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Phenolic and Enolic Hydroxyl Groups in Curcumin: Which Plays the Major Role in Scavenging Radicals?

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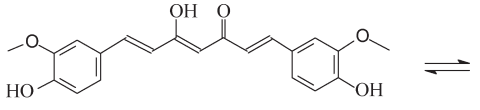
The aim of this work is to clarify the antioxidant abilities of phenolic and enolic hydroxyl groups in curcumin. 1,7-Bis(4-benzyloxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (BEC), 1,7-bis(4-hydroxy-3-methoxyphenyl)heptane-3,5-diol (OHC), 1,7-bis(4-hydroxy-3-methoxyphenyl)heptane-3,5-dione (THC), and 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (BDC) are synthesized to determine the antioxidant activities by using antiradical assays against 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical, galvinoxyl radical, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cation radical ($ABTS^{\bullet+}$) and by protecting DNA and erythrocyte against 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) induced oxidation. The phenolic hydroxyl is the main group for curcumin to trap DPPH, galvinoxyl, and $ABTS^{\bullet+}$ radicals. The conjugative system between enolic and phenolic hydroxyl groups is beneficial for curcumin to protect erythrocytes against hemin-induced hemolysis and to protect DNA against AAPH-induced oxidation, but is not beneficial for curcumin to protect erythrocytes against AAPH-induced hemolysis. More hydroxyl groups enhance the antioxidant effectiveness of curcumin in the experimental systems employed herein. Therefore, curcumin acts as an antioxidant through the phenolic hydroxyl group.

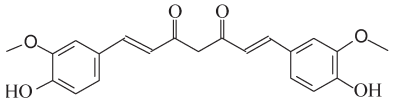
KEYWORDS: Curcumin; phenolic hydroxyl group; enolic hydroxyl group; free radical; antioxidant

INTRODUCTION

Curcuma longa is a plant, and dried *C. longa* is the source of the spice turmeric, with the potential to prevent cancer (*1*). Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptdiene-3,5-dione), as the major component, possesses various pharmacologic effects including anti-inflammatory, antioxidant, antiproliferative, and antiangiogenic activities (*2*). Although it is elucidated theoretically that the hydrogen atom at methylene in curcumin cannot be abstracted by radicals (*3*), as shown as eq 1, enol−keto tautomerism (*4*) makes it possible for curcumin to donate the hydrogen atom from the enolic hydroxyl group in polar solvents (*5*) and plays an important role in the bioavailability of curcumin (*6*).



enol tautomer of curcumin



(1)

diketone tautomer of curcumin

Another opinion regarded curcumin as a chain-breaking antioxidant able to donate its hydrogen atom from the phenolic or enolic hydroxyl group via sequential proton loss electron transfer (SPLET) or hydrogen atom transfer (HAT) (*7*,*8*). In addition, the antioxidant activities of curcumin derivatives without a methoxyl group and with a hydrogenated aliphatic carbon chain were compared by interacting with 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical and by protecting linoleic acid and erythrocytes against the oxidation induced by 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) (*9*). However, the influence of the conjugative linkage between enolic and phenolic hydroxyl groups on the free radical scavenging activity of curcumin is not clear. The aim of this work is to compare the ability of enolic and phenolic hydroxyl groups in curcumin to scavenge free radicals and to protect erythrocytes and DNA against radical-induced oxidation. Therefore, as shown in **Scheme 1**, some derivatives of curcumin have been synthesized. 1,7-Bis(4-hydroxy-3-methoxyphenyl)heptane-3,5-diol (OHC) is prepared by reducing C=C and C=O in curcumin completely. The free radical scavenging property of OHC is only derived from the phenolic hydroxyl groups. 1,7-Bis(4-hydroxy-3-methoxyphenyl)heptane-3,5-dione (THC) is prepared by reducing C=C in curcumin. The antioxidant ability of THC is regarded as the individual contribution from enolic and phenolic hydroxyl groups because there is no conjugative system contained in THC. 1,7-Bis(4-benzyloxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (BEC) is a derivative of curcumin; its phenolic hydroxyl groups are protected by benzyl groups. The antioxidant capacity of BEC is contributed only by the enolic hydroxyl group. Finally, 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (BDC) is prepared by protocatechualdehyde, which introduces four phenolic hydroxyl groups to curcumin (*10*−*13*). The creative point in this work is to apply

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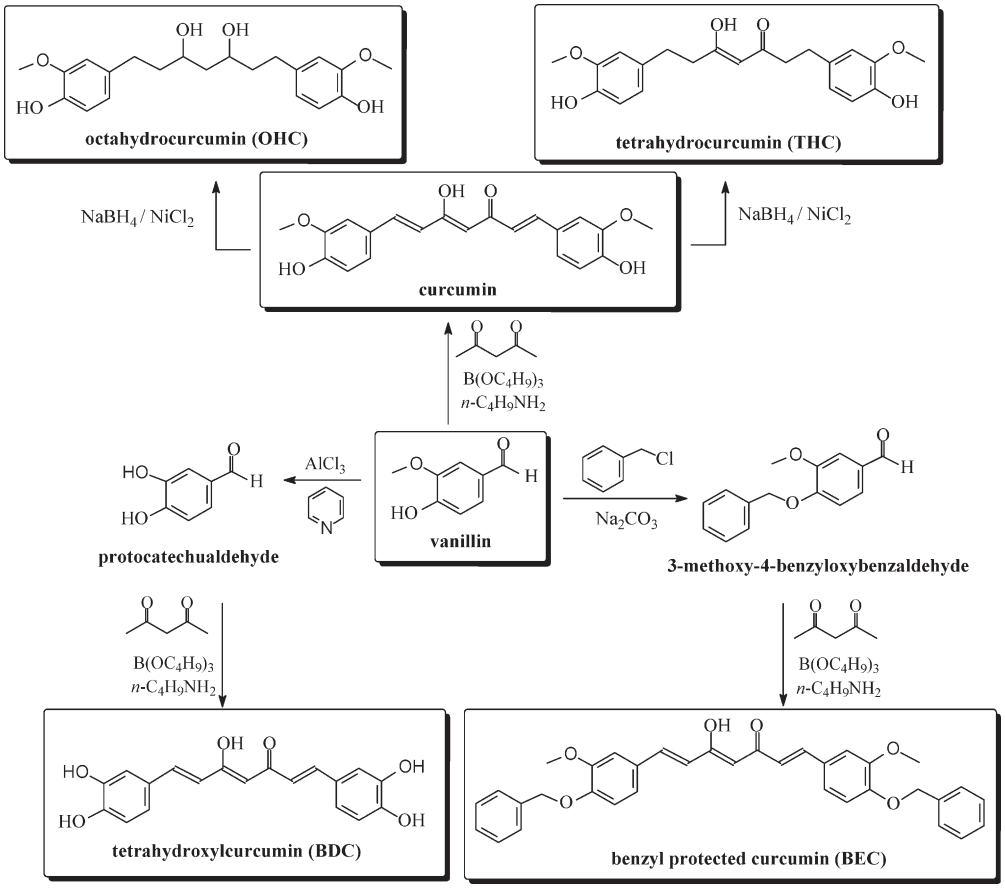
Scheme 1. Synthetic Routines for Curcumin and Its Derivatives in This Work



BEC, whose phenolic hydroxyl group is changed to an ether bond, to characterize the antioxidant effectiveness of enolic hydroxyl group in curcumin. In the literature (*9*) the antioxidant activity of THC was regarded as the contribution from the enolic hydroxyl group; however, the antioxidant activity of THC is the sum of the individual contributions from phenolic and enolic groups, whereas the antioxidant activity of OHC is contributed only by the *o*-methoxyphenolic group because the *β*-diketone moiety in THC is completely reduced to diol. The application of BDC is to test the antioxidant activity of curcumin with *o*-diphenolic hydroxyl groups conjugating with enolic groups.

The free radical scavenging properties of the aforementioned curcumin derivatives are screened by interaction with the galvinoxyl radical (*14*), DPPH radical, and the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{\bullet+}$) (*15*). Then, these curcumin derivatives are applied to the protection of erythrocytes and DNA against AAPH-induced oxidation (*16*). The obtained results are treated by chemical kinetics to give the *stoichiometric factors* (*n*) of curcumin and its derivatives in protecting erythrocytes and DNA. Finally, the stabilization effects of curcumin and its derivatives on erythrocyte membrane are evaluated in hemin-induced hemolysis of erythrocytes.

MATERIALS AND METHODS

Materials and Instrumentation. AAPH, diammonium of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), DPPH radical, galvinoxyl radical, hemin, and naked DNA sodium salt were purchased from Acros Organics, Geel, Belgium, and used as received. Other reagents of 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. The structures of curcumin and its derivatives were identified by ^{1}H and ^{13}C NMR (Varian Mercury 300 NMR spectrometer).

Synthesis of Curcumin and Its Derivatives and Characterization of Structures. Benzaldehyde (0.02 mol of vanillin, 3-methoxy-4-benzyloxybenzaldehyde, or protocatechualdehyde) and 11 mL of tributyl borate (0.04 mol) were dissolved in 10 mL of dry ethyl acetate. Then the mixture of 1.0 g of acetylacetone (0.01 mol) and 0.5 g of boric anhydride (0.007 mol) was added. The reaction mixture was stirred for 20 min, and 0.3 mL of butylamine was added dropwise during 30 min. The reaction mixture was stirred for 15 h at 80 °C, and 20 mL of 0.4 M HCl was added at 60 °C and stirred for 2 h at room temperature. The organic layers were separated, and the aqueous fraction was extracted by ethyl acetate (3 × 20 mL). The layer of ethyl acetate was washed by distilled water and dried over Na_2SO_4. After the solvent had been removed under vacuum pressure, the residue was recrystallized with acetone to obtain the final products (*17*).

The yield of curcumin was 93%: mp 181−182 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.82 (s, 6H, 2 × $CH_3OC_6H_3$−), 6.04 (s, 1H, =CH—C), 6.72 (d, J = 15.9 Hz, 2H, 2 × CH=CH−), 6.79 (d, J = 7.8 Hz, 2H, 2 × CH=CH− in phenyl), 7.12 (d, J = 7.8 Hz, 2H, 2 × −CH=CH in phenyl), 7.31 (s, 2H, 2 × −CH=C in phenyl), 7.50 (d, J = 15.9 Hz, 2H, 2 × −CH=CH), 9.66 (s, 2H, 2 × HO—C_6H_3−); ^{13}C NMR (75 MHz, DMSO-d_6) δ 183.2, 149.4, 148.0, 140.8, 126.3, 123.2, 121.1, 115.7, 111.3, 100.9, 55.7.

BEC was an orange crystal: yield 55%; mp 163−164 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.83 (s, 6H, 2 × $CH_3OC_6H_3$−), 5.14 (s, 4H, 2 × $-CH_2C_6H_5$), 6.10 (s, 1H, =CH—C), 6.80 (d, J = 15.9 Hz, 2H, 2 × CH=CH−), 7.07 (d, J = 8.4 Hz, 2H, 2 × CH=CH− in phenyl), 7.23 (d, J = 8.4 Hz, 2H, 2 × −CH=CH in phenyl), 7.33 (m, 12H, 2 × C_6H_5− and 2 × −CH=C in phenyl), 7.55 (d, J = 15.9 Hz, 2H, 2 × −CH=CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 183.2, 149.9, 149.3, 140.4, 136.7, 128.4, 128.2, 127.9, 127.8, 122.7, 122.2, 113.2, 110.8, 101.1, 69.8, 55.6.

BDC was an orange power: yield 12%; mp 304−305 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.08 (s, 1H, =CH—C), 6.55 (d, J = 15.9 Hz, 2H, 2 × CH=CH−), 6.78 (d, J = 8.4 Hz, 2H, 2 × CH=CH− in phenyl), 7.01 (d, J = 8.4 Hz, 2H, 2 × −CH=CH in phenyl), 7.08 (s, 2H, 2 × −CH=C in phenyl), 7.44 (d, J = 15.9 Hz, 2H, 2 × −CH=CH), 9.18 (s, 2H, 2 × HO—C_6H_3−), 9.64 (s, 2H, 2 × HO—C_6H_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 183.1, 148.4, 145.7, 140.8, 126.3, 121.6, 120.6, 115.9, 114.7, 101.0.

Curcumin (4.9 mmol, 1.805 g) was dissolved in 30 mL of dioxane and 60 mL of ethanol, to which 2.33 g of $NiCl_2 \cdot 6H_2O$ (9.8 mmol) was added. The solution was cooled to 0−4 °C, and 1.35 g of $NaBH_4$ (35.5 mmol) was added within 80 min. The mixture was stirred for 3.5 h at 0−4 °C and then acidified by 1.0 M HCl to pH 4. The solvent (80 mL) was removed under vacuum pressure. The aqueous fraction was extracted by ethyl acetate (3 × 30 mL). The layer of ethyl acetate was washed by saturated saline and dried over Na_2SO_4. After the solvent had been removed under vacuum pressure, the residue was purified by column chromatography (silica gel, petroleum ether/EtOAc = 3:1), 0.44 g of THC (pale yellow power) was obtained: yield 22%; mp 96−97 °C (*11*); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.54−2.77 (m, 8H, 2 × CH_2—CH_2−), 3.73 (s, 6H, 2 × $CH_3OC_6H_3$−), 5.75 (s, 1H, =CH—C), 6.57 (d, J = 8.1 Hz, 2H, 2 × CH=CH− in phenyl), 6.64 (d, J = 8.1 Hz, 2H, 2 × −CH=CH in phenyl), 6.77 (s, 2H, 2 × −CH=C in phenyl), 8.69 (s, 1H, HO—C_6H_3−), 8.71 (s, 1H, HO—C_6H_3−); ^{13}C NMR (75 MHz, DMSO-d_6) δ 204.7, 193.4, 147.4, 144.7, 131.3, 120.3, 115.3, 112.4, 99.6, 56.3, 55.5, 44.7, 30.5, 28.4.

Curcumin (5 mmol, 1.84 g) was dissolved in 90 mL of ethanol and 20 mL of distilled water, to which 1.19 g of $NiCl_2 \cdot 6\,H_2O$ (5 mmol) was added. The solution was cooled to 0−4 °C, and 1.52 g of $NaBH_4$ (40 mmol) was added within 60 min. The reaction mixture was stirred for 5 h at 0−4 °C, acidified by 0.4 M HCl to pH 4, and then extracted by CH_2Cl_2 (4 × 30 mL). The CH_2Cl_2 layer was washed by saturated saline and dried over Na_2SO_4. After the solvent had been removed under vacuum pressure, the residue was recrystallized by CH_2Cl_2, and 1.4 g of OHC (pale yellow power) was obtained: yield 75%; mp 74−75 °C (*11*); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.59−1.88 (m, 6H, 2 × CH_2—CH_2− and −CH_2−), 2.63 (m, 4H, 2 × −CH_2—CH_2), 3.74 (s, 6H, 2 × $CH_3OC_6H_3$−), 6.55 (d, J = 8.1 Hz, 2H, 2 × CH=CH− in phenyl), 6.65 (d, J = 8.1 Hz, 2H, 2 × −CH=CH in phenyl), 6.74 (s, 2H, 2 × −CH=C in phenyl); ^{13}C NMR (75 MHz, DMSO-d_6) δ 146.4, 143.7, 133.6, 120.9, 114.2, 111.0, 68.9, 55.8, 39.1, 30.8.

Curcumin and Its Derivatives Scavenge DPPH Radical, Galvinoxyl Radical, and ABTS$^{\bullet+}$. The experiments of curcumin and its derivatives to trap galvinoxyl radical, DPPH radical, and ABTS$^{\bullet+}$ were performed following previous studies (*14*, *18*). DPPH radical (∼0.1 mM) and galvinoxyl radical (∼2 μM) were dissolved in ethanol to make the absorbance (Abs_{ref}) ∼ 1.00 at 517 and 428 nm, respectively. Two milliliters of 4.0 mM ABTS aqueous solution was oxidized by 1.41 mM $K_2S_2O_8$ for 16 h, and then 100 mL of ethanol was added to make the absorbance of ABTS$^{\bullet+}$ (Abs_0) ∼ 0.70 at 734 nm (*18*). The concentration ranges of the ethanol solution of curcumin and its derivatives are shown in **Figure 1**. The absorbance of the mixtures (Abs_t) became stable after curcumin and its derivatives were added to DPPH radical and ABTS$^{\bullet+}$ for 4 h and to galvinoxyl radical for 19 h. The percentages of DPPH radical, galvinoxyl radical, and ABTS$^{\bullet+}$ scavenged by curcumin and its derivatives were calculated by $(1 - Abs_t/Abs_0) \times 100$ and plotted versus the concentration to obtain 50% inhibition concentration (IC_{50}).

Curcumin and Its Derivatives Protect DNA against AAPH-Induced Oxidation. The oxidation of DNA induced by AAPH was performed according to the literature (*19*) with a little modification (*20*). DNA and AAPH were dissolved in phosphate-buffered solution (PBSo: 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10.0 μM EDTA) to final concentrations of 2.0 mg/mL and 40 mM, respectively, to which various concentrations of curcumin and its derivatives (dissolved in dimethyl sulfoxide (DMSO) as stock solutions) were added. Then, the above solution was dispensed into test tubes to a final volume of 2.0 mL contained in each one. All of the tubes were incubated in a water bath at 37 °C to initiate the reaction. Three tubes were taken out every 2 h 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 trichloroacetic acid (3.0% aqueous solution) were added. The tubes were heated in a boiling water bath for 15 min. After cooling, 1.5 mL of *n*-butanol was added and shaken vigorously to extract thiobarbituric acid reactive substance (TBARS, λ_{max} = 535 nm). The absorbance of TBARS was plotted versus incubation time to express DNA oxidation.

Curcumin and Its Derivatives Protect Erythrocytes against AAPH- and Hemin-Induced Hemolysis. Human erythrocytes were washed by phosphate-buffered saline (PBSa: 150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10 μM EDTA) to remove plasma and centrifugated at 1700*g* for exactly 10 min to obtain a compacted volume of erythrocytes (*21*). Erythrocytes were suspended in PBSa, to which AAPH and DMSO solutions of curcumin and its derivatives were added. The final concentrations of erythrocytes and AAPH were 3.0% (v/v) and 20 mM, respectively. The above mixture was incubated at 37 °C to initiate the hemolysis. Aliquots (1.5 mL) were taken out every 1 h and centrifuged at 1700*g* to obtain the supernatant, in which hemoglobin leaked out of erythrocytes was dissolved after hemolysis took place. The absorbance of the supernatant was measured at 535 nm and plotted versus incubation time to express the hemolysis process.

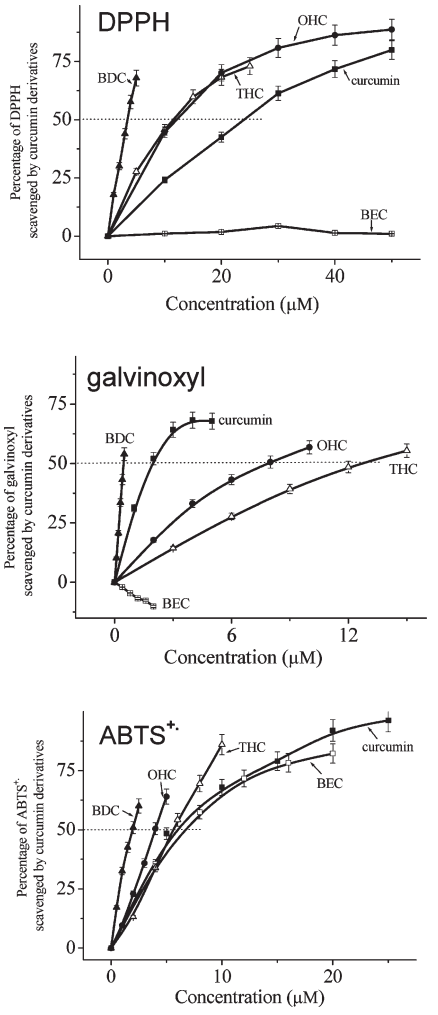


Figure 1. Percentage of DPPH radical, galvinoxyl radical, and ABTS$^{\bullet+}$ scavenged by various concentrations of curcumin and its derivatives.

Hemin was dissolved in 5 mM NaOH to reach 1.0 mM before usage. Erythrocytes were suspended in phosphate-buffered saline without EDTA (PBSe, 150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, pH 7.4), to which a DMSO solution of curcumin and its derivatives were added. This mixture was incubated at 37 °C for 30 min, and then hemin was added. The final concentrations of erythrocytes and hemin were 1.0% (v/v) and 10.0 μM, respectively. After incubation for 30 min, the mixture was centrifuged at 1700*g* to obtain the supernatant. The absorbance of the supernatant was measured at 535 nm and plotted versus the concentration of curcumin and its derivatives (*22*). It was worthy of note that the same volume of DMSO (< 1.0% to the total volume) was contained in the control experiment of hemolysis and oxidation of DNA to eliminate its influence on the experiment.

Statistical Analysis. All of the data were the average value from at least three independent measurements with the experimental error within 10%. The linear relationships between inhibition period and concentration were analyzed statistically by one-way ANOVA on Origin 6.0 professional software, and $p < 0.001$ indicated a significant difference.

Table 1. IC_{50} Values of Curcumin and Its Derivatives Reacting with DPPH Radical, Galvinoxyl Radical, and $ABTS^{\bullet+}$

	IC_{50} (μM)		
	DPPH radical	galvinoxyl radical	$ABTS^{\bullet+}$
curcumin	24.2 ± 1.0	2.0 ± 0.05	6.0 ± 0.3
BDC	3.4 ± 0.15	0.5 ± 0.02	2.0 ± 0.1
OHC	12.1 ± 0.6	7.9 ± 0.4	3.9 ± 0.2
THC	11.4 ± 0.5	12.6 ± 0.6	5.6 ± 0.3
BEC			607 ± 30.0
Trolox[a]	22.8		11.6

[a] Cited from ref *18*.

RESULTS AND DISCUSSION

Curcumin and Its Derivatives Scavenge Free Radicals. The reaction between galvinoxyl and DPPH radicals and an antioxidant indicates the ability of the antioxidant to donate its hydrogen atom to O- and N-centered radicals, respectively. The reaction between an antioxidant and $ABTS^{\bullet+}$ indicates the ability of the antioxidant to reduce the radical. **Figure 1** outlines the percentages of DPPH radical, galvinoxyl radical, and $ABTS^{\bullet+}$ scavenged by various concentrations of curcumin and its derivatives, and the concentrations for 50% radicals trapped (IC_{50}) are listed in **Table 1**.

The order of IC_{50} obtained from the interaction with DPPH radical is in agreement with that in the literature (*9*), but the data are quite different because the absorbance of DDPH was determined when the sample was mixed with DDPH for only 15 min in the literature (*9*), whereas the absorbance of DDPH is detected until the interaction between curcumin derivatives and DDPH reaches the equilibrium state (4 h) in this work. Thus, the absorbance at the equilibrium state of the interaction between samples and radicals results in an accurate value of IC_{50}. In particular, BEC cannot react with DPPH radical and galvinoxyl radical, indicating that the H atom in the enolic hydroxyl group cannot donate to N- and O-centered radicals. The low values of IC_{50} for BDC to react with DPPH and galvinoxyl radicals indicate that four phenolic hydroxyl groups enhance the ability of BDC to scavenge radicals remarkably. OHC and THC have similar IC_{50} values (12.1 and 11.4 μM) when reacting with DPPH radical, implying that they have similar abilities to donate the H atom in the phenolic hydroxyl to N-centered radicals. On the contrary, IC_{50} values of OHC and THC, 7.9 and 12.6 μM, respectively, are higher than that of curcumin (2.0 μM) when reacting with the galvinoxyl radical, indicating that OHC and THC cannot donate their H atoms in the phenolic hydroxyl group to O-centered radical as readily as curcumin. These results confirm a previous conclusion that "curcumin is a classical phenolic chain-breaking antioxidant, donating H atoms from the phenolic group not the CH_2 group" (*7*). However, curcumin and its derivatives can reduce $ABTS^{\bullet+}$ with IC_{50} ranging from 2.0 to 6.7 μM. The reaction between BEC and $ABTS^{\bullet+}$ proves that the enolic hydroxyl group is able to react with the radical by its reductive effect, not donating an H atom. Hence, the phenolic hydroxyl group behaves as an H donor, and the enolic hydroxyl group functions as a reductant in trapping radicals.

Curcumin and Its Derivatives Protect DNA against AAPH-Induced Oxidation. The decomposition of AAPH at 37 °C forms a C-centered radical that converts into peroxyl radical ($ROO^{\bullet}$) by combining with oxygen. $ROO^{\bullet}$ can abstract an H atom from the C-4′ atom of DNA and cause strand breaks (*23*). The further oxidation of linear DNA generates more than 20 carbonyl species reacting with TBA to form colorful TBARS (*19*). As our previous paper pointed out (*20*), in the absence of antioxidants TBARS form continuously with the reaction period increasing. The addition of curcumin and its derivatives hinders the formation of TBARS for a period, designated an inhibition period (t_{inh}). The relationships between t_{inh} and the concentrations of curcumin and its derivatives are outlined in **Figure 2**, and the quantitative equations of $t_{inh} \sim$ concentration are listed in **Table 2**.

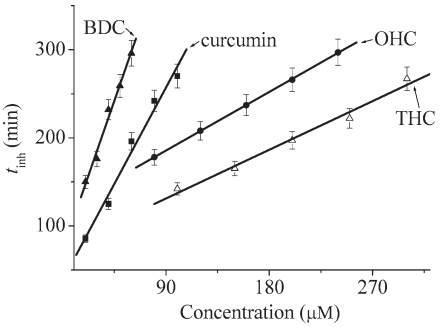


Figure 2. Relationships between t_{inh} and the concentrations of curcumin and its derivatives in AAPH-induced oxidation of DNA.

BEC cannot inhibit the formation of TBARS whatever the concentration increases, revealing that the enolic hydroxyl group does not protect DNA against AAPH-induced oxidation.

From a chemical kinetics viewpoint, t_{inh} generated by an antioxidant (AH) is linearly related to the concentration as expressed by eq 2 (*24*).

$$t_{inh} = (n/R_i)[AH] \quad (2)$$

The *stoichiometric factor* (n) is a quantitative index to express the number of the antioxidant to terminate the radical chain propagation. R_i is the initiation rate of the radical-induced reaction. In the literature (*9*) eq 2 was applied to calculate n of curcumin derivatives in protecting linoleic acid against AAPH-induced oxidation, and it was found that the values of n of hydrogenated curcumin are higher than that of curcumin, in which the formation of diene (λ_{max} = 234 nm) was employed as the index of the oxidation of linoleic acid. In the measurement of t_{inh} for the calcualtion of n, only one point of the concentration of curcumin derivatives was employed, and the values of n were obtained on the basis of the proportional relationship between t_{inh} and the concentration of the antioxidants. Actually, n is the product of R_i and the coefficient in eq 2. However, it is difficult to measure R_i directly. Therefore, Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid) is selected to be the reference antioxidant, and t_{inh} values generated by different concentrations of Trolox are measured to set up the equation of $t_{inh} \sim$ Trolox. Then, R_i is obtained by assigning n of Trolox as 2 (*25*). Another creative point in this work is to employ eq 2, an equation from chemical kinetic deduction, to treat the relationship of $t_{inh} \sim$ [AH] obtained from biological experimental systems including AAPH-induced oxidations of DNA and erthrocytes. Thus R_i should be measured first. Accordingly, Trolox is applied in AAPH-induced oxidation of DNA, but t_{inh} is not observed (data not shown). Therefore, it is impossible to obtain R_i by using Trolox as reference antioxidant in this case. However, Trolox was reported to protect DNA against radical-induced oxidation, in which electrophoresis was applied to observe supercoiled DNA not to transform to open circular or linear chains (*26*). We herein followed the oxidation of DNA by measuring the formation of TBARS. Because Trolox protects DNA by maintaining the supercoiled strand, it is reasonable not to observe the formation of carbonyl species.

To apply eq 2 to calculate n of curcumin and its derivatives, according to our previous paper (*20*), R_i is equal to the radical

Table 2. Equations of t_{inh}~ [Curcumin and Its Derivatives] and *n* of Curcumin and Its Derivatives in Protecting DNA and Erythrocytes

	protect DNA		protect erythrocytes	
AH	t_{inh} (min) = (n/R_i) [AH (μM)] + constant	*n*	t_{inh} (min) = (n/R_i) [AH (μM)] + constant	*n*
curcumin	t_{inh} = 2.43[curcumin] + 37.9	8.2	t_{inh} = 3.45[curcumin] + 18.7	5.8
BDC	t_{inh} = 3.75[BDC] + 72.6	12.6	t_{inh} = 16.5[BDC] − 2.3	27.7
OHC	t_{inh} = 0.74[OHC] + 118.8	2.5	t_{inh} = 3.32[OHC] − 9.1	5.6
THC	t_{inh} = 0.61[THC] + 75.8	2.1	t_{inh} = 6.74[THC] − 81.9	11.3
BEC			t_{inh} = 0.44[BEC] + 2.5	0.7

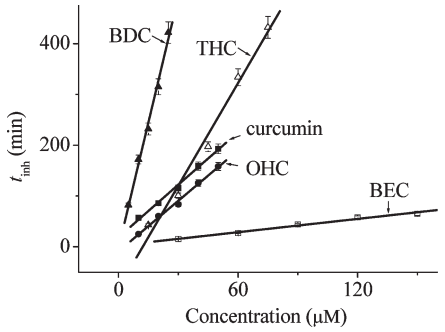


Figure 3. Relationships between t_{inh} and the concentrations of curcumin and its derivatives in AAPH-induced hemolysis of erythrocytes.

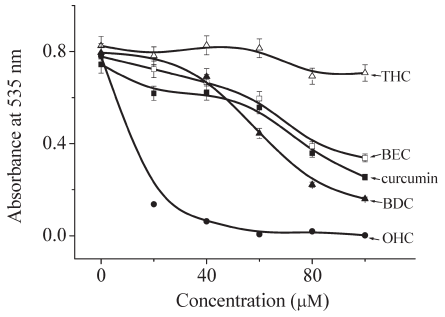


Figure 4. Relationships between t_{inh} and the concentrations of curcumin and its derivatives in hemin-induced hemolysis of erythrocytes.

generation rate ($R_g = (1.4 \pm 0.2) \times 10^{-6}$ [AAPH] s^{-1} (*27*)) when Trolox protects erythrocytes against AAPH-induced hemolysis. Both AAPH and DNA are dissolved in PBSo, and radicals generated from the decomposition of AAPH can attack DNA at the same phase. Therefore, it is safe to assume that R_i is still equal to R_g in this experimental system (*20*). As listed in **Table 2**, the values of *n* of curcumin and its derivatives are the products of coefficients in the equation of t_{inh} ~ [curcumin and its derivatives] and $R_i = R_g = 1.4 \times 10^{-6} \times 40\ mM \cdot s^{-1} = 3.36\ \mu M \cdot min^{-1}$. Therefore, *n* is a relative value to compare the antioxidant abilities among curcumin derivatives. Low values of OHC and THC, 2.5 and 2.1, respectively, reveal that the protective effect of the phenolic hydroxyl group in OHC and THC on DNA is lower than that of curcumin (8.2), indicating that no conjugative system between enolic and phenolic hydroxyl groups is not beneficial to increase the antioxidant capacity in this case. BDC has the highest value of *n* (12.6), indicating that more phenolic hydroxyl groups enhance the antioxidant capacity.

Curcumin and Its Derivatives Protect Erythrocytes against AAPH-Induced Hemolysis. AAPH-mediated hemolysis is a convenient in vitro experimental system to mimic erythrocytes undergoing oxidative stress (*28*). Hemolysis does not occur at the beginning of the reaction, because of the endogenous antioxidants systems, and takes place after the endogenous antioxidants are exhausted completely, generating a lag time (t_{lag}), being prolonged by exogenous antioxidants. The difference between the t_{lag} values in the presence and absence of exogenous antioxidant is designated the inhibition period (t_{inh}) to reveal the protective effect of exogenous antioxidant on erythrocytes, $t_{inh} = t_{lag} - t_{lag0}$ (*29*). **Figure 3** illustrates the relationships between t_{inh} and the concentrations of curcumin and its derivative, and the quantitative equations of t_{inh} ~ concentration are included in **Table 2** as well. In the literature (*9*) curcumin derivatives were also used to protect erthrocytes against AAPH-induced hemolysis, and the time of 50% erthrocytes hemolyzed was employed as the index to compare the antioxidant activities of curcumin derivatives. It was found that the antioxidant effectiveness of THC and OHC is higher than that of curcumin. In this work, we employed eq 2 to treat the relationships of t_{inh} ~ [curcumin derivatives] from AAPH-induced hemolysis of erythrocytes to obtain *n* of curcumin derivatives in protecting erthrocytes.

We have demonstrated that $R_i = R_g$ in AAPH-induced hemolysis; therefore, $R_i = R_g = 1.68\ \mu M \cdot min^{-1}$ when 20 mM AAPH is employed to hemolyze erythrocytes (*29*). The *n* values of curcumin and its derivatives are the products of the coefficients in the equation of t_{inh} ~ [curcumin and its derivatives] and $R_i = R_g = 1.68\ \mu M \cdot min^{-1}$ and are listed in **Table 2** as well. The *n* of BEC is 0.7, indicating that the protective effect of the enolic hydroxyl group on erythrocytes is very low. Because the *n* of curcumin (5.8) is similar to that of OHC (5.6), implying that the free radical scavenging property of curcumin is mainly due to a phenolic hydroxyl group such as OHC. Moreover, *n* of BDC is as high as 27.7, indicating that more phenolic hydroxyl groups enhance the protective effect on erythrocytes remarkably. The *n* of THC (11.3) is even higher than that of curcumin, indicating that the protective effect on erythrocytes is improved significantly by the nonconjugative system between enolic and phenolic hydroxyl groups. The high values of *n* imply that curcumin and its derivatives may incorporate into the endogenous antioxidant systems and play a synergistic protective role in AAPH-induced hemolysis. The partition coefficients of curcumin and its derivatives in solution and membrane are worthy of research in future work. In addition, by comparison of *n* of curcumin derivatives the obtained result is quite different from that in the literature (*9*), in which the protective effects of OHC and THC on erythrocytes are higher than that of curcumin. Thus, the usage of *n* as the index of the antioxidant ability gives an accurate result because *n* is a quantitative parameter not relating to the antioxidant concentration employed.

Curcumin and Its Derivatives Protect Erythrocytes against Hemin-Induced Hemolysis. The hydrophobic hemin is able to intercalate into the lipid part of membranes, to accelerate the potassium leakage, to dissociate skeletal proteins in membrane, and to prohibit some erythrocyte enzymes, leading eventually to hemolysis (*30*). Therefore, hemin-induced hemolysis is an in vitro experimental system to detect whether a compound can stabilize erythrocyte membrane. **Figure 4** illustrates the variation in the absorbance in the case of different concentrations of curcumin and its derivatives applied to protect erythrocytes against hemin-induced hemolysis.

With the increase of the concentration of OHC, hemin-induced hemolysis is prohibited efficiently, revealing that OHC can stabilize erythrocyte membrane remarkably. In addition to the phenolic hydroxyl group, two hydroxyl groups attached to the carbon chain make OHC a perfect membrane stabilizer. However, THC plays almost no protective role in hemin-induced hemolysis, indicating that enolic and phenolic hydroxyl groups cannot stabilize erythrocyte membrane if they are not connected with a conjugative system. However, BEC even possesses a protective capacity similar to that of curcumin. Although BDC has two phenolic hydroxyl groups, its protective effect on erythrocyte against hemin-induced hemolysis is not enhanced significantly compared with that of curcumin. Thus, the hydroxyl group attached to the carbon chain plays the major role in stabilizing erythrocyte membrane.

In conclusion, the phenolic hydroxyl group in curcumin scavenges radicals by donating its H atom to radicals, and the enolic hydroxyl group in curcumin can reduce radicals. The hydroxyl group attached to the carbon chain is beneficial for curcumin to stabilize erythrocyte membrane. Curcumin is a phenolic antioxidant; its effectiveness is mainly contributed by the hydroxyl group.

NOTE ADDED AFTER ASAP PUBLICATION

After the original ASAP posting of September 8, 2009, significant insertions were incorporated to reference previous work. These changes are included in the ASAP posting of October 30, 2009.

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