes and can trap ∼0.5 radicals when protecting LH. γ-TH can trap ∼1.2 radicals when protecting erythrocytes and LH. Therefore, the antioxidant effectiveness of γ-TH was higher than α-TH. γ-TH contained a nonconjugated diene, and the diene in α-TH was conjugated. The obtained results implied that the nonconjugated diene benefited for antioxidant capacity more than a conjugated diene. Moreover, the reactions of α- and γ-TH with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cation radical ($ABTS^{+\bullet}$) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) implicated that α- and γ-TH were able to scavenge radicals directly. However, α- and γ-TH promoted AAPH-induced hemolysis with a high concentration employed.

**KEYWORDS: Terpinene; free radical; antioxidant; kinetics; DNA; erythrocyte; linoleic acid**

## INTRODUCTION

The oxidations of cell membranes, DNA, and proteins induced by reactive oxygen species (ROS) are regarded as the etiopathogenesis of aging and some fatal diseases (*1*). Studies on the supplementation of antioxidants to maintain health and on the mechanism of antioxidants to protect biological issues have attracted much scientific attention (*2*). Antioxidants are traditionally composed of phenol- and amine-related compounds, in which the hydrogen atom in —OH and —NH is abstracted by radicals (*3*). In this decade, essential oils were proven to have a radical-scavenging effect (*4*) and even to have side effects (*5*). The antioxidant activity of the C—H bond attracts much attention. Some alkenes, such as β-caryophyllene, α-humulene, and isocaryophyllene, were recently reported to be potential anticancer drugs (*6*). α-Terpinene (α-TH) and γ-terpinene (γ-TH) (structures in **Scheme 1**), the major components in essential oils, were reported to retard the peroxidation of linoleic acid (*7*). The autoxidation of γ-TH induced by azobis(isobutyronitrile) (AIBN) can be inhibited by a very low concentration of $Fe^{3+}$, which is regarded as a simple model of superoxide dismutase (*8*). The autoxidation of γ-TH induced by AIBN provides a useful model for the research on the solvent effect (*9*). Although both α- and γ-TH can be oxidized to form *p*-cymene (*10*), it is still an attractive topic for the difference of the bioactivity of α- and γ-TH in biological experimental systems. Presented here is a comparison of the antioxidant effects of α- and γ-TH on the oxidation of methyl linoleate (LH), DNA, and erythrocytes induced by 2,2′-azobis (2-amidinopropane hydrochloride) [AAPH, R—N═N—R, R = —$CMe_2C(=NH)NH_2$]. AAPH oxidizes the super-coiled DNA strand to form carbonyl species (*11*) and hemolyze erythrocytes (*12*). Thus, AAPH can be used as a radical resource to mimic DNA and membrane undergoing oxidation induced by radicals. The results are dealt with a chemical kinetic method to obtain the number of radicals trapped by α- and γ-TH when α- and γ-TH protect DNA and erythrocytes.

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## MATERIALS AND METHODS

**Materials.** α-TH, γ-TH, AAPH, and naked DNA sodium salt were purchased from ACROS ORGANICS (Belgium) and used as received. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Fluka Chemie GmbH (Switzerland) and used as received. LH (>98%) was purchased from Tokyo Chemical Industry Co. (Japan), and other reagents with analytical grade were purchased from Beijing Chemical Reagent Co. (China) and used without further purification. Human erythrocytes were provided by the Red Cross Center for Blood, Changchun, China.

α- and γ-TH scavenge $ABTS^{+\bullet}$ and DPPH. ABTS (2.00 mL, 4.0 mM) was oxidized by 1.41 mM $K_2S_2O_8$ for 16 h to generate $ABTS^{+\bullet}$, and then 100 mL of ethanol was added to make the absorbance ($Abs_{ref}$) ∼ 0.70 at 734 nm. DPPH was dissolved in ethanol to make the absorbance ($Abs_{ref}$) ∼ 1.00 at 517 nm. The experiments of α- and γ-TH to trap $ABTS^{+\bullet}$ and DPPH were performed according to our previous report (*13*). Briefly, various concentrations of ethanol solution of α- or γ-TH were mixed with $ABTS^{+\bullet}$ solution for 7 h to obtain a stable absorbance ($Abs_{detect}$). α-TH was mixed with DPPH for 8 h to obtain

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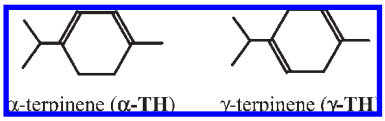
**Scheme 1.** Structure of α- and γ-TH




$Abs_{detect}$, and γ-TH was mixed with DPPH for 26 h to obtain $Abs_{detect}$. The percentages of DPPH or $ABTS^{+\bullet}$ scavenged by α- or γ-TH were calculated by $(1 - Abs_{detect}/Abs_{ref}) \times 100$ and plotted versus the concentration of terpinenes to obtain 50% inhibition concentration ($IC_{50}$).

**α- and γ-TH Protect LH against AAPH-Induced Oxidation.** The concentration of LH was detected by gas chromatography (Hewlett-Packard 1890 equipped with a SE-54 30 m × 0.25 mm capillary column, 0.25 μm film thickness, $N_2$, chamber temperature at 250 °C, and injector temperature at 280 °C) connected with a hydrogen flame ionization detector (detector temperature at 300 °C) (*14*). LH (14.3 mM as the final concentration) and AAPH (40 mM as the final concentration) were dissolved in *t*-butanol/$H_2O$ (1:1, v/v) (*15*) in a test tube with α- or γ-TH added to a final concentration at 1.14 mM. To quantitate the variety of the concentration of LH, methyl palmitate (9.3 mM as the final concentration) was added to the aforementioned solution as an internal standard because, as an ester of saturated fatty acid, it cannot be oxidized during the oxidation of LH. Then, the test tube was incubated at 37 °C in a thermostat to initiate the oxidation. Aliquots were taken out, and the concentration of LH was analyzed by gas chromatography (GC). The concentration of LH was obtained by comparing it to the concentration of methyl palmitate and plotted versus the reaction period.

**α- and γ-TH Protect DNA against AAPH-Induced Oxidation.** AAPH-induced oxidation of DNA was performed according to the literature (*16*), with a little modification (*17*). DNA and AAPH were dissolved in phosphate-buffered solution [PBSo: 8.1 mM $Na_2HPO_4$, 1.9 mM $NaH_2PO_4$, and 10.0 μM ethylenediaminetetraacetic acid (EDTA)] to a final concentration at 2.0 mg/mL and 40 mM, respectively, to which various concentrations of dimethyl sulfoxide (DMSO) solution of α- or γ-TH were added. Then, the above solution was dispatched into test tubes with 2.0 mL solution contained in every one. All of the test tubes were incubated in a water bath at 37 °C to initiate the reaction. Three test tubes were taken out at appropriate intervals and cooled immediately, to which 1.0 mL of thiobarbituric acid (TBA) solution (1.00 g of TBA and 0.40 g of NaOH dissolved in 100 mL of PBSo) and 1.0 mL of trichloroacetic acid (3.0 wt % aqueous solution) were added. The test tubes were heated in a boiling water bath for 15 min, and 1.5 mL of *n*-butanol was added and shaken vigorously to extract thiobarbituric acid reactive substances (TBARS). After cooling, the absorbance of the *n*-butanol layer was measured at 535 nm.

**α- and γ-TH Protect Erythrocytes against AAPH-Induced Oxidation.** The solvent employed to dissolve AAPH and to suspend erythrocytes was phosphate-buffered saline (PBSa: 150 mM NaCl, 8.1 mM $Na_2HPO_4$, 1.9 mM $NaH_2PO_4$, and 10 μM EDTA). After washed by PBSa to remove residual plasma, human erythrocytes with a compacted volume were obtained by centrifugation at 1700*g* for exactly 10 min (*18*). Hemolysis was performed as described in our previous reports (*19*). Briefly, AAPH and erythrocytes were mixed in PBSa to a final concentration at 20 mM and 3.0% (v/v), respectively, to which various concentrations of α- or γ-TH (dissolved in DMSO or ethanol as stock solutions) were added. The above mixture was incubated at 37 °C to initiate hemolysis. Aliquots (1.5 mL) were taken out at appropriate intervals and centrifuged at 1700*g* to obtain the supernatant, in which hemoglobin escaped from erythrocytes was dissolved. The absorbance of the supernatant ($\lambda_{max}$ = 535 nm) was measured and plotted versus the reaction time. It is worthy to note that the same volume of DMSO or ethanol (less than 1.0% to the total volume) was contained in the control experiment to eliminate the influence of organic solvents on hemolysis (*19*).

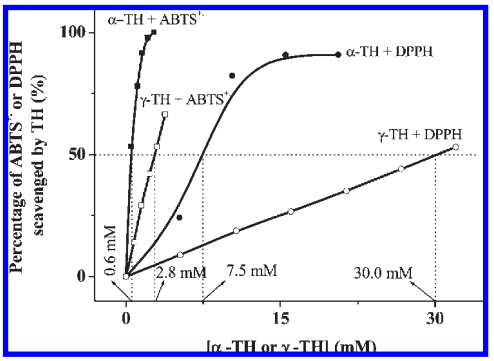



**Figure 1.** Percentage of $ABTS^{+\bullet}$ and DPPH scavenged by different concentrations of α- and γ-TH.

**Statistical Analysis.** All of the data were average values from three independent measurements, with the experimental error less than 10%. Equations were analyzed by one-way analysis of variation (ANOVA) using Origin 6.0 professional Software, and $p < 0.001$ indicated a significant difference.

## RESULTS

**Evidence for α- and γ-TH To Scavenge Radicals Directly. Figure 1** outlines the percentage of $ABTS^{+\bullet}$ and DPPH scavenged by various concentrations of α- and γ-TH.

As shown in **Figure 1**, $IC_{50}$ values of α- and γ-TH to trap $ABTS^{+\bullet}$ (0.6 and 2.8 mM, respectively) are lower than the corresponding values of α- and γ-TH to trap DPPH (7.5 and 30.0 mM, respectively). The reaction between $ABTS^{+\bullet}$ and an antioxidant indicates that the antioxidant is able to reduce $ABTS^{+\bullet}$ (*20*), and the reaction between DPPH and an antioxidant indicates that the hydrogen atom in the antioxidant can be abstracted by DPPH (*21*). Thus, low $IC_{50}$ values of α-TH to trap $ABTS^{+\bullet}$ and DPPH elucidate that its abilities to be a reducer and to donate its hydrogen atom are higher than those of γ-TH. The $IC_{50}$ of γ-TH has been reported as 122 ± 2.5 μg/mL when γ-TH reacts with DPPH (*22*). In our work, the absorbance was recorded after terpinenes were mixed with $ABTS^{+\bullet}$ and DPPH for a long period (see the second paragraph in the Materials and Methods) to obtain a stable absorbance at the end of the reaction between terpinene and radicals, but the reaction period was not mentioned in ref *22*.

**α- and γ-TH Protect LH against AAPH-Induced Oxidation.** The oxidation process of linoleic acid can be followed by high-performance liquid chromatography (HPLC) (*15*) or GC. If GC is applied to detect the decay of the concentration of linoleic acid, methyl ester of linoleic acid is employed because LH is readily evaporated and detected in GC (*14*). As shown in **Figure 2A**, methyl palmitate is added to the reaction system because an ester of a saturated fatty acid cannot be oxidized and will keep an invariable concentration during the decay of the concentration of LH. Thus, methyl palmitate can be an internal standard to measure the concentration of LH.

As shown in line a of **Figure 2**, in the presence of AAPH, the concentration of LH decreases with the increase of the reaction time. However, as shown in line b, the addition of α-TH retards the decrease of the concentration of LH at the beginning of the reaction and then the concentration of LH decreases similar to line a. As illustrated as the cross-point of two dot tangents, the addition of 1.14 mM α-TH generates an inhibition period ($t_{inh}$) of 165 min. Also, as line c shows,

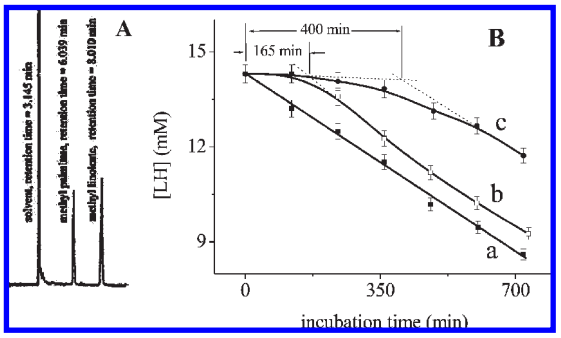



**Figure 2.** (A) GC chart and (B) decay of the concentration of 14.3 mM LH undergoing the oxidation induced by 40 mM AAPH in the (a) absence and presence of 1.14 mM (b) α-TH or (c) γ-TH.

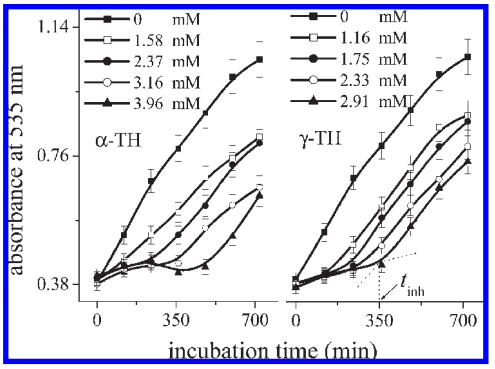



**Figure 3.** Increase of the absorbance at 535 nm in the absence (■) and presence (□, ●, ○, and ▲) of different concentrations of α- and γ-TH when DNA (2.0 mg/mL) is subjected to the oxidation induced by AAPH (40 mM) at 37 °C.

the same concentration of γ-TH leads to 400 min of $t_{inh}$ generated, revealing that the antioxidant ability of γ-TH is 2.4-fold (400/165 min) higher than α-TH in protecting LH.

**α- and γ-TH protect DNA against AAPH-induced oxidative damage.** In the presence of AAPH, the super-coiled DNA is transformed into the open circular and the linear forms and eventually more than 20 carbonyl species (*11*). Carbonyl species are readily determined after reacting with TBA to form TBARS (*16*). As **Figure 3** shows, in the control experiment, the increase of the absorbance of TBARS indicates that much more carbonyl species are generated with the reaction time increasing.

The additions of α- and γ-TH (dissolved in DMSO as the stock solution) slow the formation of TBARS, demonstrating that they are able to protect DNA against the oxidation induced by AAPH. Moreover, a turning point can be found in the process of the formation of TBARS, as shown in **Figure 3**. Consequently, the addition of γ-TH generates $t_{inh}$. A $t_{inh}$ is also found in the presence of α-TH, but the shape of the line within $t_{inh}$ is wavy when a high concentration of α-TH is employed. Although a wavy line is related to the pro-oxidant effect of an antioxidant as previous literature pointed out (*23*), the equations between $t_{inh}$ and the concentrations of α- and γ-TH are listed in **Table 1** (*vide post*). The coefficient in the equations reveals the sensitivity of $t_{inh}$ to the variety of the concentration for α- and γ-TH. The similarity of the coefficients of $t_{inh} \sim$ [α-TH] and $t_{inh} \sim$ [γ-TH] reveals that α- and γ-TH possess a similar antioxidant effect on DNA.

**α- and γ-TH Protect Erythrocytes against AAPH-Induced Hemolysis. Figure 4** outlines the variety of the absorbance

**Table 1.** Equations of $t_{inh} \sim$ [α- or γ-TH] and *n* of α- and γ-TH in Protecting DNA, Erythrocytes, and LH

| $t_{inh}$ (min) = A [α- or γ-TH (μM)] + C | *n* |
|---|---|
| in Protecting DNA[a] | |
| $t_{inh}$ = 0.115 [α-TH] − 3.94 | 0.39 |
| $t_{inh}$ = 0.122 [γ-TH] + 2.87 | 0.41 |
| in Protecting Erythrocytes[a] | |
| $t_{inh}$= 0.42 [α-TH] + 210.09 | 0.71 |
| $t_{inh}$ = 0.72 [γ-TH] + 208.17 | 1.21 |
| in Protecting LH[b] | |
| $t_{inh}$ of α-TH = 165 min | 0.49 |
| $t_{inh}$ of γ-TH = 400 min | 1.18 |

[a] The rate of radical initiation ($R_i$) is equal to the rate of radical generation ($R_g$); $R_i = R_g = 1.4 \times 10^{-6}$ [AAPH] $s^{-1} = 3.36$ μM $min^{-1}$ when [AAPH] = 40 mM in protecting DNA and $R_i = R_g = 1.68$ μM $min^{-1}$ when [AAPH] = 20 mM in protecting erythrocytes. Thus, the *stoichiometric factor* (*n*) is the product of the coefficient in the equation of $t_{inh} \sim$ [terpinene] and $R_i$, $n = A \times R_i$. [b] The *n* of α- and γ-TH in protecting LH can be calculated by $t_{inh} = (n/R_i)$ [terpinene] when [terpinene] = 1.14 mM and $R_i = R_g = 1.4 \times 10^{-6}$ [AAPH] $s^{-1} = 3.36$ μM $min^{-1}$ when [AAPH] = 40 mM.

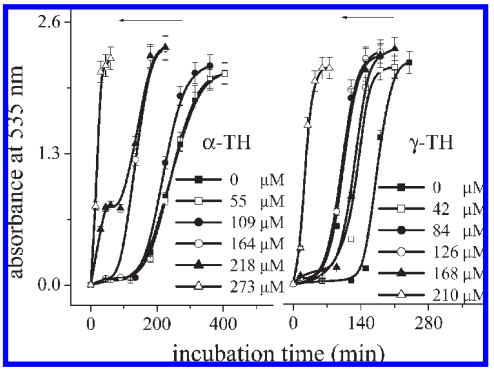



**Figure 4.** Increase of the absorbance at 535 nm in the absence and presence of various concentrations of α- and γ-TH (dissolved in DMSO as the stock solution) when erythrocytes (3.0% suspension in PBSa) are subjected to the oxidation induced by AAPH (20 mM) at 37 °C.

when erythrocytes are subjected to AAPH-induced hemolysis. As shown in the blank experiment ([terpinene] = 0 μM), hemolysis does not take place at the first period because of the protective effect of endogenous antioxidants on erythrocyte membranes. After the endogenous antioxidants are completely exhausted, hemolysis takes place, resulting in a $t_{inh}$. The additional antioxidant can prolong $t_{inh}$ (*24*). However, as shown in **Figure 4**, the addition of α- or γ-TH (dissolved in DMSO as the stock solution) does not prolong $t_{inh}$. Thus, **Figure 5** illustrates the relationship between $t_{inh}$ and the concentration of α- and γ-TH.

As shown in **Figure 5**, $t_{inh}$ is similar to the blank experiment when the concentration of α-TH is below 110 μM, indicating that α-TH cannot affect hemolysis with the concentration lower than 110 μM. However, $t_{inh}$ decreases rapidly when the concentration of α-TH exceeds 110 μM, indicating that α-TH promotes hemolysis with the concentration increasing. In addition, $t_{inh}$ decreases promptly when γ-TH is added, although $t_{inh}$ increases when [γ-TH] = 168 μM (the reason is not clear and will be explored in further work). It is still confirmed that $t_{inh}$ decreases with the concentration of γ-TH increasing.

**Figure 6** illustrates the protective effects of α- and γ-TH on erythrocytes when they are dissolved in ethanol as the stock

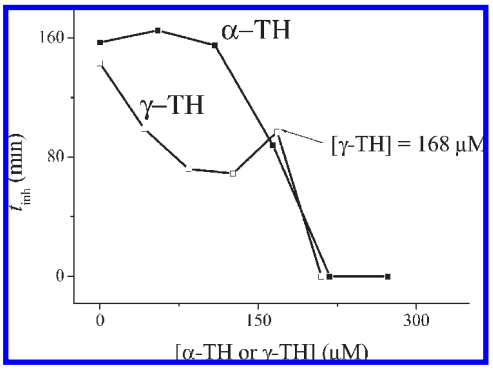



**Figure 5.** Relationships between $t_{inh}$ and the concentration of α- and γ-TH (dissolved in DMSO as the stock solution) in protecting erythrocytes.

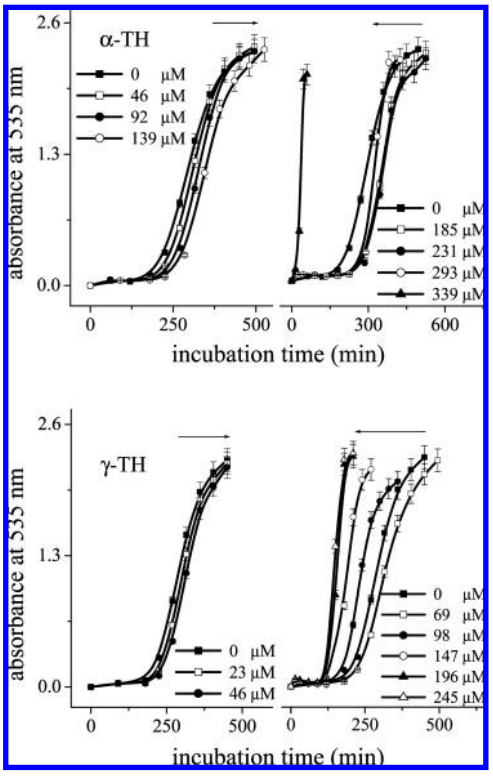



**Figure 6.** Increase of the absorbance at 535 nm in the absence and presence of various concentrations of α- and γ-TH (dissolved in ethanol as the stock solution) when erythrocytes (3.0% suspension in PBSa) are subjected to the oxidation induced by AAPH (20 mM) at 37 °C.

solution. The $t_{inh}$ increases when low concentrations of α- and γ-TH are employed and then decreases with the concentration of α- and γ-TH increasing. **Figure 7** shows the relationship between $t_{inh}$ and the concentration of α- and γ-TH.

α-TH prolongs $t_{inh}$ when its concentration is below 140 μM, demonstrating that α-TH acts as an antioxidant to protect erythrocytes, with the concentration ranging from 0 to 140 μM. The concentration for γ-TH to be an antioxidant ranges from 0 to 45 μM, and γ-TH accelerates hemolysis when the concentration exceeds 45 μM. Thus, α- and γ-TH dissolved in ethanol exhibit antioxidant activities below a certain concentration, and the range of the concentration for α-TH to be an antioxidant is much wider than that of γ-TH. Moreover, the relationships between $t_{inh} \sim$ [α- and γ-TH] are expressed by equations and included in

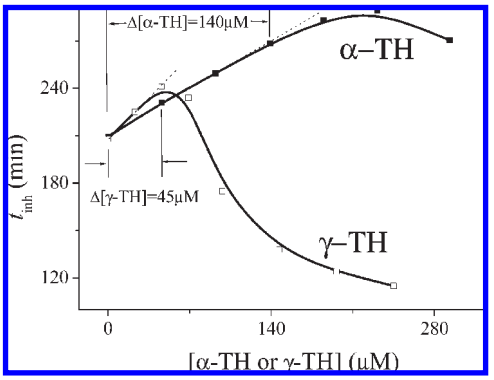



**Figure 7.** Relationships between $t_{inh}$ and the concentration of α- and γ-TH (dissolved in ethanol as the stock solution) in protecting erythrocytes.

**Table 1** (*vide post*), namely, equations of the dot tangents in **Figure 7**. A relatively high coefficient in the equation of $t_{inh} \sim$ [γ-TH] implicates that $t_{inh}$ is more sensitive to the variety of the concentration for γ-TH than that for α-TH. The efficiency of γ-TH to protect erythrocytes is higher than α-TH within the available range of the concentration.

## DISCUSSION

The pharmaceutical and therapeutic potentials of essential oils regarding the prevention of a variety of diseases and the bioactivity, such as antibacterial, antiviral, antioxidative, and antidiabetic agents, were recently reviewed (*25*). The antioxidant activity of γ-TH is higher than that of α-TH and even than that of 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) (*26*). However, different determination methods and mixed extraction employed always lead to inconsistent results (*27*). The properties of α- and γ-TH to scavenge free radical are still worthy to be discussed because the chemical meaning of the equations of $t_{inh} \sim$ [α- or γ-TH] derived from DNA and erythrocytes is an attractive topic.

The relationship between $t_{inh}$ and the concentration of an antioxidant (AH) can be expressed as eq **1** (*28*).

$$t_{inh} = (n/R_i)[AH] \quad (1)$$

The $n$ is a *stoichiometric* factor that can be regarded as the number of radical propagation chains terminated by one molecule of an antioxidant, and $R_i$ is the initiation rate of a radical-induced reaction (*3*). Consequently, $n$, the product of $R_i$ multiplying the coefficient of eq **1**, behaves as a quantitative index to express the ability of an antioxidant to terminate radical propagation chains. Nevertheless, it is difficult to measure $R_i$ directly because not all of the radicals generated from the decomposition of AAPH can initiate a radical propagation chain. Thus, to apply eq **1** to calculate $n$ of an antioxidant, trolox is always selected to be the reference antioxidant and the relationship of $t_{inh} \sim$ [trolox] is first measured. Then, $R_i$ is obtained when $n$ of trolox is taken as 2 (*29*). Accordingly, trolox is applied to protect DNA against AAPH-induced oxidation in the present work, but $t_{inh}$ is not observed (data not shown). Although it was reported that trolox can protect DNA against radical-induced oxidation (*30*), it just hinders the conversion of super-coiled DNA to open-circular or linear form. It cannot prohibit DNA to form carbonyl species. Thus, the measurement of TBARS is not appropriate to characterize the antioxidant effect of trolox on DNA. On the other hand, our previous work (*19*) proved that $R_i$ is equal to the rate of the radical generated ($R_g$)

from the decomposition of AAPH ($R_g = (1.4 \pm 0.2) \times 10^{-6}$ [AAPH] $s^{-1}$; *23*) when trolox was employed to be the reference antioxidant to protect erythrocytes against AAPH-induced hemolysis. Thus, it is safe to assume that $R_i$ is still equal to $R_g$ to apply eq **1** to calculate *n* of terpinenes in the oxidation of DNA and erythrocytes, and the results are listed in **Table 1**.

Because these two terpinenes are isomers, **Table 1** reveals that *n* of α-TH approaches that of γ-TH when they protect DNA, indicating that they possess a similar protective effect on DNA. The *n* of γ-TH (1.21) is higher than that of α-TH (0.71) when they protect erythrocytes, indicating that γ-TH protects erythrocytes more efficiently than α-TH, although the available range of concentrations for γ-TH to be an antioxidant is not as wide as α-TH. However, *n* of γ-TH (1.18) is much higher than that of α-TH (0.49) when they protect LH, indicating that γ-TH serves as an efficient antioxidant more than α-TH in this case. The aforementioned results reveal that γ-TH possesses a relatively high antioxidant effect on erythrocytes and LH than α-TH. The difference between the structure of these two isomers is that γ-TH is a nonconjugative cyclohexadiene, whereas α-TH is a conjugative one. Thus, γ-TH forms a radical with the H atom on the methylene at the bisallyl position abstracted, whereas α-TH forms a radical by the H atom on the methylene at the allyl position abstracted. It can deduce that the methylene at the bisallyl position benefits for increasing the antioxidant ability more than a conjugated diene.

In summary, α- and γ-TH exhibit a similar protective effect on the oxidation of DNA induced by AAPH and promote AAPH-induced hemolysis of erythrocytes with the concentration increasing. Low concentrations of α- and γ-TH (dissolved in ethanol as stock solutions) protect erythrocytes against AAPH-induced hemolysis, in which the activity of γ-TH is higher than α-TH. The ability of γ-TH to protect LH against AAPH-induced peroxidation is also higher than α-TH. Therefore, methylene at the bisallyl position in a nonconjugated diene rather than in a conjugated diene increases the antioxidant ability.

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