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Free radical scavenging ability and antioxidant efficiency of curcumin and its substituted analogue

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Abstract

Free radical reactions of curcumin and its ethoxy substituted derivative (C1) 1,7-bis-(4-hydroxy-3-ethoxy phenyl)-1,6-heptadiene-3,5-dione have been studied using a pulse radiolysis technique in homogeneous aqueous–organic solutions like acetonitrile–water and isopropanol–water mixtures, as well as in neutral TX-100 and cationic CTAB micellar solutions. The phenoxyl radicals of curcumin or C1 were generated by one-electron transfer to several oxidants like $N_3^{\bullet}$, $Br_2^{-\bullet}$, $CCl_3O_2^{\bullet}$, glutathione radicals which exhibit absorption from a 300–600-nm wavelength region with the maximum at 490–500 nm. Other important properties of the phenoxyl radicals such as extinction coefficient, radical lifetime and their formation and decay rate constants were also determined in these systems. The antioxidant property of curcumin and C1 were estimated in terms of their ability to inhibit the lipid peroxidation in liposomes and also in terms of trolox equivalent antioxidant capacity (TEAC). The results were compared with α-tocopherol.

Keywords: Curcumin and its ethoxy derivative (C1); Free radical; Pulse radiolysis; Antioxidant

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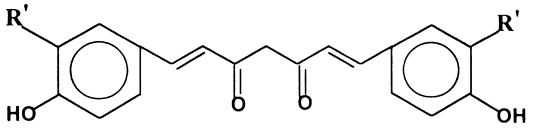
1. Introduction

Curcumin, a yellow orange dye derived from the rhizomes of *Curcuma longa* (turmeric) which is used as a spice and food coloring in Indian cooking is well known for its medicinal properties since ancient times. Curcumin shows antioxidant activity as seen by its ability to inhibit lipid peroxidation, strand break formation in DNA, etc. [1–13]. The antioxidant activity of curcumin arises mainly from scavenging of several biologically relevant free radicals that are produced during physiological processes [2,3,9,13]. Although a lot of work has been reported in the literature on the potential use of curcumin as an antioxidant, the search for new synthetic derivatives is on to develop compounds that show better antioxidant activities. In the present work, we studied curcumin and its synthetic derivative (C1) whose chemical structures are given below.

In order to see their ability to scavenge several biologically important free radicals, pulse radiolysis studies of C1 and curcumin were carried out. As both curcumin and C1 are insoluble in water, aqueous–organic solution mixtures were used to study their reactions with oxidants. They are lipophilic and are expected to be localised in the membranous portion of living cells. For this surfactant, solutions like non-ionic Triton X-100 (TX-100) and cationic cetyl trimethyl ammonium bromide (CTAB) micelles were used as models for cell membranes [14,15]. These surfactants when solubilised in water at concentrations greater than the critical micellar concentration, form micelles, having a hydrophobic core that provides a solubilisation site for curcumin and C1 [14–16]. In the case of TX-100, the micelles are neutral, whereas in the case of CTAB, the micelles are positively charged. In this paper we present some of the reactions of curcumin and C1 with different oxidising radicals using the nanosecond pulse radiolysis technique in homogeneous aqueous–organic solutions as well as in micellar solutions. With a view to understand their lipophilic character, the binding constant for their association with micelles was estimated by following the absorption and fluorescence spectral changes of curcumin and C1 in the presence of surfactants. Also we present here data supporting the antioxidant ability of curcumin and C1 in liposomes.



Curcumin: $R' = OCH_3$
C1: $R' = OC_2H_5$.

2. Experimental

TX-100, CTAB were received from Aldrich. All other chemicals were of the highest purity available and used without any further purification. Curcumin was from Sigma. C1 was synthesised by condensing 4-hydroxy-3-ethoxy benzaldehyde with acetylacetone-boron oxide complex in the presence of tributyl borate and *n*-butyl amine followed by acid hydrolysis. The method is similar to that of curcumin synthesis as described by Pabon [17]. The antioxidant property of curcumin and C1 was determined by two methods: (a) measuring its ability to inhibit lipid peroxidation induced by Ferric-ascorbate system in liposomes in terms of IC_{50} (in μmol dm^{-3}) value as reported earlier [18], where IC_{50} is the concentration of antioxidant required to inhibit 50% lipid peroxidation; and (b) measuring its trolox equivalent antioxidant capacity (TEAC), i.e. comparing their ability of scavenging the radical cation of 2,2′-azino-bis (3-ethylbenzothiazoline)-6-sulphinate (ABTS) with that of trolox as described by Miller and Rice Evans [19,20].

Reactions of curcumin and C1 with several important free radicals were studied by using the nanosecond pulse radiolysis technique. Here the transient species having lifetimes of a few microseconds to nanoseconds were produced by em-

ploying 50-ns electron pulses from a 7-MeV linear electron accelerator with absorbed doses of 8–15 Gy and were detected by kinetic spectrophotometry [21,22]. The absorbed dose was measured by using aerated thiocynate dosimetry monitoring $(SCN)_2^{-\bullet}$ at 500 nm [23]. All the experiments were carried out at pH 7 using equimolar mixture of phosphates (5–10 mM) as buffers. The details regarding the generation of important oxidising radicals like $N_3^\bullet$, $CCl_3O_2^\bullet$, $Br_2^{-\bullet}$, etc., are given below [24,25].

Radiolysis of water produces three major reactive species like $H^\bullet$, $^\bullet OH$, and e_{aq}^-. In N_2O-saturated solutions e_{aq}^- is quantitatively converted to $^\bullet OH$ radicals ($N_2O + e_{aq}^- \rightarrow {}^\bullet OH + OH^- + N_2$). The specific one-electron oxidising azide radicals ($N_3^\bullet$) were generated by the reaction of $^\bullet OH$ radicals with N_3^- ($N_3^- + {}^\bullet OH \rightarrow OH^- + N_3^\bullet$). Thus, in N_2O-saturated solutions containing 0.1 mol dm^{-3} NaN_3, all the hydroxyl radicals are exclusively converted to $N_3^\bullet$ radicals with a radiation chemical yield of 5.6×10^{-7} mol J^{-1}. Similarly on irradiation of oxygen saturated aqueous solutions containing CCl_4 (4%) and 2-propanol (48%), primary $^\bullet OH$ radicals react with 2-propanol to produce acetone ketyl radicals. These radicals and e_{aq}^- react with CCl_4 to produce $CCl_3^\bullet$ radicals, which in turn react with oxygen under aerated conditions to produce halocarbonperoxyl radicals ($CCl_3^\bullet + O_2 \rightarrow CCl_3O_2^\bullet$). $Br_2^{-\bullet}$ radicals were generated by irradiating N_2O-saturated CTAB solution (1×10^{-2} mol dm^{-3}) containing 0.01 mol dm^{-3} Br^-. The bromide ions react with $^\bullet OH$ to produce $Br^\bullet$, ($Br^- + {}^\bullet OH \rightarrow Br^\bullet + OH^-$) which subsequently reacted with Br^- to give $Br_2^{-\bullet}$ ($Br^\bullet + Br^- \rightarrow Br_2^{-\bullet}$). Reaction of glutathione radicals ($GS^\bullet$) were studied by irradiation of N_2O-saturated 50% methanol–water mixture containing 2×10^{-3} mol dm^{-3} glutathione as given in the literature [26–28]. To determine the rate constants for the reactions of curcumin and C1 with several oxidising radicals, the change in the rate of formation of the transient absorption at 490–500 nm was followed as a function of their parent concentration (either curcumin or C1). Typically the concentrations were varied from 2×10^{-5} to 2×10^{-4} mol dm^{-3}.

3. Results

3.1. Estimation of binding constant of curcumin and C1 with TX-100

Binding constants (K_a) of curcumin and C1 with TX -100 [Eq. (1)] were calculated by using the Benesi–Hildebrand Eq. (3) [29]. The equilibrium constant (K_a) for the reaction between the host (X) and the guest (A) is

$$X + A \rightleftharpoons XA \tag{1}$$

$$K_a = \frac{\Delta a}{C_X(C_A\varepsilon_o - \Delta a)} \tag{2}$$

This can also be rewritten as

$$\frac{C_A}{\Delta a} = \frac{1}{\varepsilon_o K_a C_X} + \frac{1}{\varepsilon_o} \tag{3}$$

Where Δa is the difference in the absorbance intensity of the complex (AX) in the presence and in the absence of the host (X) at a particular wavelength. C_A and C_x are the total concentration of the guest and the host, respectively. Hence to determine the binding constant of curcumin and C1 with TX-100, concentration of curcumin or C1 was kept fixed at $\sim 1 \times 10^{-5}$ mol dm^{-3} and the concentration of TX-100 was varied from 4×10^{-4} to 2×10^{-2} mol dm^{-3}. The changes in the absorption spectrum of curcumin and C1 were monitored at 425 and 427 nm, respectively. From the slope and intercept of the linear plot for $1/\Delta a$ vs. $1/[TX-100]$ the binding constant was calculated to be $1.5 \pm 0.2 \times 10^3$ and $4.9 \pm 0.2 \times 10^3$ dm^3 mol^{-1} for C1 and curcumin, respectively. K_a was also determined by the fluorescence method under these conditions. For this the solutions of C1 or curcumin in TX-100 were excited at the isosbestic point in the absorption spectra, i.e. 380 and 370 nm, respectively, where all the solutions were having identical absorption intensity and fluorescence intensity was monitored at 506 nm. Change in the fluorescence intensity ΔF as shown in Fig. 1 for C1, was measured and a graph of $1/\Delta F$ vs. $1/[TX-100]$ was plotted (inset of Fig. 1). From this the binding constants for

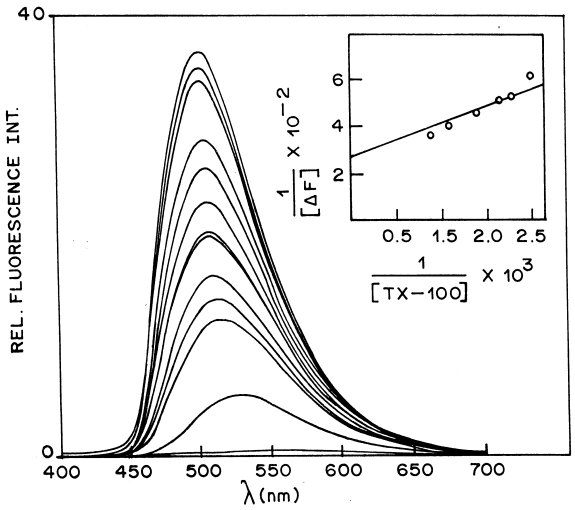


Fig. 1. Fluorescence spectra obtained after exciting the solutions of C1 (9.6×10^{-6} mol dm^{-3}) at 380 nm indicating changes in fluorescence intensity of C1 at varying concentrations of TX-100 from 4×10^{-4} to 2×10^{-2} mol dm^{-3}. Inset: Linear plot for the reciprocal change in the fluorescence intensity with the reciprocal concentration of TX-100.

C1 and curcumin were estimated to be $1.9 \pm 0.2 \times 10^3$ and $2.0 \pm 0.4 \times 10^3$ dm^3 mol^{-1}, respectively. From these two studies the average values of $1.7 \pm 0.2 \times 10^3$ and $3.4 \pm 0.3 \times 10^3$ dm^3 mol^{-1} were obtained for the binding constants of C1 and curcumin with TX-100, respectively.

3.2. Pulse radiolysis studies

3.2.1. In aqueous–organic solution

Earlier it was reported [9,30] that curcumin on reaction with $N_3^\bullet$ radical in a 20% acetonitrile–water system produced a phenoxyl radical by a diffusion-controlled reaction with maximum absorption at 480–490 nm, with an extinction coefficient of 7300 ± 200 dm^3 mol^{-1} cm^{-1}. The decay of the radical followed second order kinetics with the $2k$ value of $7.5 \pm 0.5 \times 10^8$ dm^3 mol^{-1} s^{-1}. We studied the reaction of C1 with $N_3^\bullet$ radical by irradiating an N_2O-saturated 20% acetonitrile–water solution containing 5×10^{-5} mol dm^{-3} C1, 0.1 mol dm^{-3} azide solution at pH 7. A transient attributed to the phenoxyl radical of C1 having a strong absorption in the 400–600-nm region with a maximum absorption at 490–500 nm, as shown in Fig. 2, was observed. The reaction with $N_3^\bullet$ radicals can be represented as shown in Eq. (4) where CR–OH and CR–O$^\bullet$ represent two curcuminoids (curcumin or C1) and the phenoxyl radical, respectively.

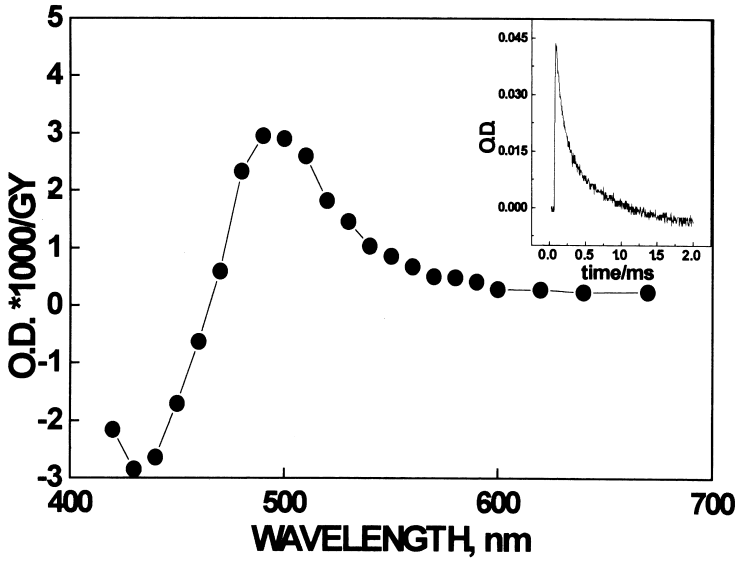


Fig. 2. Transient spectra obtained after pulse radiolysis of an N_2O-saturated 20% acetonitrile–water system containing 0.1 mol dm^{-3} sodium azide and 5×10^{-5} mol dm^{-3} C1 at pH 7. Inset: Absorption–time plot indicating the decay of the phenoxyl radical of C1 at 500 nm. Absorbed dose: 10 Gy.

$$CR{-}OH + N_3^{\bullet} \rightarrow CR{-}O^{\bullet} + N_3^- + H^+ \quad (4)$$

The rate constant for the above reaction was estimated to be $4.6 \pm 0.7 \times 10^9$ dm^3 mol^{-1} s^{-1} for Cl. The extinction coefficient of the radical at 500 nm after correcting for the parent absorption of C1 was estimated to be 7000 ± 200 dm^3 mol^{-1} cm^{-1}. The phenoxyl radical of C1 decayed by second order kinetics with a $2k$ value of $8.1 \pm 0.2 \times 10^8$ dm^3 mol^{-1} s^{-1} which was found to be much similar to that of curcumin. A typical absorption–time plot showing the decay of the phenoxyl radical of C1 in the acetonitrile–water system is given as an inset of Fig. 2.

Reactions of halocarbonperoxyl radicals with curcumin was reported earlier [9] in aqueous 2-propanol solutions. The phenoxyl radical of curcumin appeared in a few microseconds with the maximum absorption at 480–500 nm with a rate constant of $1.0 \pm 0.2 \times 10^8$ dm^3 mol^{-1} s^{-1}. Similarly the reaction of C1 with halocarbonperoxyl radicals was studied in aqueous 2-propanol solutions containing 5×10^{-5} mol dm^{-3} C1 at pH 7 where phenoxyl radicals with maximum absorption at 490–500 nm appeared in 120 μs with a rate constant of $1.5 \pm 0.6 \times 10^8$ dm^3 mol^{-1} s^{-1}.

Reactions of glutathione radicals with curcumin and C1 were studied. On pulse radiolysis of N_2O-saturated 50% aqueous–methanol solutions of 2×10^{-3} mol dm^{-3} glutathione and 5×10^{-5} mol dm^{-3} C1 or curcumin, transient species having absorption in the wavelength region from 300 to 650 nm with a maximum at 490–500 nm and simultaneous bleaching in the 400–450 nm region was observed as shown in Fig. 3. The rate constant for the reaction of glutathione radicals with curcumin and C1 were determined to be $1.0 \pm 0.3 \times 10^8$ and $1.4 \pm 0.1 \times 10^8$ dm^3 mol^{-1} s^{-1}, respectively. Although the radical absorption showed a very similar spectrum, the decay of the radicals did not match with that of the phenoxyl radical. While the phenoxyl radical produced in acetonitrile and water decayed by second order radical–radical reaction, the radical produced from the reaction of glutathione radical with C1 and curcumin decayed by the first order kinetics with a rate constant of $1.0 \pm 0.2 \times 10^2$ s^{-1}. One of the reasons for this first order decay may be due to the presence of excess methanol or due to formation of radical adduct [Eq. (6)]. The reaction with thiyl radicals may be represented as,

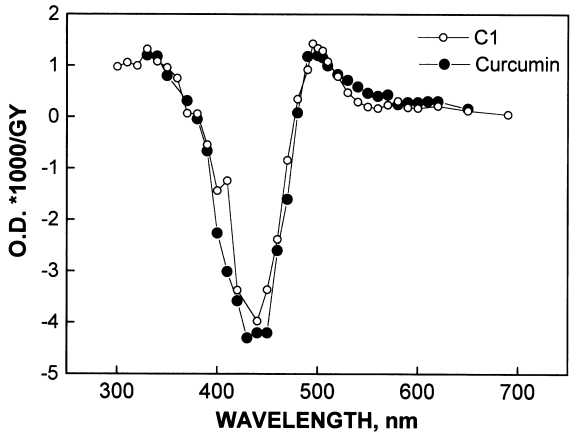


Fig. 3. Transient spectra obtained on pulse radiolysis of 2×10^{-3} mol dm^{-3} glutathione and 5×10^{-5} mol dm^{-3} of C1 (○) or curcumin (●) in N_2O-saturated 50% methanol–water solution at pH 7.

$$GS^{\bullet} + CR{-}OH \rightarrow GSH + CR{-}O^{\bullet} \quad (5)$$

$$\rightarrow (CR{-}OH \cdots GS)^{\bullet} \quad (6)$$

3.2.2. In micellar solutions

In TX-100 micellar solution, the phenoxyl radicals of C1 were generated by electron transfer to $N_3^{\bullet}$ radical produced on radiolysis of N_2O-saturated aqueous solutions containing 0.01 mol dm^{-3} TX-100, 1×10^{-4} mol dm^{-3} C1 and 0.1 mol dm^{-3} azide at pH 7. The phenoxyl radicals thus produced absorb in the same wavelength region as in aqueous–organic solutions and the band is much sharper (Fig. 4). The rate constant for the formation of the phenoxyl radical in TX-100 micelles was determined to be $1.1 \pm 0.2 \times 10^9$ dm^3 mol^{-1} s^{-1}. The extinction coefficient of the radical in TX-100 micelles is much higher than that in the acetonitrile–water system and was estimated to be $14\,900 \pm 400$ dm^3 mol^{-1} cm^{-1} at 500 nm after correcting for the parent absorption of C1. Unlike the homogeneous aqueous–organic solution, it did not show any appreciable decay in the maximum detectable time range of 10 ms. Thus at a radical concentration of 7×10^{-6} mol

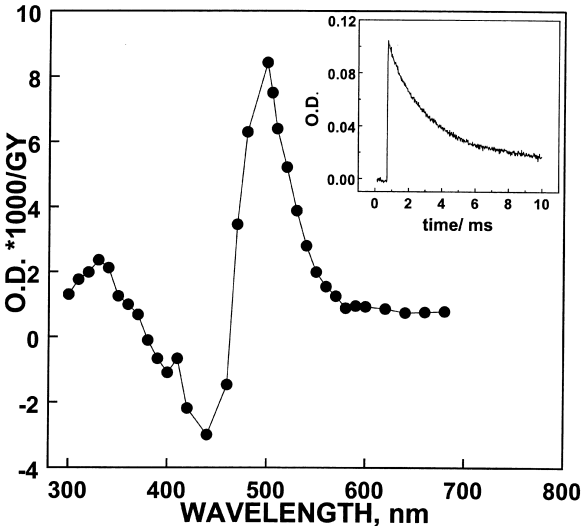


Fig. 4. Transient spectra obtained after pulse radiolysis of N_2O-saturated aqueous solution containing 0.01 M TX-100, 0.1 mol dm^{-3} N_3^- and 5×10^{-5} mol dm^{-3} C1 at pH 7. Inset: Absorption–time plot indicating the decay of the phenoxyl radical at 500 nm. Absorbed dose: 16 Gy.

dm^{-3}, the first half-life of the radical was 1.8 ms. The inset of Fig. 4 shows the time-variation of the transient signal of C1 in TX-100 micelles at 500 nm. The reaction of curcumin with $N_3^{\bullet}$ radical in TX-100 was reported earlier [9] where the phenoxyl radical with the absorption maximum at 490 nm and the extinction coefficient of $14\,800 \pm 250$ dm^3 mol^{-1} cm^{-1} was determined with the bimolecular rate constant of $1.2 \pm 0.1 \times 10^9$ dm^3 mol^{-1} s^{-1}. Similar to C1 the radicals of curcumin were found to be much longer lived in TX-100 micelles.

C1 and curcumin solubilised in CTAB micelles also showed similar properties. The phenoxyl radicals of C1 or curcumin were produced by electron transfer to Br_2^- radicals on pulse radiolysis of N_2O-saturated solution containing 1×10^{-2} mol dm^{-3} CTAB, 0.01 mol dm^{-3} Br^- at pH 7. The rate constant for the formation of the radical at 500 nm was determined to be $2.2 \pm 0.3 \times 10^9$ dm^3 mol^{-1} s^{-1} and $1.4 \pm 0.7 \times 10^9$ dm^3 mol^{-1} s^{-1} for C1 and curcumin, respectively. In both cases the radicals thus produced did not show complete decay in the time scales monitored. The reaction with $Br_2^{-\bullet}$ may be represented as.

$$Br_2^{-\bullet} + CR{-}OH \rightarrow CR{-}O^{\bullet} + 2Br^- + H^+ \qquad (7)$$

3.3. Antioxidant activity of C1

The antioxidant activity of C1 and curcumin was determined by comparing with that of α-tocopherol as given in Section 2. The IC_{50} values for the inhibition of lipid peroxidation in liposomes for C1, curcumin and α-tocopherol were found to be 1.11 ± 0.10, 1.30 ± 0.13 and 12.53 ± 0.85 μmol dm^{-3}, respectively. Similarly TEAC values for C1, curcumin and α-tocopherol were estimated to be 2.36 ± 0.25, 2.61 ± 0.15 and 0.97 ± 0.01, respectively. Thus both these studies indicate that C1 and curcumin are more effective antioxidants than α-tocopherol.

4. Discussion

Both curcumin and C1 are phenolic antioxidants. While curcumin is obtained from the natural product, C1 is the synthetic derivative. They are insoluble in water, and are expected to be localised in the hydrophobic pockets of the cell membrane. Thus an increased solubility was observed in the presence of surfactants. Also a large binding constant for C1 and curcumin with TX-100 micelles determined by following the changes in absorption and fluorescence spectra suggest that they are highly lipophilic and may show irreversible binding with lipids. Furthermore, their reactions with many oxidants were studied to test their free radical scavenging ability and antioxidant activity. Pulse radiolysis offers a unique system to study reactions of short-lived free radicals in a micro to millisecond time scale. Curcumin and C1 react with many reactive oxidants like azide radicals, haloperoxyl radicals, $Br_2^{-\bullet}$ radicals, etc., by electron transfer and the most possible site of attack by the oxidant is the phenolic OH group. At pH 7, the radical cations produced by electron transfer loose a proton immediately to give the corresponding phenoxyl radicals, as the pK_a of most of the phenoxyl radicals is < 0 [31]. The phenoxyl radicals are not reactive towards most of the important biomolecules and

thus render protection towards cellular components. The spectral properties of the phenoxyl radicals are somewhat different in aqueous micellar solutions as compared to aqueous–organic solutions. In micelles, the absorption spectrum is much sharper and more intense suggesting that the phenoxyl radical is located inside the micelle as observed in the case of the parent. This was further supported by the increase in the lifetimes of the phenoxyl radicals in micelles as compared to the homogeneous solutions, because in micelles the probability of the inter-micellar radical–radical reaction is much less. A significant increase in the lifetime of the phenoxyl radicals in micelles is an advantage that, in real systems, where curcumin or C1 is located in the cell membrane the lifetimes may be still higher. Therefore their regeneration by other water-soluble antioxidants like ascorbate and other enzyme systems becomes possible. Like α-tocopherol, curcumin and C1 effectively scavenge peroxyl radicals, a property making them likely candidates as chain breaking antioxidants.

The test for antioxidant activity can be seen by protecting the cell membrane from lipid peroxidation. Lipid peroxidation is a free radical-mediated chain reaction leading to membrane damage. The free radical scavenging ability of curcumin and C1 as seen by pulse radiolysis should lead to the inhibition of lipid peroxidation. Thus the IC_{50} value for the inhibition of lipid peroxidation in liposomes for curcumin and C1 and comparing these values with those of α-tocopherol, suggest that both of them are as efficient as α-tocopherol. Similarly TEAC values confirm this.

In conclusion both curcumin and its synthetic derivative show very similar antioxidant activity.

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