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Electron Spin Resonance Estimation of Hydroxyl Radical Scavenging Capacity for Lipophilic Antioxidants

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Hydroxyl radical scavenging capacity estimation for lipophilic antioxidants is a challenge due to their poor solubility in aqueous radical generating and measuring systems. In this study, an electron spin resonance (ESR) method was developed and validated for its application in estimating the relative hydroxyl radical (HO•) scavenging capacity for lipophilic antioxidants under physiological pH using a Fenton Fe^{2+}/H_2O_2 system for radical generation and acetonitrile as a solvent. The Fenton Fe^{2+}/H_2O_2 system generates a constant flux of pure HO• under the assay conditions. The method was validated by linearity, precision, and reproducibility using selected known lipophilic antioxidants including α-tocopherol, lutein, β-carotene, and BHT. The potential effects of commonly used water-miscible and water-immiscible organic solvents on the Fenton Fe^{2+}/H_2O_2 HO• generating system as well as their possible interactions with the fluorescent and spectroscopic probes were also reported. In addition, the limitation of the ESR assay was described.

KEYWORDS: Hydroxyl radical; lipophilic antioxidant; ESR; Fenton reaction; solvent effect

INTRODUCTION

Antioxidants are well recognized for their potential role in reducing the risk of several aging-associated human diseases such as cardiovascular disorder and cancer (*1*−*3*). Free radical medicated oxidative chain reaction is a commonly accepted mechanism of lipid peroxidation in the biological systems, and this mechanism may apply to oxidation of other cellular components such as cholesterol and proteins. Antioxidants may suppress the initiation and propagation of the oxidative chain reaction through a number of mechanisms including but not limited to chelating transition metals, quenching free radicals, and reducing peroxides. Generally, antioxidants are classified into hydrophilic and lipophilic antioxidants according to their solubility in water or nonpolar organic solvents. It is well accepted that hydrophilic or hydrophobic antioxidants may act synergistically and provide stronger protection to biological systems and reduce the damage caused by reactive oxygen species (ROS) including free oxygen centered radicals (*4*). In contrast to hydrophilic antioxidants, lipophilic antioxidants have a tendency to be distributed in or penetrate into the hydrophobic domain of cell membranes and biological particles such as the nonpolar core of lipoproteins. Tocopherols, the most important representatives of lipophilic antioxidants, may act mainly as oxygen radical scavengers thus to inhibit the initiation and propagation of the oxidative chain reactions in hydrophobic systems. It was also suggested that tocopherols incorporated in the cellular membrane might interact with and scavenge hydrophilic ROS in the surrounding aqueous phase outside of cells because their chroman head groups might orient near the surface of cell membranes (*5*). Lipophilic antioxidants may provide stronger protection against lipid peroxidation and other free radical mediated oxidative damage to important biological molecules because of their better cellular availability (*4*). Rapid, simple, reliable, and physiologically relevant in vitro assays are in high demand for screening and development of novel lipophilic antioxidants.

Among all known ROS, hydroxyl radical (HO•) is the most damaging one, which may be produced under the physiological conditions and reacts rapidly with almost any biological molecule and may be involved in pathology of many human diseases (*6*). The production of HO• in biological systems is mainly attributed to the decomposition of hydrogen peroxide and superoxide catalyzed by transition metals including Fe and Cu. Fenton and Fenton-like reactions generating HO• through a similar chemical mechanism may serve as in vitro models for investigating the HO• scavenging properties of lipophilic antioxidants. In 2006, a novel in vitro fluorometric assay was developed for HO• scavenging capacity (HOSC) estimation using a Fenton-like Fe^{3+}/H_2O_2 system at the physiological pH (*7*). This assay may be performed in aqueous acetone and is superior to other existing HO• scavenging capacity assays because pure HO• was generated at a constant concentration under physiologically relevant conditions during the entire

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testing period. However, this assay cannot be used to estimate HO• scavenging capacity of lipophilic antioxidants due to their poor solubility in aqueous acetone.

In vitro antioxidant property estimation is still a challenge for lipophilic antioxidants. Antioxidant properties of lipophilic components might be underestimated or could not be measured in aqueous systems because of their poor solubility in these testing systems such as that in the oxygen radical absorbing capacity (ORAC), HOSC, and total peroxyl radical-trapping potential (TRAP) assays (*7*−*9*). Efforts have been made to enhance the solubility of lipophilic antioxidants in aqueous phases to be able to examine their antioxidant properties. These included the utilization of amphiphilic solvents such as DMSO (*10*) and solubilizing agents such as randomly methylated β-cyclodextrin (RMCD) (*11*, *12*). It was reported that RMCD might reduce the total HO• concentration in the assay system when using the Fenton-like Fe^{3+}/H_2O_2 system for radical generation (*7*), suggesting its capacity to directly react with and quench HO•, which may lead to overestimation of antioxidant capacity for lipophilic components. These previous studies also indicated that solubilizing agents might alter the radical purity in the testing systems and lead to inaccurate estimation of HO• scavenging capacity for lipophilic antioxidants (*7*). Several organic solvents including DMSO, methanol, and ethanol were able to generate carbon-centered radicals (*7*, *13*, *14*), showing possible solvent interference in HOSC assay, which measures HO• scavenging capacity using the Fenton-like Fe^{3+}/H_2O_2 system. Therefore, this study was undertaken to develop a practical assay for examining HO• scavenging capacity of lipophilic antioxidants such as lutein and β-carotene under the physiological pH.

MATERIALS AND METHODS

Chemicals. Disodium ethylenediaminetetraacetate (EDTA), iron(III) chloride, iron(II) sulfate heptahydrate, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), fluorescein (FL), nitro blue tetrazolium (NBT), 5,5-dimethyl *N*-oxide pyrroline (DMPO), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), butylated hydroxytoluene (BHT), α-, δ-, and γ-tocopherols, lutein, and β-carotene were all purchased from Sigma-Aldrich (St. Louis, MO). 30% hydrogen peroxide (H_2O_2) was purchased from Fisher Scientific. Randomly methylated β-cyclodextrin (RMCD) was purchased from Cyclolab R&D Ltd. (Budapest, Hungary). Ultrapure water was used for all experiments from an ELGA (Lowell, MA) A Purelab Ultra Genetic polishing system with <5 ppb TOC and resistivity of 18.2 mΩ. All other chemicals and solvents were of the highest commercial grade and used without further purification.

The Fenton Reaction System. The classical Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^-$) was used to generate HO• at pH 7.4. The typical spin-trapping reaction mixture consisted of 30 μL of 250 mM DMPO, 30 μL of 1.0 mM H_2O_2, 30 μL of 1.0 mM EDTA, 22.5 μL of 100 mM sodium phosphate buffer (PBS, pH 7.4), 7.5 μL of lipophilic antioxidants or acetonitrile for blank, and 30 μL of 1.0 mM $FeSO_4$, added in that order. The solution of $FeSO_4$ was freshly prepared daily and kept on ice under N_2. The reaction mixture was vortexed, and the electron spin resonance (ESR) spectrum was recorded after 2 min subsequent to addition of $FeSO_4$ at the ambient temperature.

The Fenton-like Reaction System. The Fenton-like reaction mixture contained 40 μL of 400 mM DMPO, 40 μL of 100 mM H_2O_2, 32 μL of 100 mM PBS (pH 7.4), 8 μL of acetonitrile, and 40 μL of 2.72 mM $FeCl_3$, added in that order (*7*). Final concentrations were 100 mM DMPO, 25 mM H_2O_2, 20 mM PBS, 0.68 mM $FeCl_3$, and 5% (v/v) solvent blank in each reaction mixture. All reaction mixtures were vortexed, and the ESR spectra were recorded at 5, 10, and 20 min of the reactions at the ambient temperature.

ESR Spin-Trapping Assay. All ESR experiments were conducted at ambient temperature using a Varian E-109 X-band spectrometer (Varian, Inc., Palo Alto, CA). Individual samples were placed in the ESR cavity using two 50 μL glass capillaries for the measurements. Typical spectrometer parameters were: scan range, 100 G; receiver gain, 8×10^3; microwave power, 15 mW; and field modulation, 1 G. Results were reported as means of duplicate measurements.

Fluorometric and Spectrophotometric Measurements of HO• Scavenging Capacity for Lipophilic Antioxidants in Fenton Reaction System. Fluorometric and spectrophotometric measurements were performed using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland). All components of Fenton reaction were identical to the ESR experiments, except that a fluorescent or spectroscopic probe was used to replace DMPO, the spin trapping agent. Fluorescein and dichlorofluorescein (DCFH) were tested for their potentials to be used for fluorometric determination of HO• scavenging capacity (*7*, *15*), whereas nitro blue tetrazolium (NBT) was evaluated as a possible spectroscopic probe (*16*). DCFH was prepared from dichlorofluorescin diacetate (DCF-DA) by hydrolysis with 1.0 mM KOH under the conditions previously described (*15*). The Fenton reaction conditions were the same as those for ESR determination, while the total volume of each reaction mixture was 250 μL. The 96-well plate was covered with a lid to prevent solvent evaporation during determination. All experiments were carried out in duplicate measurements.

RESULTS AND DISCUSSION

Selecting a Hydroxyl Radical Generation System. Pure HO• at constant concentration is required during the entire assay period for examining radical scavenging capacity of lipophilic antioxidants. In 2006, Moore and others reviewed available HO• generating systems and classified them into five categories including the classic Fenton reaction, the superoxide-driven Fenton reaction, Fenton-like system, pulse radiolysis of water, and the photo-Fenton system, based on the chemical reactions involved in the radical generation (*7*). A Fenton-like Fe^{3+}/H_2O_2 reaction system was developed to generate pure hydroxyl radicals at constant concentration for measuring HO• scavenging capacity of antioxidants that have adequate solubility in aqueous acetone at physiological pH (*7*). Attempt was first made to identify a solvent that may dissolve lipophilic antioxidants and is compatible with the aqueous Fenton-like Fe^{3+}/H_2O_2 testing system. Acetonitrile is an organic solvent compatible with water and has significant solubility for lipophilic compounds including tocopherols and β-carotene. The possible effect of acetonitrile on hydroxyl radicals generated by the Fenton-like Fe^{3+}/H_2O_2 system was thus evaluated because some organic solvents may interfere with the radical generation reactions (*7*). **Figure 1** shows the time-dependent ESR spectra of DMPO/HO• spin adducts formed during the Fe^{3+}/H_2O_2 reaction with the presence of 5% acetonitrile (v/v). The ESR spectra of DMPO/HO• spin adducts formed at the different concentrations of acetonitrile in the Fe^{3+}/H_2O_2 reaction system are presented in **Figure 2**. Acetonitrile may reduce the total hydroxyl radical concentration in the assay mixture at low dose and may generate carbon-centered radicals at doses of 10−20% (v/v). These data suggested that acetonitrile is not a suitable solvent for measuring HO• scavenging capacity of antioxidants using the Fenton-like Fe^{3+}/H_2O_2 system.

It was shown that the HO• production was reliable and stable using the classic Fenton Fe^{2+}/H_2O_2 radical generating system, and the amounts of DMPO/HO• formed in this system were proportional with ESR peak height or signal intensity, which was measured at the second peak of the ESR curve in the spectra (*17*). The antioxidant capacities may be determined by measuring and comparing the decrease of ESR peak height with and without the presence of antioxidants using the Fenton Fe^{2+}/H_2O_2 radical generating system. The Fenton Fe^{2+}/H_2O_2 system coupled with ESR measurements was successfully used to

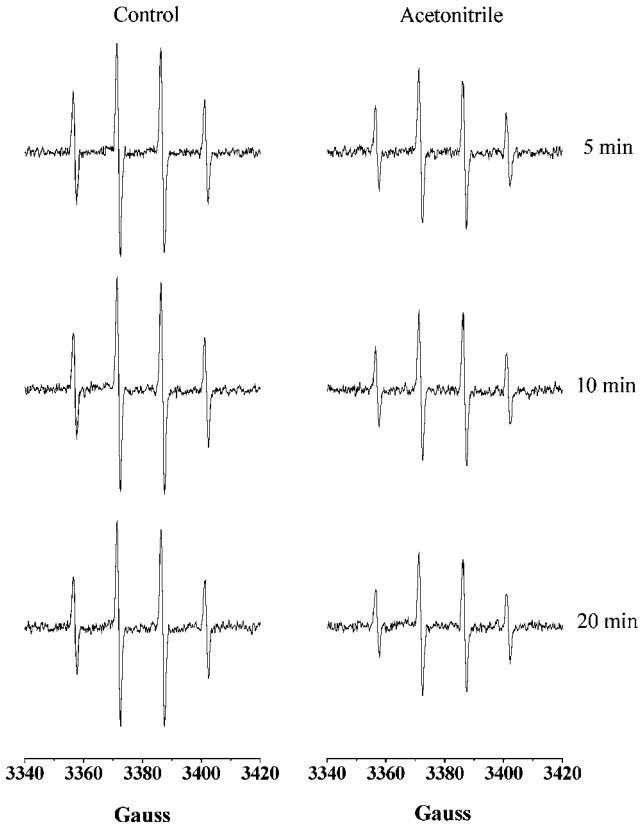

Figure 1. Time-dependent effect of acetonitrile on DMPO/HO• production in Fenton-like reaction (Fe^{3+}/H_2O_2) system measured by ESR. Reactions were performed at ambient temperature in sodium phosphate buffer (PBS, 100 mM, pH 7.4). All reaction mixtures contained 40 μL of 400 mM DMPO, 40 μL of 100 mM H_2O_2, and 40 μL of 2.72 mM Fe^{3+} in the absence (control: 5% PBS) or presence of 5% acetonitrile (v/v).

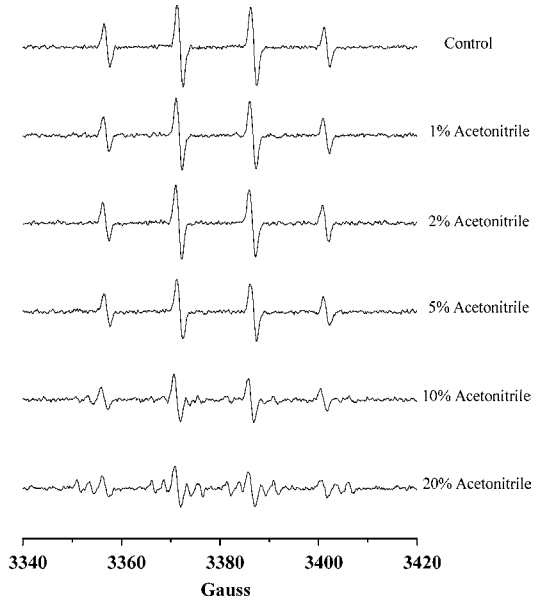

Figure 2. Dose-dependent effect of acetonitrile on DMPO/HO• production in Fenton-like reaction (Fe^{3+}/H_2O_2) system measured by ESR. Reactions were performed at ambient temperature in PBS (100 mM, pH 7.4), and detected at 20 min after the reaction was initiated. All reaction mixtures contained 40 μL of 400 mM DMPO, 40 μL of 100 mM H_2O_2, and 40 μL of 2.72 mM Fe^{3+} in the absence (control: PBS) or presence of different percents of acetonitrile (v/v).

evaluate the HO• scavenging capacity of several phenolic acids (*18*, *19*). In the present study, the Fenton Fe^{2+}/H_2O_2 system was further investigated for the purity and concentration consistency of HO• generated at pH 7.4. Typical ESR spectra of the Fenton Fe^{2+}/H_2O_2 system at 1, 5, 10, 15, and 20 min of the reaction in the absence of any antioxidant or organic solvent showed that the ESR peak height remained consistent (data not shown) during the measurement period with a relative standard deviation (RSD) value of 3.30% ($n = 5$), indicating its possible application for evaluating antioxidant properties against HO•. Further investigations were performed using the Fenton Fe^{2+}/H_2O_2 system to develop a HO• scavenging capacity assay for lipophilic antioxidants.

Effect of Commonly Used Solvents on the Fenton Fe^{2+}/H_2O_2 HO• Generation System. Previous studies have detected the possible interaction of organic solvents with HO•, and the effect of this interaction on antioxidant capacity estimation (*7*, *13*, *14*). Acetone was a recommended solvent for HO• scavenging capacity estimation of hydrophilic antioxidants using the Fenton-like Fe^{3+}/H_2O_2 reaction system (*7*), but had interference with the Fenton Fe^{2+}/H_2O_2 system during our preliminary examination, suggesting that the effect of a selected solvent on HO• scavenging capacity evaluation depends on the reaction systems used for radical generation. Different organic solvents and their mixtures have been used for extraction and evaluation of natural lipophilic and hydrophilic antioxidants. To use the Fenton Fe^{2+}/H_2O_2 system for estimating the HO• scavenging capacity of lipophilic antioxidants, it is necessary to understand the possible effect of these solvents on this HO• generation system.

A number of commonly used organic solvents were selected and tested for their potential interaction with the Fenton Fe^{2+}/H_2O_2 reaction system. Based on solubility in water, organic solvents may be generally classified into two groups: the water-immiscible (immiscible) ones such as chloroform, hexane, benzene, ethyl acetate, and *n*-butanol, as well as the water-miscible (miscible) ones mainly including acetone, acetonitrile, methanol, and ethanol. **Figure 3** shows the ESR spectra of DMPO-radical adducts in the Fenton Fe^{2+}/H_2O_2 reaction system with individual immiscible solvents. Slight reduction of the ESR signal intensity at about 3.1%, 11.2%, and 4.6% was observed in the presence of hexane, cyclohexane, and chloroform, respectively, at a final concentration of 5% (v/v) in the Fenton reaction system (**Figure 3**). Under the same experimental conditions, benzene, ethyl acetate, and toluene significantly decreased the ESR signal intensity to about 66.1%, 63.9%, and 23.7% of that in the control reaction without any organic solvent. Also noted was that carbon-centered radicals were produced during the reactions in the presence of chloroform, ethyl ether, and *n*-butanol (**Figure 3**). These solvent effects cannot be explained by their chemical structures or their different partition coefficients between oil and water.

Further investigation was performed to evaluate and compare hexane and chloroform for their potential utilization in HO• scavenging capacity examination for lipophilic antioxidants. The ESR results showed that α-tocopherol dissolved in hexane or chloroform did not quench HO• generated by the Fenton Fe^{2+}/H_2O_2 reaction system (**Figure 4**). Further increase of α-tocopherol concentration in hexane or chloroform did not significantly increase its radical scavenging capacity as measured by ESR with the Fenton system (data not shown). The presence of these lipophilic solvents may suppress the diffusion of lipophilic antioxidant molecules into the aqueous phase where the radicals are formed and available for antioxidant-radical reactions. These data suggest that immiscible organic solvents are not suitable for evaluation of lipophilic antioxidants against HO• because

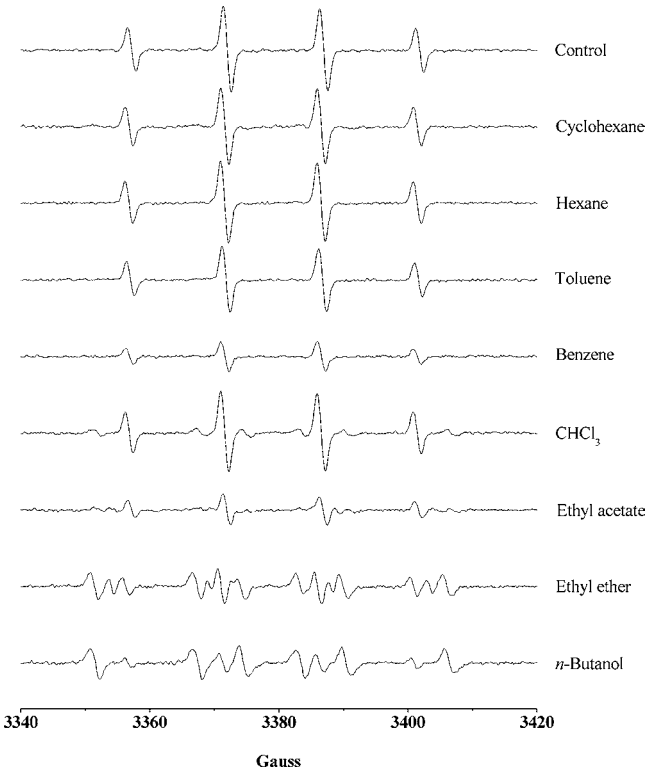

Figure 3. Effect of selected immiscible solvents on DMPO/HO• production. Measurements were conducted via ESR in Fenton Fe^{2+}/H_2O_2 reaction system 20 min after the reaction was initiated. Reactions were performed at ambient temperature in PBS (100 mM, pH 7.4). All reaction mixtures contained 30 μL of 250 mM DMPO, 30 μL of 1 mM H_2O_2, 30 μL of 1 mM EDTA, and 30 μL of 1 mM Fe^{2+} in the absence (control: 5% PBS) or presence of 5% solvents (v/v).

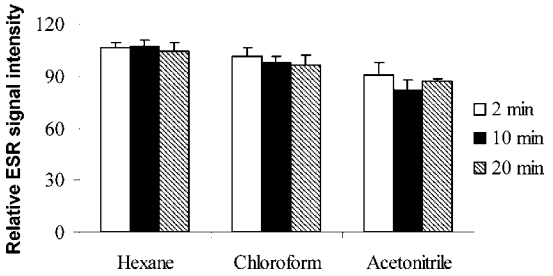

Figure 4. Effect of 0.55 mM α-tocopherol on DMPO/HO• production in three different solvents detected by ESR in Fenton Fe^{2+}/H_2O_2 reaction system. Reactions were performed at ambient temperature in PBS (100 mM, pH 7.4). All reaction mixtures contained 30 μL of 250 mM DMPO, 30 μL of 1 mM H_2O_2, 30 μL of 1 mM EDTA, and 30 μL of 1 mM Fe^{2+} in the absence (control: 5% acetonitrile) or presence of 5% α-tocopherol (v/v).

of suppressed diffusion of lipophilic antioxidants into the aqueous Fenton reaction systems, although they have better solubility for lipophilic antioxidants and may not interfere with the Fenton Fe^{2+}/H_2O_2 reaction system.

DMSO, ethanol, methanol, acetone, and acetonitrile are miscible organic solvents commonly used in antioxidant extraction and evaluation. The effect of these solvents on the Fenton Fe^{2+}/H_2O_2 HO• generating system is reported in **Figure 5**. Methanol, ethanol, and DMSO had strong interaction with this HO• generating system, showing HO• scavenging capacities and the formation of carbon-centered free radicals, which was consistent with the previous reports (*13*, *14*). Formation of carbon-centered radicals may lead to the inaccurate estimation

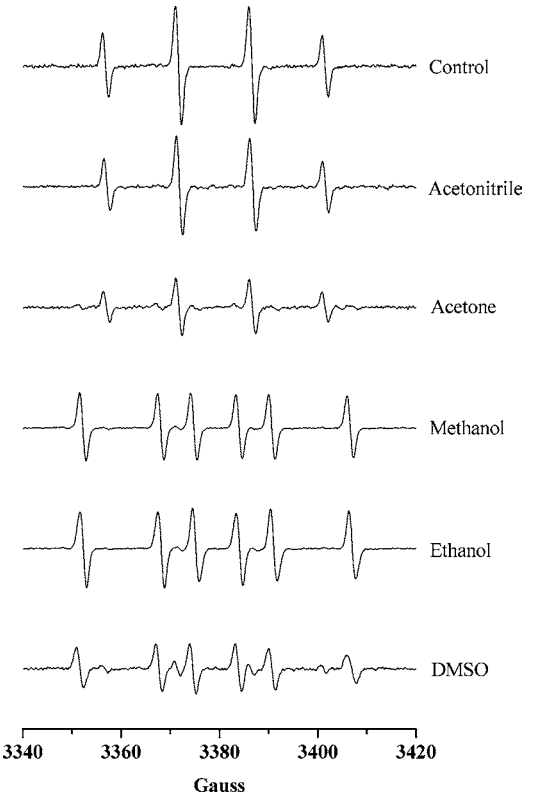

Figure 5. Effect of selected miscible solvents on DMPO/HO• production detected by ESR in Fenton Fe^{2+}/H_2O_2 reaction system at 20 min of the reaction. Reactions were performed at ambient temperature in PBS (100 mM, pH 7.4). All reaction mixtures contained 30 μL of 250 mM DMPO, 30 μL of 1 mM H_2O_2, 30 μL of 1 mM EDTA, and 30 μL of 1 mM Fe^{2+} in the absence (control: 5% PBS) or presence of 5% solvents (v/v).

of HO• scavenging capacity for a selected antioxidant sample. Furthermore, these three solvents were found to significantly inhibit the ESR signal intensity in a time- and dose-dependent manner without addition of any antioxidant (data not shown), indicating that these solvents cannot be used in evaluation of HO• scavenging capacities. In contrast, the presence of acetone and acetonitrile did not generate carbon-centered radicals in the Fenton Fe^{2+}/H_2O_2 system, and acetone at the same concentration resulted in a larger reduction of ESR signal intensity, which may be associated with a lower measuring sensitivity of HO• scavenging capacity (**Figure 5**). A 39.4% decrease of ESR signal intensity was observed 2 min after acetone was added in the Fenton reaction system, although no further decrease in the ESR signal was noted from 2 to 20 min measurements (average decrease of 43.3%, RSD = 3.93%). Additional investigation showed no obvious effect on DMPO/HO• production by increasing acetonitrile concentration from 1% to 20% (v/v) in the Fenton Fe^{2+}/H_2O_2 reaction system (five concentrations, RSD = 5.93%) (**Figure 6**), although the ESR signal intensity decreased 7.7% on an average in 20 min (**Figure 7**). This is highly consistent with the finding that acetonitrile has the lowest rate constant for reacting with HO• among commonly used organic solvents (*20*). It is well known that acetonitrile has excellent solubility for lipophilic compounds including carotenoids and tocopherols. Taken together, these results indicated that the Fenton Fe^{2+}/H_2O_2 reaction system may produce a constant flux of pure HO• in the presence of acetonitrile at physiological pH, and acetonitrile may be used as a solvent for HO• scavenging capacity estimation of lipophilic antioxidants using this radical generating system.

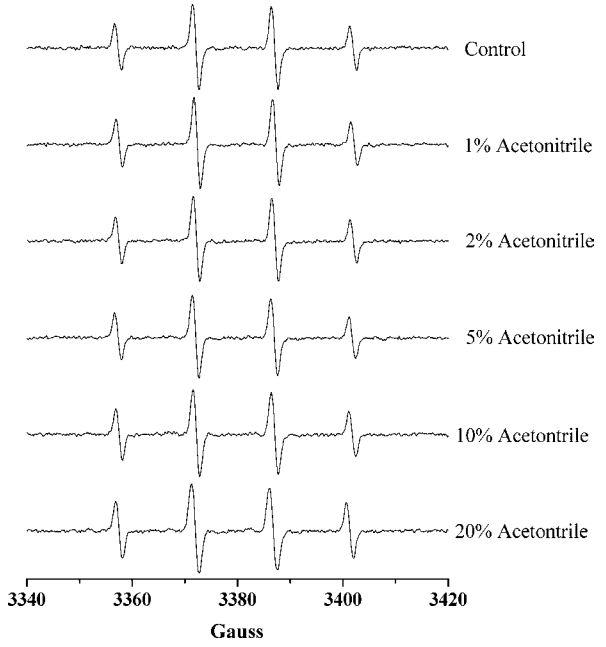

Figure 6. Dose-dependent ESR spectra of acetonitrile on DMPO/HO• production in Fenton Fe^{2+}/H_2O_2 reaction system at 20 min of the reaction. Reactions were performed at ambient temperature in PBS (100 mM, pH 7.4). All reaction mixtures contained 30 μL of 250 mM DMPO, 30 μL of 1 mM H_2O_2, 30 μL of 1 mM EDTA, and 30 μL of 1 mM Fe^{2+} in the absence (control: PBS) or presence of 1–20% acetonitrile (v/v).

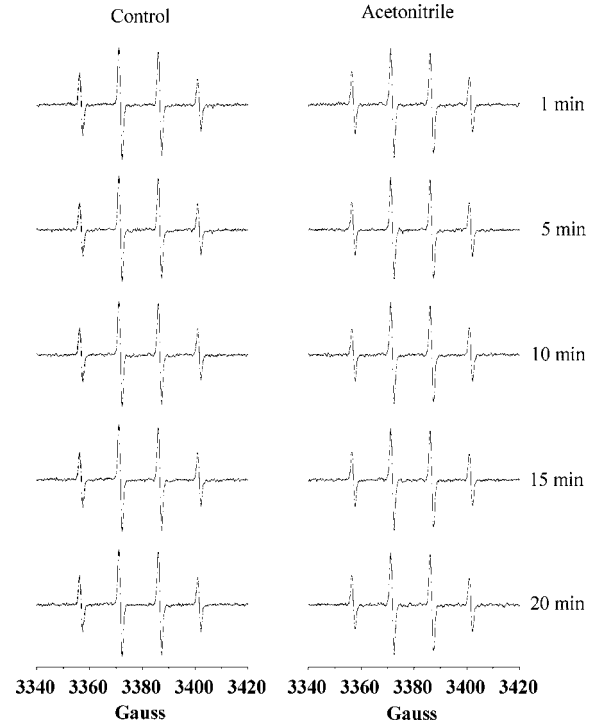

Figure 7. Time-dependent effect of acetonitrile on DMPO/HO• production in Fenton Fe^{2+}/H_2O_2 reaction system measured by ESR. Reactions were performed at ambient temperature in PBS (100 mM, pH 7.4). All reaction mixtures contained 30 μL of 250 mM DMPO, 30 μL of 1 mM H_2O_2, 30 μL of 1 mM EDTA, and 30 μL of 1 mM Fe^{2+} in the absence (control: 5% PBS) or presence of 5% acetonitrile (v/v).

Attempt To Develop a High-Throughput Fluorometric or Spectrometric Assay. ESR is a useful analytical technique for determining free radicals and estimating the free radical scavenging capacity of antioxidants. However, the instrument

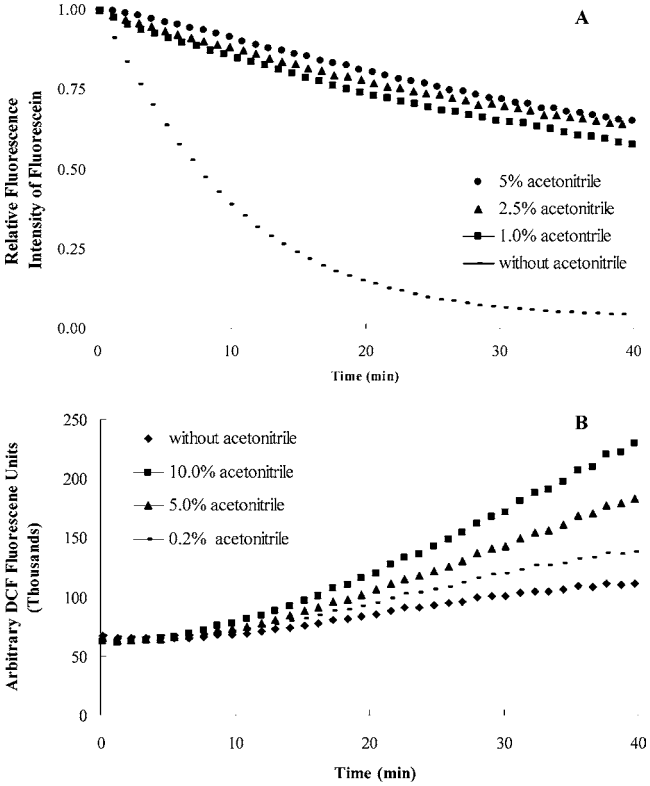

Figure 8. Dose-dependent effect of acetonitrile on the intensity of fluorescent probes. (**A**) Fluorescein and (**B**) DCFH in Fenton Fe^{2+}/H_2O_2 reaction systems measured using a 96-well plate reader. Reactions were carried out at ambient temperature in PBS (100 mM, pH 7.4). All reaction mixtures contained 50 μL of 0.3 μM fluorescein or 35.0 μM DCF-DA, 50 μL of 5 mM H_2O_2, 50 μL of 5 mM EDTA, and 50 μL of 5 mM Fe^{2+} in the absence (control: PBS) or presence of 0.2–10% acetonitrile (v/v).

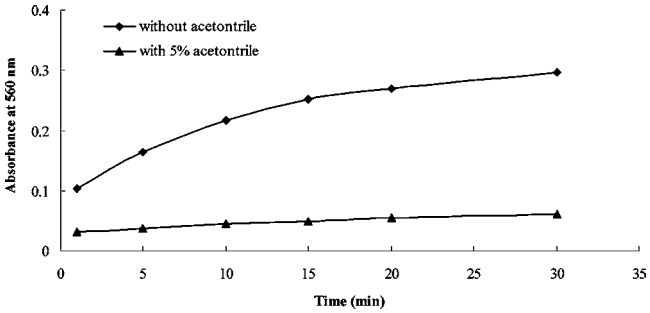

Figure 9. Effect of acetonitrile on the spectrophotometric signal intensity for NBT. Reactions were carried out at ambient temperature in PBS (100 mM, pH 7.4). All reaction mixtures contained 50 μL of 0.42 mM NBT, 50 μL of 5 mM H_2O_2, 50 μL of 5 mM EDTA, and 50 μL of 5 mM Fe^{2+} in the absence (control: 5% PBS) or presence of 5% acetonitrile (v/v).

is not widely available because of the cost. Additionally, sensitivity of ESR method is generally much lower than that of a fluorometric assay. A fluorometric protocol with potential to be adapted to high-throughput assay is preferred for screening and evaluating natural lipophilic antioxidative components because of the instrumental availability. Recently, a number of free radical scavenging capacity assays have been adapted to fluorometric determination by involving an appropriate probe. These included the novel fluorometric HO• scavenging capacity (HOSC) and oxygen radical absorbing capacity (ORAC) assays using fluorescein (FL) as the fluorescent probe (*7*) and the peroxyl radical scavenging capacity (PSC) assay using 2′,7′-dichlorofluorescin (DCFH), a derivative of 2′,7′-dichlorofluo-

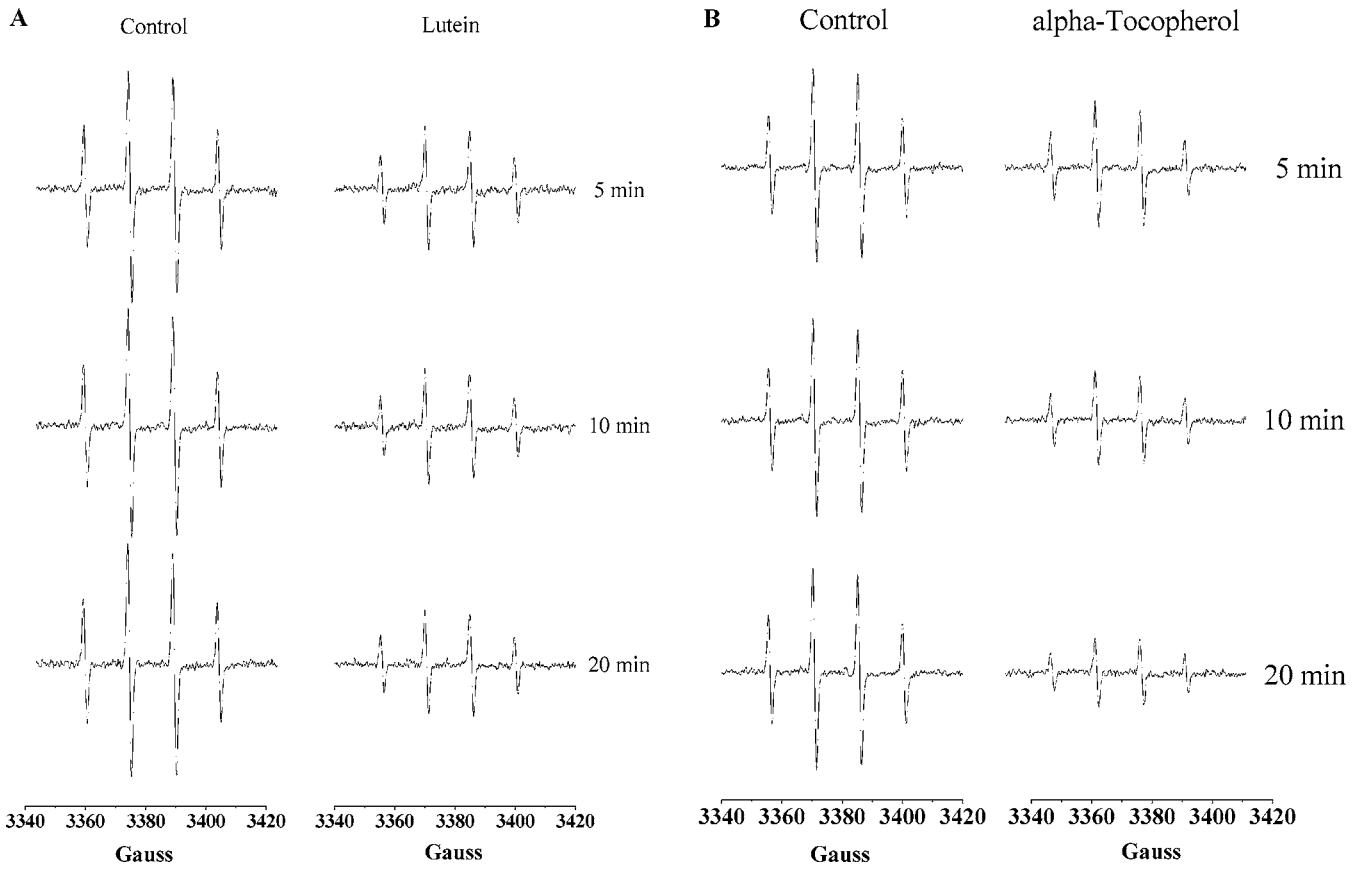

Figure 10. Time-dependent effects of (**A**) 0.369 mM lutein and (**B**) 2.0 mM α-tocopherol on DMPO/HO• production in Fenton Fe^{2+}/H_2O_2 reaction system. Reactions were performed at ambient temperature in PBS (100 mM, pH 7.4), and detected at 5, 10, and 20 min, respectively, after the reaction was initiated. All reaction mixtures contained 30 μL of 250 mM DMPO, 30 μL of 1 mM H_2O_2, 30 μL of 1 mM EDTA, and 30 μL of 1 mM Fe^{2+} in the absence (control: 5% acetonitrile) or presence of 5% lutein or α-tocopherol (v/v).

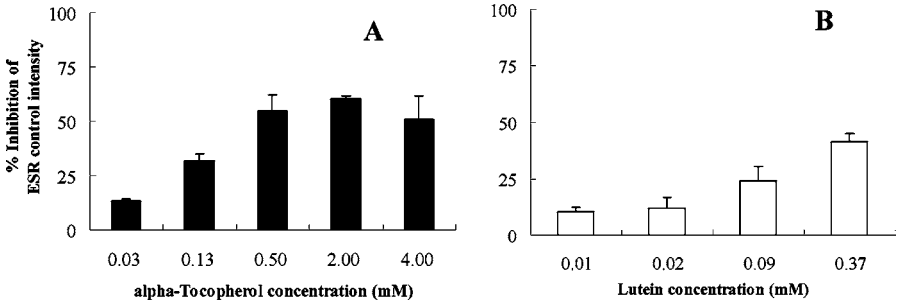

Figure 11. Dose response of lipophilic antioxidants for DMPO/HO• signal intensity in Fenton Fe^{2+}/H_2O_2 reaction system. (**A**) α-Tocopherol and (**B**) lutein. DMPO/HO• signals were measured at 20 min after the reaction was initiated. Reactions were performed at ambient temperature in PBS (100 mM, pH 7.4). All reaction mixtures contained 30 μL of 250 mM DMPO, 30 μL of 1 mM H_2O_2, 30 μL of 1 mM EDTA, and 30 μL of 1 mM Fe^{2+} in the absence (control: 5% acetonitrile) or presence of 0.006–0.369 mM lutein or 0.031–4.0 mM α-tocopherol.

rescin diacetate (DCF-DA), as the fluorescent probe (*15*). Both FL and DCFH were tested for their potential as fluorescent probes to examine HO• scavenging capacity for lipophilic antioxidants using the Fenton Fe^{2+}/H_2O_2 reaction system and acetonitrile as the solvent. When FL is used as the fluorescent probe in the HOSC or ORAC assay, the antioxidant capacity of a selected antioxidant sample is estimated as its ability to prevent the rapid decay of fluorescence due to the probe-radical reaction. In contrast, DCFH dose not have fluorescence, but may be converted to highly fluorescent 2′,7′-dichlorofluorescein (DCF) by reacting with hydroxyl radicals. Antioxidants may suppress the radical attack on DCFH and inhibit the fluorescence increase of the assay system. The results in **Figure 8** showed that acetonitrile dose-dependently suppressed the fluorescence decay of FL or the fluorescence development of DCFH induced by HO• attack, indicating the possible interaction between acetonitrile and both probes. Neither FL nor DCFH may serve as the fluorescent probe for further developing fluorometric assay for lipophilic antioxidants using the Fenton Fe^{2+}/H_2O_2 system. Further research is required to fully understand the chemical and physical mechanisms involved in this phenomenon.

It is well known that nitro blue tetrazolium (NBT) may serve as an electron acceptor and it changes color upon receiving an electron. NBT is used as the spectrometric probe for determination of superoxide anion ($O_2^{\bullet -}$) and estimating antioxidant activity of botanical extracts against $O_2^{\bullet -}$ (*16*). In the present study, NBT was evaluated for its potential to serve as a spectrometric probe to develop a colorimetric assay using the Fenton Fe^{2+}/H_2O_2 reaction system. As shown in **Figure 9**, the

presence of 5% acetonitrile in the Fenton reaction system strongly inhibited the interaction between NBT and HO• and resulted in a minor color change in the reaction mixture. This result indicates that it is not possible to develop a spectrometric assay using NBT and the Fenton Fe^{2+}/H_2O_2 reaction system to determine the HO• scavenging capacity for lipophilic antioxidants.

Results of these attempts suggest that ESR assay without additional detection probe may be a better way for estimating hydroxyl radical scavenging capacity of lipophilic antioxidants using the Fenton Fe^{2+}/H_2O_2 reaction system and acetonitrile as the solvent. Additional study was conducted to further investigate the potential of ESR determination of HO• scavenging capacity for lipophilic antioxidants.

ESR Determination of Hydroxyl Radical Scavenging Capacities for Lipophilic Antioxidants. Several known lipophilic antioxidants including α-tocopherol, lutein, β-carotene, and BHT were selected to evaluate the ESR determination of HO• scavenging capacity for lipophilic antioxidants using acetonitrile as the solvent and the Fenton Fe^{2+}/H_2O_2 reaction system to generate HO•. These lipophilic antioxidants exhibited excellent solubility in acetonitrile, and no phase separation was observed when the antioxidant solution was added to the hydroxyl radical generating system. At least three concentrations of each antioxidant were prepared and tested under the experimental conditions. To better understand the ESR measurement of antioxidant−HO• interactions under the Fenton Fe^{2+}/H_2O_2 reaction conditions with acetonitrile as the solvent, time effects were investigated for each antioxidant at 5, 10, and 20 min of reaction. No measurement was conducted beyond 20 min of antioxidant−HO• reaction because of the short lifetime of the spin trapping agent, DMPO (*21*). All of the tested lipophilic antioxidants quenched more hydroxyl radicals in the reaction system when the reaction lasted longer except BHT. The time-dependence of DMPO/HO• signal intensity for lutein and α-tocopherol is shown in **Figure 10A** and **B**. The reductions of the ESR signal intensity for lutein and α-tocopherol under the experimental conditions were about 5.7% and 26.2%, respectively, at 20 min as compared to that at 5 min (**Figure 10**). These data indicated that the time of antioxidant−HO• reaction might alter the ESR measurement results.

All of the tested lipophilic antioxidants also reacted and quenched HO• in a dose-dependent matter except BHT, determined at 20 min of antioxidant−HO• reaction under the experimental conditions. Typical dose-dependent ESR spectra of α-tocopherol and lutein are shown in **Figure 11A** and **B**, respectively. The reductions of DMPO/HO• signal intensity by 13.2−60.4% and 10.3−41.2% were observed for α-tocopherol and lutein, respectively, when their corresponding concentrations were increased from 0.031 to 4.0 mM and 0.006 to 0.369 mM, respectively, in the reaction mixtures. Note that, for BHT, there was no obvious reduction of ESR signal intensity observed even at a final concentration of 50 mM.

Reproducibility of the ESR Protocol and Limitation of Its Application. A plot of ESR peak intensity (y) versus concentrations of trolox (x) at 5 min of antioxidant−HO• reaction in **Table 1** showed that the DMPO/HO• signal intensity may be proportional to the concentrations of a selected antioxidant. Similar calibrated curves $y = 5.1828x + 414.97$ ($r^2 = 0.97$) and $y = 6.1334x + 173.32$ ($r^2 = 0.94$) were obtained at 10 and 20 min of the trolox−HO• reactions, respectively. The precision of this ESR protocol was also measured as RSD, which was obtained by calculating the ratio of standard deviation to the mean for six replicate measurements. The RSD values were 2.94%, 4.78%, and 6.21% for estimating the HO• scavenging capacity of trolox measured at 5, 10, and 20 min of the antioxidant−HO• reactions, respectively. Better linearity or precision was obtained when measurements were conducted at shorter antioxidant−HO• reaction time, suggesting that the hydroxyl radical scavenging capacities of lipophilic antioxidants should be determined within 20 min. These data demonstrate the possibility to quantify or quantitatively compare HO• scavenging capacity for lipophilic antioxidants with acetonitrile as the solvent using ESR and the Fenton Fe^{2+}/H_2O_2 reaction system. Trolox has been used as the standard antioxidant in several antioxidant activity assays such as ORAC, HOSC, and PSC assays. Trolox is selected because it is an analogue of tocopherols with adequate solubility in both lipophilic and hydrophilic solvents and has been reported to partite 20−45% into the lipid phase of liposomes (*22*, *23*), although α-tocopherol is sometimes employed as a standard for lipophilic antioxidants (*15*). Results shown in **Table 1** also suggest the potential use of trolox as the standard compound to report the HO• scavenging capacity of lipophilic antioxidants as trolox equivalents, which is a relative antioxidant activity and may be used to compare the antioxidant samples analyzed across different laboratories and at different times.

Table 1. Reproducibility of the ESR assay[a]

	day	slope	intercept	r^2
	1	3.5829	442.50	0.97
	2	3.5252	361.31	0.97
5 min	3	3.2869	382.60	0.97
	4	4.9256	229.20	0.98
	5	5.3339	328.27	1.00
SD		0.9298	70.43	
RSD (%)		22.51	20.19	
	1	3.6461	228.11	0.90
	2	3.5285	525.23	0.97
10 min	3	3.5378	465.81	1.00
	4	5.0021	302.40	0.99
	5	5.1828	414.97	0.97
SD		0.8372	120.94	
RSD (%)		20.03	31.23	
	1	4.6349	660.65	0.99
	2	4.8110	657.94	0.98
20 min	3	5.0972	602.82	0.99
	4	6.4932	412.65	0.98
	5	6.1334	173.32	0.94
SD		0.8293	209.56	
RSD (5%)		15.26	41.79	

[a] Reactions were carried out at ambient temperature in PBS (100 mM, pH 7.4) and detected at 5, 10, and 20 min, respectively, after the reaction was initiated. All reaction mixtures contained 30 μL of 250 mM DMPO, 30 μL of 5 mM H_2O_2, 30 μL of 5 mM EDTA, and 30 μL of 5 mM Fe^{2+} in the absence (control: acetonitrile) or presence of 5–100 mM trolox dissolving in acetonitrile (v/v).

Reproducibility of the ESR assay for measuring the HO• scavenging capacity of lipophilic antioxidants was evaluated using trolox with acetonitrile as the solvent and the Fenton Fe^{2+}/H_2O_2 reaction system. Five concentrations of trolox standard samples were tested for their HO• scavenging capacities in duplicate at 5, 10, and 20 min of antioxidant−HO• reactions in 5 consecutive days, and the results were used to prepare the regression lines. The slope, y-intercept, and r^2 values of each regression line were reported in **Table 1**. The RSD values were 22.5%, 20.0%, and 15.3% for slope, and 20.2%, 31.2%, and 41.8% for y-intercept determined using the ESR data obtained at 5, 10, and 20 min of antioxidant−HO• reactions, respectively, suggesting the huge day-to-day variations of the assay. These data indicate that it is hard to obtain reproducible relative HO• scavenging capacity values using this ESR assay. In other words,

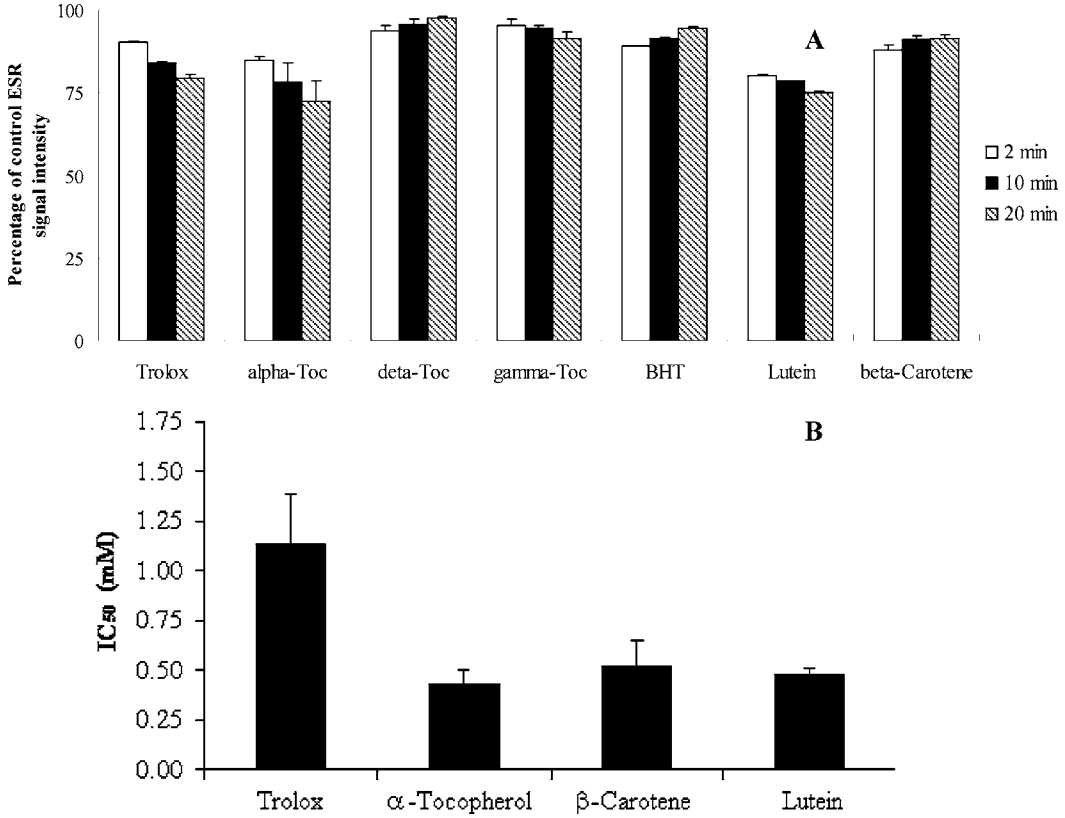

Figure 12. Effect of several selected lipophilic antioxidants on ESR signal intensity in Fenton Fe^{2+}/H_2O_2 reaction system. (**A**) Percentage of control E ESR signal intensity, (**B**) IC_{50} values (mM). Reactions were performed at ambient temperature in PBS (100 mM, pH 7.4) and detected at 2–20 min (for **A**) or 20 min (for **B**) after the reaction was initiated. All reaction mixtures contained 30 μL of 250 mM DMPO, 30 μL of 1 mM H_2O_2, 30 μL of 1 mM EDTA, and 30 μL of 1 mM Fe^{2+} in the absence (control: 5% acetonitrile) or presence of 0.55 mM individual antioxidants.

one may be able to obtain a relative HO• scavenging capacity value for a selected lipophilic antioxidant, but this value cannot be used to quantitatively compare this antioxidant with other antioxidative samples measured using the same protocol from different laboratories or that measured at different times. This is the limitation of this ESR assay.

In contrast, r^2 values ranged from 0.9 to 1.0, indicating the excellent linearity of the measurements and the stability of the assay within a day (**Table 1**). Furthermore, a lack-of-fit test was performed using all data in **Table 1** to determine whether these data fitted a linear model. The calculated F value was 0.029, which is much less than the critical value of 2.32 for (*3, 23*) degree of freedom at 1% level of significance, confirming the excellent linearity of the assay. This also suggests that it is possible to compare a group of lipophilic antioxidants for their relative capacities in quenching HO• under the ESR assay conditions. **Figure 12A** presents the HO• scavenging capacity of a group of lipophilic antioxidants. Their HO• scavenging capacities were in the order of α-tocopherol > lutein > trolox > β-carotene > γ-tocopherol > BHT > δ-tocopherol determined at 20 min after the reaction was initiated. The order of trolox, α-tocopherol, and lutein is similar to that of the previous observation (*22*). The IC_{50} values of trolox, α-tocopherol, β-carotene, and lutein were also determined and shown in **Figure 12B**, which demonstrated the similar order of the hydroxyl radical scavenging capacities as shown in **Figure 12A**. IC_{50} value is the required concentration of a selected antioxidant to quench 50% of the hydroxyl radicals in the ESR assay system under the testing conditions. A stronger antioxidant has a smaller IC_{50} value. These data indicate the potential application of this ESR assay in measuring and comparing HO• scavenging capacity of lipophilic antioxidants.

In summary, an ESR protocol was developed and validated for its application in measuring HO• scavenging capacity for lipophilic antioxidants. This study also discussed the effects of commonly used organic solvents on the Fenton Fe^{2+}/H_2O_2 HO• generating system as well as their possible interactions with the fluorescent and spectroscopic probes. In addition, the limitation of the ESR assay was also described.

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