# Free Radical Scavenging Properties of Conjugated Linoleic Acids

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Conjugated linoleic acids (CLA) were investigated for free radical scavenging properties against the stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH•) by electron spin resonance (ESR) spectrometry and spectrophotometric methods. ESR results demonstrated that CLA directly reacted and quenched free DPPH radicals in benzene, while spectrophotometric analysis showed the radical scavenging capacity of CLA in ethanol. Dose and time effects of CLA-DPPH• reactions were observed in both tests. The $ED_{50}$ of CLA was 18 mg/mL under experimental conditions. CLA are much weaker radical scavengers as compared to vitamin E, vitamin C, and BHT. Kinetics of CLA-DPPH• reactions was different to that of linoleic acid (LA)-DPPH• reactions. CLA reacted and quenched DPPH radicals at all tested levels without a lag phase, while LA had a lag phase and showed no radical quenching activity at levels of 5−80 mg/mL in 30 min. These data indicated that CLA can provide immediate protection against free radicals, but LA cannot.

**Keywords:** *Conjugated linoleic acids; antioxidant; free radical scavenging activity; electron spin resonance*

## INTRODUCTION

Conjugated linoleic acids (CLA) are a group of octadecadienoic acids containing conjugated double bonds that are separated by one single bond. Much attention has been given to CLA because they are natural components of foods and have interesting health benefits (*1*). CLA have been investigated for their physiological and pharmacological activities, appearance in foods, separation, and characterization methods, and procedures to prepare them. The observed physiological and pharmacological activities of CLA include anticarcinogenic, antiatherosclerotic, antioxidant, immunomodulatory, antibacterial, altering tissue fatty acid composition and metabolism, influencing signal transduction, and effects on body composition and metabolism (*1*−*7*). However, the mechanism(s) of their biological actions is still poorly understood.

It has been accepted that free radicals and radical-mediated oxidation play a role in many pathological processes, such as carcinogenesis and atherosclerosis (*8*). CLA were showed to prevent cancer and atherosclerosis in both animal and cell models (*1*, *9*, *10*), and the antioxidant activity of CLA has been investigated by several research groups since it was considered as a possible explanation for some of their biological actions (*3*, *4*, *7*, *8*). Conflicting results were obtained from previous studies of the antioxidant properties of CLA (*7*, *8*, *11*−*15*).

Ha et al. (*7*) reported that CLA were more effective in preventing linoleic acid (LA) oxidation than α-tocopherol but was comparable to butylated hydroxytoluene (BHT) as measured by the thiocyanate method. This observation was supported by a later study conducted by Ip et al. (*11*). IP et al. (*11*) found that CLA supplementation inhibited lipid peroxidation in mammary gland of the rats. Yurawecz and co-workers (*12*) found that CLA could be converted to furan fatty acids by air oxidation in methanol when enough water was present and should be considered as a source of furan fatty acids in biological systems. Formation of furan fatty acids may contribute to the antioxidant properties of CLA (*16*).

In contrast, van den Berg et al. (*8*) reinvestigated the antioxidant properties of CLA under the conditions of metal ion dependent and independent models and concluded that CLA did not act as effective radical scavengers in any way comparable to that of vitamin E or BHT, nor did CLA appear to be converted to metal chelators. The results indicated that CLA acted in a manner similar to other polyunsaturated fatty acids under oxidative stress. The oxidation of CLA in air was also compared to LA since LA is considered as the natural fatty acid with the most similar chemical structure of CLA and may be involved in the mechanism(s) of CLA's biological actions (*15*, *17*). CLA had higher oxidative susceptibility than that of LA (*8*).

Until Leung and Liu (*15*) reported that *trans*-10,*cis*-12-conjugated linoleic acid (t10,c12-CLA) had strong oxyradical scavenging capacity as detected by total oxyradical scavenging capacity (TOSC) assay, it had almost been concluded that CLA might not have antioxidant capacity although CLA might produce antioxidative substances (*1*). Leung and Liu (*15*) showed that the antioxidant activity of t10,c12-CLA was higher than c9,t11-CLA and α-tocopherol at lower concentrations of 2 and 20 μM. Furthermore, c9,t11-CLA acted as a weak antioxidant at lower concentration but possessed strong pro-oxidant activity at 200 μM. The mechanism by which CLA isomers interact with free radicals becomes very interesting because of the structural similarities between them. There was no direct evidence that CLA directly reacted and quenched free radicals, although

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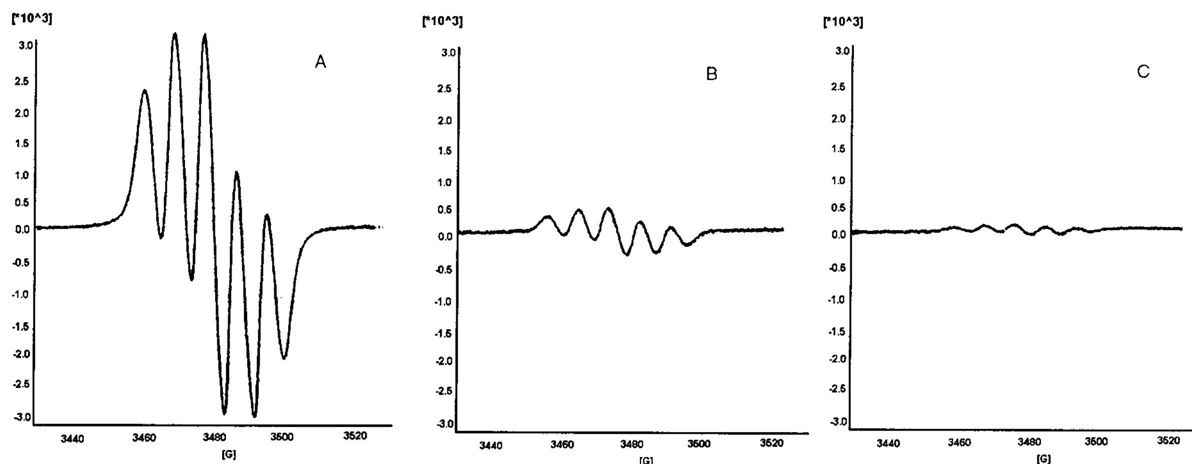


**Figure 1.** Free radical scavenging activity of CLA determined by ESR. The final concentration of DPPH was 2.2 mM in all three tested samples. A, B, and C represented final CLA concentrations of 0, 5, and 10 mg/mL, respectively.

Winston and co-researchers (*18*) believed that individual antioxidants might compete for peroxyl radicals in different ways to inhibit ethylene formation from α-keto-γ-methiolbutyric acid oxidation. In other words, free radical scavenging agents can reduce ethylene formation but this does not mean that reduced ethylene formation could be caused only by radical scavengers. More studies are needed to elucidate the reactions between CLA and free radicals.

Electron spin resonance (ESR) spectrometry is considered to be the least ambiguous method for the detection of free radicals (*19–21*). ESR has been successfully used to study the free radical scavenging activities of antioxidants by using stable free radicals, such as 2,2-diphenyl-1-picryhydrazyl radical (DPPH·) (*20*, *22*). In addition to ESR measurement, the stable DPPH radical can be used to study the reaction kinetics of antioxidants, quantify, and compare the free radical scavenging capacities of different antioxidants (*21*, *23*). In this study, ESR and spectrophotometric methods were employed (i) to determine whether CLA could directly react and quench stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH·) and (ii) to compare CLA with vitamin E, vitamin C, and BHT, and LA for their radical quenching properties.

## MATERIALS AND METHODS

Conjugated linoleic acids (CLA) were kindly provided as a gift by Pharmanutrients Company (Lake Bluff, IL). The CLA preparation contains 35.0% *cis*-9,*trans*-11-CLA (c9,t11-CLA) and 35.8% *trans*-10,*cis*-12-CLA (t10,c12-CLA), as analyzed by the manufacturer and confirmed in our laboratory. Linoleic acid, 2,2-diphenyl-1-picryhydrazyl radical (DPPH·), vitamin E, vitamin C, and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were the highest commercial grade and used without further purification.

**ESR Analysis.** Solutions of CLA and DPPH· in benzene were mixed and incubated at 38–40 °C for 15 h, followed by ambient temperature for 10 h. The ESR analysis was performed before and after incubation. The ESR was conducted using a Bruker EMX ESR spectrometer (Bruker Instruments, Inc., Billerica, Massachusetts) with a modulation frequency of 100 kHz, a sweep width of 100.00 G, 3480 G center field, and $3.17 \times 10^5$ receiver gain. The final concentration of DPPH· was 2.2 mM, and CLA concentrations were 0, 5, and 10 mg/mL in the reaction mixtures (*20*, *22*).

**Comparison of Radical DPPH· Scavenging Capacity**. Total free radical scavenging capacity of CLA was estimated and compared to vitamin E, vitamin C, and BHT according to the previously reported procedure using the stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH·) (*23*, *24*). Briefly, freshly made DPPH· solution was added into ethanol solutions of CLA, vitamin E, vitamin C, and BHT to start the reaction. The final concentration was 100 μM for DPPH· and 50 mM for antioxidants in each sealed reaction vial, except for the control containing no antioxidant. The absorbance at 517 nm was measured against a blank of pure ethanol after the reaction was carried out at ambient temperature for 60 min. Radical DPPH· scavenging capacity was estimated from the difference in absorbance with or without antioxidants and expressed as percent DPPH· remaining. All tests were conducted in triplicate.

**Kinetics of CLA-Radical and LA-Radical Reactions**. The kinetics of CLA-DPPH· and LA-DPPH· reactions were investigated by measuring the disappearances of DPPH radicals in the reaction mixtures at room temperature. Seven levels of CLA or LA were used in the kinetic study. One milliliter of CLA or LA solution was mixed into 1 mL of 200 μM DPPH· ethanol solution. Absorbance of each reaction mixture at 517 nm was measured against an ethanol blank at 0, 5, 10, 20, 40, 80, 160, 320, and 2000 min. The percent DPPH· remaining at each tested time point was calculated using a DPPH· standard curve (*25*). The dose and time dependences of CLA and DPPH· reactions were demonstrated by plotting the percent DPPH· remaining against time for each level of CLA tested. The $ED_{50}$ of CLA was obtained by plotting the percent DPPH· remaining at steady state of the reaction against corresponding CLA concentration. The $ED_{50}$ is the concentration of CLA required to quench 50% DPPH· radical under experimental conditions. Triplicate reactions were carried out for each level of CLA and LA.

**Statistic Analysis**. Data were reported as mean ± SD for triplicate measurements, except ESR analysis. An independent samples t-test (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) was conducted to identify differences among means ($n = 3$). A $p < 0.05$ was considered statistically significant.

## RESULTS

**ESR Results.** The free radical scavenging capacity of CLA was detected by ESR (Figure 1A–C). No differences in ESR absorbance were observed among samples containing 0, 5, and 10 mg/mL CLA before incubation of the reaction mixtures (data not shown). After incubating with 2.2 mM DPPH· radicals, CLA resulted in the disappearance of the ESR absorbance at both 5 and 10 mg/mL (17.8 and 35.7 mM, respectively) levels in benzene (Figure 1A–C). Furthermore, higher CLA

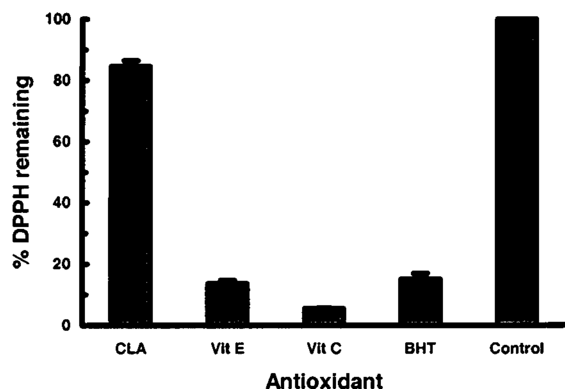


**Figure 2.** Comparison of radical DPPH scavenging capacity. Radical DPPH scavenging capacities of conjugated linoleic acids (CLA), vitamin E (Vit E), vitamin C (Vit C), butylated hydroxytoluene (BHT), as compared to the control containing no antioxidant. All antioxidants were tested at 50 mM final concentration against 100 $\mu$M DPPH radical. Vertical bars represent the standard deviation of each data points ($n = 3$).

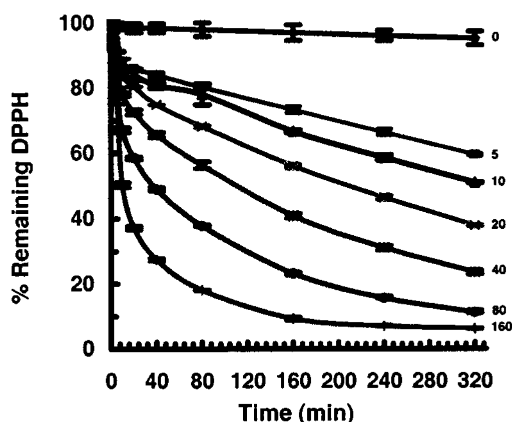


**Figure 3.** Kinetics of CLA–DPPH radical reaction. 0, 5, 10, 20, 40, 80, and 160 represent the final CLA concentrations of 0, 5, 10, 20, 40, 80, and 160 mg/mL in the reaction mixtures. The DPPH radical concentration was 100 $\mu$M in all reaction mixtures. Vertical bars represent the standard deviation of each data points ($n = 3$).

concentration corresponded to greater suppression of ESR absorbance.

**Comparison of DPPH· Scavenging Capacity**. Total DPPH· scavenging capacity of CLA was measured and compared with other compounds. CLA had a much weaker radical scavenging activity compared to vitamin E, vitamin C, and BHT (Figure 2). After 1 h, CLA quenched 15.4 ± 1.1% DPPH·, while vitamin E, vitamin C, and BHT quenched 86.3 ± 1.1, 94.5 ± 0.7, and 84.9 ± 1.9%, respectively, on the same per mole basis. All antioxidants were tested at a level of 50 mM against 100 $\mu$M DPPH·.

**Reaction Kinetics of CLA and LA against DPPH Radicals**. The kinetics of the CLA-DPPH· reaction is shown in Figure 3. With CLA, free radical scavenging activity was detected at all tested concentrations, and the lowest concentration was 5 mg of CLA/mL in ethanol (Figure 3). Dose dependence was observed. Higher concentrations of CLA were more effective in quenching free radicals in the system. The percent DPPH remaining at steady state was plotted against the corresponding CLA concentration to obtain Figure 4. The $ED_{50}$ of CLA to quench DPPH radical is approximately 18 mg CLA/mL (Figure 4). $ED_{50}$ is the concentration of CLA required to quench 50% DPPH radical under the

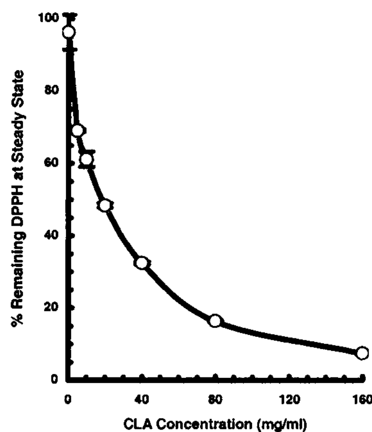


**Figure 4.** Disappearance of DPPH as a function of CLA concentration. $ED_{50}$ was measured as the CLA concentration that was correlated to 50% remaining DPPH radical at steady state of reaction. Vertical bars represent the standard deviation of each data points ($n = 3$).

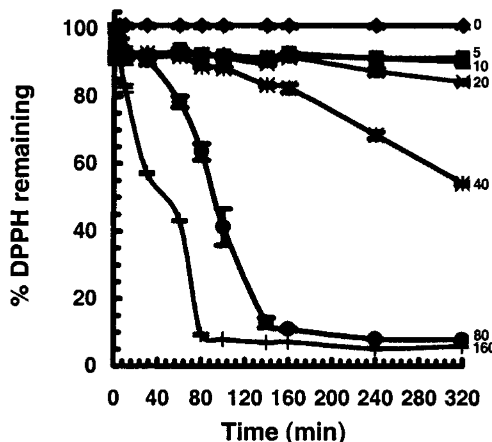


**Figure 5.** Kinetics of LA-DPPH radical reaction. 0, 5, 10, 20, 40, 80, and 160 represent the final LA concentrations of 0, 5, 10, 20, 40, 80, and 160 mg/mL in the reaction mixtures. The DPPH radical concentration was 100 $\mu$M in all reaction mixtures. Vertical bars represent the standard deviation of each data points ($n = 3$).

experimental conditions. CLA was observed to immediately react and quench DPPH radicals at all tested levels, and no lag phase was observed in CLA-DPPH· reactions.

The kinetics of LA-DPPH· reactions is illustrated in Figure 5. The characteristic of the kinetic curves of LA-DPPH· is different from that of the CLA-DPPH· reactions. LA quenched DPPH radicals at concentrations of 160, 80, 40, and 20 mg/mL in a 320 min reaction period (Figure 5). No significant radical scavenging activity was observed for all tested concentrations of LA in 5 min. There was no significant radical scavenging capacity detected for 5 and 10 mg/mL of LA in 320 min, and no significant radical scavenging activity detected for LA at concentrations of 5–80 mg/mL in 30 min. Higher concentrations of LA corresponded to a more rapid disappearance of DPPH radical.

## DISCUSSION

To better understand the beneficial actions of CLA, it is critical to clarify whether CLA can act as antioxidant by any possible mechanisms, such as directly quenching free radicals to terminate the radical chain reaction, chelating transition metals to suppress the

initiation of radical formation or stimulating the antioxidative defense enzyme activities. This study was conducted to address (i) whether CLA can directly react and quench free radicals, (ii) the strength of their radicalscavenging activity, and (iii) whether CLA and LA act differently in the presence of free radicals.

Electron spin resonance (ESR) spectrometry has been considered as a reliable method for radical detections (*20*). ESR detects the spins of the unpaired electron, the free radical. An unpaired electron may have a spin of either +1/2 or −1/2 and behaves as a small magnet in electromagnetic field. ESR spectrometers are set up to display the first-derivative spectra of the radicals in an electromagnetic field. ESR has also been adapted to evaluate antioxidants for their radical scavenging activity (*20*, *22*). Free radical scavengers donate an electron to the free radical. This electron donation is associated with the disappearance of the ESR absorbance. The lower the ESR absorbance, the stronger is the free radical scavenging activity. This study demonstrated that CLA directly reacted with radicals in benzene, a nonpolar solvent. The higher levels of CLA caused a greater suppression of ESR absorbance. These findings indicate that CLA have radical scavenging capacity. Yurawecz et al. (*12*) detected furan fatty acids (FFA) from CLA oxidation in methanol containing significant amount of water. The water was essential for air oxidation of CLA since the FFA could not be obtained when CLA oxidation was performed in methanol (*12*). FFAs were shown to scavenge free radicals (*16*) and are considered to be the active form of CLA to act as radical scavengers (*15*). In this study, we attempted to detect the FFA methyl esters and free FFA acids in the CLA-DPPH• reaction mixtures by gas chromatography−mass spectrometry (GC/MS) and high performance liquid chromatography−mass spectrometry (LC-MS), respectively. No FFAs were detected (data are not shown). Our data suggested that CLA directly reacted and quenched the DPPH radicals because the reaction mixture contained only DPPH radicals, CLA, and solvent, which was benzene for ESR analysis and ethanol for spectrophotometric analysis. No water was added to the reaction vials. The findings in this study do not necessarily conflict with the results reported by Yurawecz and others (*12*), since different chemical mechanisms may be involved in CLA-radical and CLA-$O_2$ reactions. Radical scavenging capacity of CLA was also confirmed by the results from kinetic studies of CLA-DPPH• reactions.

Leung and Liu reported (*15*) that *trans*-10,*cis*-12-conjugated linoleic acid (t10,c12-CLA) was more effective as an antioxidant than vitamin E and *cis*-9,*trans*-11-conjugated linoleic acid (c9,t11-CLA) at lower concentrations (2−20 $\mu$M) only. In this study, CLA, at a level of 50 mM, were much less effective radical scavengers as compared to vitamin E, vitamin C, and BHT, although CLA quenched a significant amount of DPPH radicals. This may be because the CLA preparation used in this study was a mixture of isomers, and the concept of the assay was also different from that involved in the previous test (*15*). In this study, CLA, at concentrations of 5−40 mg/mL, were more effective than LA in quenching DPPH radicals. This observation is consistent only with the data for t10,c12-CLA isomer but not c9,t11-CLA in the study performed by Leung and Liu (*15*). However, our observation supported the protective effects of CLA on LA peroxidation and reduced lipid peroxidation in mammary gland as reported by Ha et al. (*7*) and Ip et al. (*11*), respectively. Ip and coinvestigators (*11*) measured thiobarbituric acid-reactive substances (TBARS) as the indicator of lipid peroxidation in mammary gland. Radical scavenging activity of CLA could well explain the reduced TBARS observed by Ip and others in their rat feeding study (*11*).

Kinetic data indicated that CLA reacted and quenched DPPH radicals at all tested levels without a lag phase, while LA showed a lag phase. The lag phase indicated the delay of radical quenching actions of LA. Kinetic data also showed the dose and time effects of CLA against DPPH radicals. Both CLA and LA, at higher concentration for longer time, had greater scavenging capacity against DPPH radical. Higher concentrations of LA corresponded to a shorter lag phase, while low concentrations of LA (5 and 10 mg/mL) had no significant scavenging activity against DPPH• in 320 min. These data provided the evidence that CLA acted differently in the presence of free radicals as compared to LA. CLA can provide immediate protection against free radicals, but LA cannot.

The chemical mechanism for CLA-radical reaction cannot be clearly elucidated without identifying the reaction product(s) and/or intermediates. However, it is clear that the conjugated double bonds made contributions to the radical scavenging capacity of CLA. The difference in the reaction kinetics between CLA-radical and LA-radical perhaps provide a reasonable explanation why CLA are anticarninogenic while increased LA was observed to promote tumor formation (*1*, *26*).

In conclusion, CLA were shown to exhibit their capacity to directly react and quench free radicals as measured by ESR and spectrophotometric methods. CLA had much weaker free radical scavenging activities as compared to vitamin E, vitamin C, and BHT on a molarity basis. The kinetics of CLA-DPPH• and LA-DPPH• reactions were very different. CLA immediately quenched DPPH radicals while LA did not quench DPPH radicals before a lag phase. CLA can provide immediate prevention against free radicals but LA cannot. Additional studies are necessary to show free radical scavenging activity of CLA in different radical systems and under physiological conditions, and to determine whether there is any link between their radical scavenging properties and their biological effects, including anticarcinogenesis and antiatherosclerosis.

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