Food Chemistry 119 (2010) 1435–1442

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant capacity of curcumin-directed analogues: Structure–activity relationship and influence of microenvironment

Ya-Jing Shang, Xiao-Ling Jin, Xian-Ling Shang, Jiang-Jiang Tang, Guo-Yun Liu, Fang Dai *, Yi-Ping Qian, Gui-Juan Fan, Qiang Liu, Bo Zhou *

State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou, Gansu 730000, China

ARTICLE INFO

Article history:
Received 19 May 2009
Received in revised form 29 June 2009
Accepted 8 September 2009

Keywords:
Curcumin
Antioxidant
DPPH·
Haemolysis
Structure–activity relationship

ABSTRACT

Curcumin is the active ingredient of turmeric powder with a variety of biological activities including antioxidative activity. In order to find more active antioxidants with curcumin as the lead compound we synthesised a series of enone analogues of curcumin. The present work studied and compared the capacity of curcumin-directed analogues to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH·) and protect human red blood cells (RBCs) from oxidative haemolysis. It was found that these compounds which bear *o*-diphenoxyl and *o*-dimethoxyphenoxyl groups exhibited significantly higher DPPH·-scavenging and anti-haemolysis activities than those which bear no such groups. In contrast to curcumin analogues that retained the 7-carbon spacer, the compounds with a 5-carbon linker had lower activity. In the case of the latter, the introduction of a ring further decreased DPPH·-scavenging activity. However, the introduction of a ring did increase anti-haemolysis activity, suggesting that the lipophilicity of these compounds might play an important role in the antioxidant activity.

1. Introduction

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, diferuloylmethane, **1a**) is a naturally occurring phenolic compound isolated as a yellow pigment from turmeric (dry rhizomes of *curcuma longa* Linn) which is commonly used as a food colourant, spice, and traditional medicine in India and China (Goel, Kunnumakkara, & Aggarwal, 2008; Sharma, Gescher, & Steward, 2005). This compound has been the focus of many recent biochemical investigations due to its various biological and pharmacological activities, including antioxidant (Daniel, Limson, Dairam, Watkins, & Daya, 2004; Lee et al., 2009; Naidu & Thippeswamy, 2002; Patro et al., 2002; Priyadarsini et al., 2003; Sardjiman, Reksohadiprodjo, Hakim, van deer Goot, & Timmerman, 1997; Sun, Zhang, Chen & Liu, 2002; Venkatesan & Rao, 2000; Weber, Hunsaker, Abcouwer, Deck, & Vander Jagt, 2005; Wright, 2002) and anticancer activities (Aggarwal, Kumar, & Bharti, 2003).

The cancer preventive activity of curcumin is linked to its indirect antioxidant ability to activate Kelch-like ECH-associated protein 1 (Keap 1)/the nuclear factor erythroid 2-related factors (Nrf2) pathway resulting in transcriptional induction of a battery of phase 2 enzymes, and its direct antioxidant ability to eliminate free radicals and to reduce oxidative stress (Dinkova-Kostova & Talalay, 2008). Consequently, the past few years has witnessed tremendous interest in the antioxidant activity of curcumin. Concerning indirect antioxidant property of curcumin, it has the α–β unsaturated carbonyl moiety and hence acts as a Michael reaction acceptor to covalently bind with sulfhydryl-rich proteins (Keap 1), signaling the up-regulation of phase 2 enzymes. In the case of direct antioxidant action, it has been suggested that the activity of curcumin depends upon the presence of the phenolic groups (Barclay et al., 2000). On the other hand, other studies concluded that the central methylene hydrogens of curcumin are important for antioxidant activity (Jovanovic, Boone, Steenken, Trinoga, & Kaskey, 2001). Recently, Litwinienko and Ingold demonstrated that both the phenolic groups and the central methylene hydrogens may be involved in the mechanism of formation of the phenoxyl radical, depending on reaction conditions (Litwinienko & Ingold, 2004).

The structural simplicity and nontoxicity of curcumin, along with its defects such as poor bioavailability (Anand, Kunnumakkara, Newman, & Aggarwal, 2007), make this molecule a promising lead compound for development of potential antioxidant and cancer chemopreventive agents (Anand et al., 2008). On our ongoing research project on kinetics and mechanisms of natural antioxidants, we found recently that simple structural modification of curcumin could significantly enhance its antioxidative activity (Chen, Deng, Zhou, Yang, & Liu, 2006; Deng, Chen, Zhou, Yang, & Liu, 2006; Wei, Chen, Zhou, Yang, & Liu, 2006). Therefore, it is of interest to extend this research and study the structure–activity relationship (SAR) of other curcumin analogues. In this study, four

* Corresponding authors. Fax: +86 931 8915557 (B. Zhou).
E-mail addresses: daifang@lzu.edu.cn (F. Dai), bozhou@lzu.edu.cn (B. Zhou).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.09.024

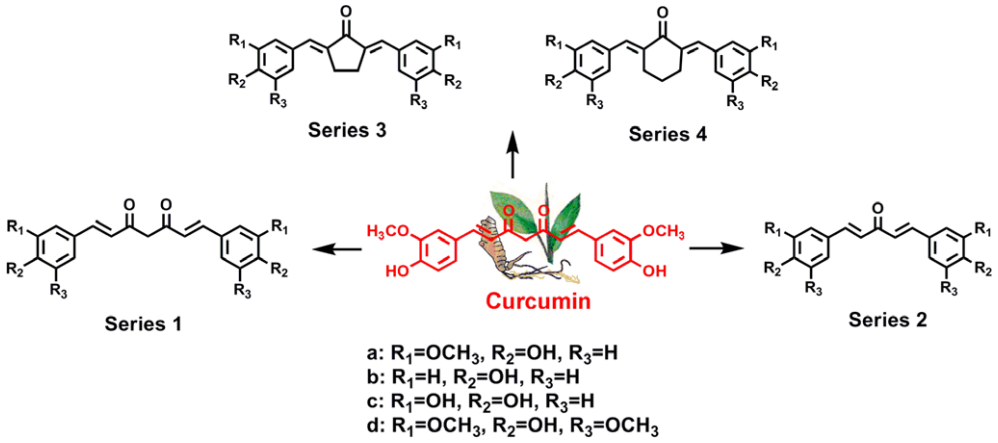


Fig. 1. Molecular structures of curcumin-directed analogues.

series of enone analogues of curcumin were synthesised (Fig. 1) and their antioxidant activities were assessed by DPPH·-scavenging and anti-haemolysis experiments. To explore the substituent effect, we obtained the analogues in series 1 where the 7-carbon spacer was retained and hydroxyl and methoxy groups were introduced into the ortho position of the 4-hydroxyl group. The monoketone analogues in series 2 were designed to test the importance of the length of the spacer between the two aryl rings. To increase rigidity as well as to create molecules which are more lipophilic, a saturated ring was introduced in the compounds in series 3 and 4.

2. Materials and methods

2.1. Materials

^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury plus-300 spectrometer or on a Bruker Avance 400 spectrometer with TMS as the internal standard. EIMS spectra were measured on an HP 5988A spectrometer. Electron-spray ionisation mass spectra in positive mode (ESIMS) data were recorded on a Bruker Esquire 6000 spectrometer.

The compounds **1a–1d** were synthesised by condensation of 2,4-pentanedione with two equivalents of substituted benzaldehyde based on the available methods (Mazumder et al., 1997; Nurfina et al., 1997; Roughley & Whiting, 1973). Generally, 2,4-pentanedione (0.01 mol) and boron oxide (0.007 mol) were dissolved in ethyl acetate (EtOAc) (10 ml) and stirred for 0.5 h at 40 °C followed by addition of the corresponding benzaldehyde (0.02 mol) and tributyl borate (0.02 mol) and stirred for an additional 0.5 h. Then *n*-butylamine (1 ml) in EtOAc (10 ml) was added dropwise over 30 min. After further stirring for 4 h at 40 °C the mixture was allowed to stand overnight to complete the reaction. The mixture was hydrolysed with HCl (0.4 M, 15 ml) and the aqueous layer was extracted three times with EtOAc. The combined organic layers were washed with water and dried over Na_2SO_4, and evaporated to *ca.* 8 ml. Then 5 ml of methanol were added, followed by cooling at 0 °C for 3 h. The curcumin or its derivative was filtered off, washed with cold methanol and dried, then recrystallised from petroleum ether and ethyl acetate to give pure compounds.

1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin, **1a**): 74% yield from vanillin. ^{1}H NMR (300 MHz, $CDCl_3$), δ 3.93 (6H, s), 5.8 (1H, s), 5.85 (2H, br s, –OH), 6.45 (2H, d, J = 15.9 Hz), 6.92 (2H, d, J = 8.4 Hz), 7.05 (2H, s), 7.1 (2H, d, J = 8.1 Hz), 7.56 (2H, d, J = 15.9 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.0, 101.2, 109.5, 114.8, 121.7, 122.8, 127.6, 140.5, 146.7, 147.8, 183.2; EIMS, *m/z* 368 $[M]^+$.

1,7-Bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (**1b**): 69% yield from 4-hydroxy-benzaldehyde. ^{1}H NMR (300 MHz, CD_3COCD_3), δ 5.98 (1H, s), 6.64 (2H, d, J = 15.9 Hz), 6.89 (4H, d, J = 8.7 Hz), 7.55 (4H, d, J = 8.7 Hz), 7.58 (2H, d, J = 15.9 Hz); ^{13}C NMR (75 MHz, CD_3COCD_3), δ 101.8, 116.9, 122.1, 127.7, 131.0, 141.1, 160.6, 184.6; EIMS, *m/z* 308 $[M]^+$.

1,7-Bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (**1c**): 51% yield from 3,4-dihydroxy-benzaldehyde. ^{1}H NMR (300 MHz, CD_3COCD_3), δ 5.98 (1H, s), 6.58 (2H, d, J = 15.9 Hz), 6.87 (2H, d, J = 8.4 Hz), 7.06 (2H, dd, J = 8.4, 1.8 Hz), 7.18 (2H, d, J = 1.8 Hz), 7.51 (2H, d, J = 15.9 Hz); ^{13}C NMR (75 MHz, CD_3COCD_3), δ 101.7, 115.2, 116.4, 122.1, 122.6, 128.3, 141.3, 146.3, 148.6, 184.4; ESIMS, *m/z* 363.1 $[M + Na]^+$.

1,7-Bis(4-hydroxy-3,5-dimethoxyphenyl)-1,6-heptadiene-3,5-dione (**1d**): 57% yield from 4-hydroxy-3,5-dimethoxybenzaldehyde. ^{1}H NMR (300 MHz, CD_3COCD_3), δ 3.93 (12H, s), 5.81 (1H, s), 6.44 (2H, d, J = 15.9 Hz), 6.78 (4H, s), 7.53 (2H, d, J = 15.9 Hz); ^{13}C NMR (75 MHz, CD_3COCD_3), δ 56.3, 101.2, 105.1, 122.0, 126.5, 137.0, 140.7, 147.2, 183.1; EIMS, *m/z* 428 $[M]^+$.

The compounds **2a–2d**, **3a–3d** and **4a–4d** were prepared using the available methods (Du, Bao et al., 2006; Du, Liu et al., 2006). Briefly, a mixture of the appropriate aldehyde (0.01 mol) and the ketone (0.005 mol) was dissolved in glacial acetic acid saturated with anhydrous hydrogen chloride and heated in a water bath at 25–30 °C for 2 h. After standing for 2 d, the mixture was treated with cold water and filtered. The solid obtained was then washed and dried. The crude product was recrystallised from appropriate solvents (methanol or ethanol) to give pure compounds.

1,5-Bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadiene-3-one (**2a**): 53% yield from 4-hydroxy-3-methoxybenzaldehyde. ^{1}H NMR (400 MHz, CD_3COCD_3), δ 3.93 (6H, s), 6.88 (2H, d, J = 8.0 Hz), 7.1 (2H, d, J = 15.9 Hz), 7.21 (2H, dd, J = 8.4, 1.6 Hz), 7.37 (2H, d, J = 2 Hz) 7.65 (2H, d, J = 16 Hz), 8.17 (2H, br s, –OH); ^{13}C NMR (100 MHz, CD_3COCD_3), δ 55.5, 110.7, 115.3, 123.2, 123.3, 127.3, 142.5, 147.9, 149.2, 187.7; EIMS, *m/z* 326 $[M]^+$.

1,5-Bis(4-hydroxyphenyl)-1,4-pentadiene-3-one (**2b**): 51% yield from 4-hydroxybenzaldehyde. ^{1}H NMR (300 MHz, CD_3COCD_3), δ 6.90 (4H, d, J = 8.7 Hz), 6.93 (2H, d, J = 15.6 Hz), 7.60 (4H, d, J = 8.7 Hz), 7.67 (2H, d, J = 15.6 Hz), 8.89 (2H, br s, –OH); ^{13}C NMR (75 MHz, CD_3COCD_3), δ 116.1, 123.2, 115.3, 123.2, 127.1, 159.9, 188.1; EIMS, *m/z* 266 $[M]^+$.

1,5-Bis(3,4-dihydroxyphenyl)-1,4-pentadiene-3-one (**2c**): 30% yield from 3,4-dihydroxybenzaldehyde. ^{1}H NMR (300 MHz, CD_3COCD_3), δ 6.88 (2H, d, J = 8.4 Hz), 7.00 (2H, d, J = 15.9 Hz), 7.10

(2H, dd, J = 8.1, 1.2 Hz), 7.24 (2H, s), 7.60 (2H, d, J = 16.5 Hz), 8.41 (4H, br s, –OH); ^{13}C NMR (75 MHz, CD_3COCD_3), δ 115.5, 116.5, 122.9, 124.0, 128.4, 143.5, 146.4, 148.8, 188.8; ESIMS, m/z 321.1 $[M+Na]^+$.

1,5-Bis(4-hydroxy-3,5-dimethoxyphenyl)-1,4-pentadiene-3-one (**2d**): 17.2% yield from 4-hydroxy-3,5-dimethoxybenzaldehyde. ^{1}H NMR (400 MHz, $CDCl_3$), δ 3.95 (12H, s), 5.85 (2H, br s, –OH), 6.87 (4H, s), 6.95 (2H, d, J = 15.6 Hz), 7.66 (2H, d, J = 15.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$), δ 56.4, 105.4, 123.6, 126.3, 137.4, 143.4, 147.2, 188.4; ESIMS, m/z 387.2 $[M+H]^+$.

2,6-Bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone (**3a**): 90% yield from vanillin. ^{1}H NMR (300 MHz, DMSO-D_6), δ 3.05 (4H, s), 3.82 (6H, s), 6.86 (2H, d, J = 8.1 Hz), 7.12 (2H, d, J = 8.4, 1.6 Hz), 7.23 (2H, s), 7.34 (2H, s), 8.29 (1H, s, –OH), 9.63 (1H, s, –OH); ^{13}C NMR (75 MHz, DMSO-D_6), δ 25.9, 55.6, 114.5, 115.9, 124.7, 127.1, 132.8, 134.7, 147.7, 148.5, 194.7; ESIMS, m/z 375.1 $[M+Na]^+$.

2,6-Bis(4-hydroxybenzylidene)cyclopentanone (**3b**): 90% yield from 4-hydroxybenzaldehyde. ^{1}H NMR (300 MHz, DMSO-D_6), δ 2.99 (4H, s), 6.84 (4H, d, J = 8.7 Hz), 7.32 (2H, s), 7.50 (4H, d, J = 8.7 Hz), 10.01 (2H, br s, –OH); ^{13}C NMR (75 MHz, DMSO-D_6), δ 25.9, 115.9, 126.6, 132.3, 132.7, 134.5, 158.9, 194.8; ESIMS, m/z 315.1 $[M+Na]^+$.

2,6-Bis(3,4-dihydroxybenzylidene)cyclopentanone (**3c**): 85% yield from 3,4-dihydroxybenzaldehyde. ^{1}H NMR (300 MHz, DMSO-D_6), δ 2.88 (4H, s), 6.83 (2H, d, J = 8.1 Hz), 6.99 (2H, dd, J = 8.1,1.2 Hz), 7.12 (2H, s), 7.25 (2H, s), 9.21 (2H, s, –OH), 9.56 (2H, s, –OH); ^{13}C NMR (75 MHz, DMSO-D_6), δ 26.0, 116.0, 117.4, 123.9, 127.1, 132.7, 134.5, 145.4, 147.5, 194.8; ESIMS, m/z 325.1 $[M+H]^+$.

2,6-Bis(4-hydroxy-3,5-dimethoxybenzylidene)cyclopentanone (**3d**): 92% yield from 4-hydroxy-3,5-dimethoxybenzaldehyde. ^{1}H NMR (400 MHz, $CDCl_3$), δ 3.14 (4H, s), 3.95 (12H, s), 5.81 (2H, br s, –OH), 6.88 (4H, s), 7.52 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$), δ 26.5, 56.3, 108.0, 127.5, 134.2, 135.2, 136.6, 147.1, 190.2; ESIMS, m/z 413.2 $[M+H]^+$.

2,6-Bis(4-hydroxy-3-methoxybenzylidene)cyclohexanone (**4a**): 78% yield from 3,4-dimethoxybenzaldehyde. ^{1}H NMR (400 MHz, $CDCl_3$), δ 1.80 (2H, q, J = 6.0 Hz), 2.92 (4H, t, J = 6.0 Hz), 3.92 (6H, s), 5.8 (2H, s), 6.75 (4H, s), 6.96 (2H, d, J = 8.7 Hz), 9.99 (2H, d, J = 8.7 Hz), 7.07 (2H, dd, J = 8.0, 1.6 Hz), 7.74 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$), δ 23.0, 28.5, 55.9, 111.2, 114.3, 124.4, 128.5, 134.2, 136.9, 146.0, 146.3, 190.1; EIMS, m/z 366 $[M]^+$.

2,6-Bis(4-hydroxybenzylidene)cyclohexanone (**4b**): 79% yield from 4-hydroxybenzaldehyde. ^{1}H NMR (300 MHz, CD_3COCD_3), δ 1.76 (2H, q, J = 6.4 Hz), 2.91 (4H, t, J = 6.4 Hz), 6.91 (4H, d, J = 9.0 Hz), 7.42 (4H, d, J = 9.0 Hz), 8.75 (2H, s); ^{13}C NMR (75 MHz, CD_3COCD_3), δ 22.7, 115.2, 127.4, 132.1, 133.6, 135.4, 157.8, 188.1; ESIMS, m/z 307.1 $[M+H]^+$.

2,6-Bis(3,4-dihydroxybenzylidene)cyclohexanone (**4c**): 46.7% yield from 3,4-dihydroxybenzaldehyde. ^{1}H NMR (300 MHz, CD_3COCD_3), δ 1.75 (2H, q, J = 6.6 Hz), 2.90 (4H, t, J = 6.6 Hz), 6.88 (2H, d, J = 8.1 Hz), 6.91 (2H, d, J = 8.1 Hz), 7.08 (2H, s), 7.58 (2H, s), 8.22 (4H, br s, –OH); ^{13}C NMR (100 MHz, CD_3COCD_3), δ 23.9, 116.3, 118.3, 124.6, 129.2, 134.9, 137.0, 145.9, 147.2, 189.4; ESIMS, m/z 339.1 $[M+H]^+$.

2,6-Bis(4-hydroxy-3,5-dimethoxybenzylidene)cyclohexanone (**4d**): 91.7% yield from 4-hydroxy-3,5-dimethoxybenzaldehyde. ^{1}H NMR (400 MHz, $CDCl_3$), δ 1.81 (2H, q, J = 6.4 Hz), 2.94 (4H, t, J = 6.4 Hz), 3.92 (12H, s), 5.79 (2H, br s, –OH), 6.75 (4H, s), 7.73 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$), δ 23.0, 28.4, 56.4, 107.8, 127.4, 134.3, 135.9, 137.3, 146.8, 189.8; ESIMS, m/z 427.2 $[M+H]^+$.

2,2-diphenyl-1-picrylhydrazyl radical (DPPH·), 2,2′-azobis (2-amidinopropane hydrochloride) (AAPH) and trolox were purchased from Sigma–Aldrich, Inc. (St. Louis, MO) and used as received. Other chemicals used were of analytical grade.

2.2. Assay for DPPH·-scavenging activity

The DPPH·-scavenging activity assay was carried out by monitoring the absorbance of an ethanolic solution of DPPH· (100 μM) at 517 nm in the presence and absence of the test compounds at room temperature with a Hitachi 557 spectrophotometer. The concentration (IC_{50}) of the test compounds at which absorbance decreased by 50% of a unit during a 30 min observation was taken as the free radical scavenging potency.

2.3. Preparation of RBCs

Human red blood cells were separated from heparinised blood that was drawn from a healthy donor. The blood was centrifuged at 2000 rpm for 10 min to separate the RBCs from plasma, then the RBCs were washed three times with phosphate-buffered saline (PBS) at pH 7.4. During the last washing the cells were centrifuged at exactly 2000 rpm for 10 min to obtain a constantly packed cell volume.

2.4. Assay for haemolysis

The 5% suspension of RBCs in PBS (pH 7.4) was incubated under air atmosphere at 37 °C for 5 min, into which a PBS solution of AAPH was added to initiate haemolysis. The reaction mixture was shaken gently while being incubated at 37 °C. The extent of haemolysis was determined spectrophotometrically as described previously (Kuang, Wang, Zheng, Liu, & Liu, 1994). Briefly, aliquots of the reaction mixture were taken out at appropriate time intervals, diluted with 0.15 M NaCl, and centrifuged at 2000 rpm for 10 min to separate the RBCs. The percentage haemolysis was determined by measuring the absorbance of the supernatant at 540 nm and compared with that of complete haemolysis by treating the same RBC suspension with distilled water. In case of anti-haemolysis experiments curcumin and its enone analogues, dissolved in dimethyl sulfoxide (DMSO) were added and incubated before addition of AAPH. The final concentration of DMSO was 0.1% (v/v) that did not interfere with the determination. Every experiment was repeated three times and the results were reproducible within 10% deviation.

2.5. Calculation of CLogP

The calculation of the octanol–water coefficient logP (CLogP) was made using Bio-Loom software (Biobyte Corp. version 5) (Hansch & Leo, 1995; Selassie, Kapur, Verma, & Rosario, 2005).

3. Results and discussion

3.1. DPPH·-scavenging activity of curcumin and its enone analogues

DPPH· is a relatively stable nitrogen radical and is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH· assay has been widely used to assess radical-scavenging activity of phenolic compounds and their abilities to transfer labile H atoms to radicals (Goupy, Dufour, Loonis, & Dangles, 2003) (Eq. (1)). DPPH·-scavenging activity of curcumin and its analogues at room temperature was evaluated by UV–Vis spectroscopy. Upon addition of curcumin or its analogues to an ethanol solution of DPPH·, the absorption at 517 nm due to DPPH· decreased immediately. Excellent linear correlations for concentration vs. absorbance were obtained for all the compounds tested (Fig. 2) and the concentrations giving 50% reduction in the absorbance of 100 μM DPPH· solution (IC_{50}) were determined from the linear curves. The IC_{50} values of curcumin and its analogues are given in Table 1.

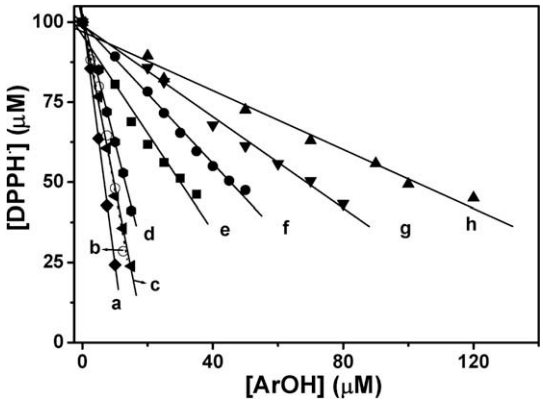


Fig. 2. DPPH (100 μM)-scavenging activity of curcumin and its analogues in ethanol at room temperature. The reaction time was 30 min. (a) **1c**; (b) **3c**; (c) **2c**; (d) **4c**; (e) **1a**; (f) **2a**; (g) **4a**; and (h) **3a**.

It can be seen from Fig. 2 and Table 1 that the activity of these compounds depends significantly on the introduction of electron-donating groups (hydroxyl and methoxy) in the ortho position of the 4-hydroxyl group. In series 1, compound (**1c**) bearing *o*-diphenoxyl groups is the most active one (IC_{50} = 6.73 μM) followed by compound (**1d**) bearing *o*-dimethoxyphenoxyl groups (IC_{50} = 22.63 μM). Curcumin (**1a**) exhibits similar but somewhat weak activity (IC_{50} = 29.99 μM) and compound (**1b**) is the least active one (IC_{50} > 300 μM) in series 1. A similar structure–activity relationship was also obtained in the compounds of other series, that is, these compounds (**2c**, **2d, 3c, 3d, 4c** and **4d**) bearing *o*-diphenoxyl and *o*-dimethoxyphenoxyl groups exhibit significantly higher DPPH·-scavenging activity than those bearing no such groups (**2a**, **2b, 3a, 3b, 4a** and **4b**). By comparing the IC_{50} values for the compounds (**1a–1d** and **2a–2d**) in series 1 and 2 (Table 1), it is clear that the decrease of the length of the spacer between the two aryl rings reduces the DPPH·-scavenging activity. The relatively low IC_{50} values for the compounds in series 2 compared with those in series 3 and 4 (Table 1), clearly indicates that the introduction of ring further decreases the activity, with two exceptions (**3c** and **3d**). It is also noticeable that DPPH·-scavenging activity of **1c**, **2c**, **3c**, **3d**, **4c** is significantly higher than that of ascorbic acid (VC, a famous antioxidant, positive control) and curcumin.

$$DPPH^{\cdot} + ArOH \rightarrow DPPH_2 + ArO^{\cdot} \quad (1)$$

3.2. Inhibition of RBC haemolysis by curcumin and its enone analogues

Although DPPH· assay has been widely used to conveniently test the free radical-scavenging activity of phenolic compounds by hydrogen abstraction reactions (Eq. (1)), the method is only chemical relevance and the system used (ethanol) is homogenous solution. It has been recognised that the antioxidant activity in homogenous solutions may not parallel that in heterogeneous media, let alone the activity *in vivo* (Pryor, Strickland, & Church, 1988; Zhou, Miao, Yang, & Liu, 2005). Human RBCs are heterogeneous media, and are particularly exposed to endogenous oxidative damage because of their specific role as oxygen carriers. Therefore, the antioxidative effect of curcumin and its analogues was investigated in RBC model to bridge the gap between chemical and biological activities and to evaluate the influence of microenvironment on the antioxidative activity.

RBC membrane is rich in polyunsaturated fatty acids which are very susceptible to free radical mediated peroxidation. Since AAPH is water-soluble and the rate of free radical generation from AAPH can be easily controlled and measured, it has been extensively used as a free radical initiator for biological studies. Thermal decomposition of AAPH in the aqueous dispersion of RBCs produces an initiating radical (R·) which can attack the polyunsaturated lipids (LH) in RBC membranes to induce lipid peroxidation (Eqs. (2)–(7)). Since the lipid peroxidation is a free radical chain reaction, the RBC membrane is quickly damaged, leading to haemolysis. On the other hand, if antioxidants (ArOH) are present or added to RBCs they would react with the chain propagating peroxyl radicals (LOO·) to stop the peroxidation (Eq. (8)), hence inhibit haemolysis.

$$\text{Initiation:} \quad R{-}N{=}N{-}R \rightarrow 2R^{\cdot} + N_2 \quad (2)$$

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \quad (3)$$

$$ROO^{\cdot} + LH \rightarrow ROOH + L^{\cdot} \quad (4)$$

$$\text{Propagation:} \quad L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (5)$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (6)$$

$$\text{Termination:} \quad LOO^{\cdot} + LOO^{\cdot} \rightarrow \text{molecular products} \quad (7)$$

$$\text{Antioxidation:} \quad LOO^{\cdot} + ArOH \rightarrow LOOH + ArO^{\cdot} \quad (8)$$

$$LOO^{\cdot} + ArO^{\cdot} \rightarrow \text{molecular products} \quad (9)$$

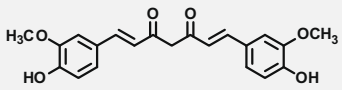
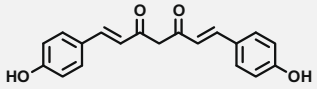
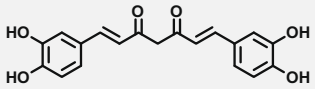
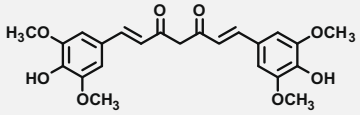
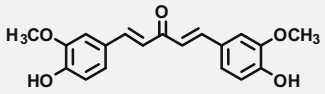
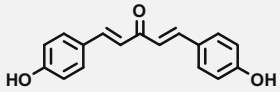
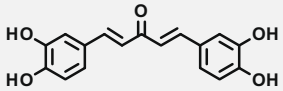
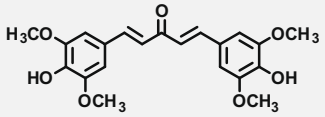
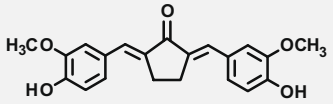
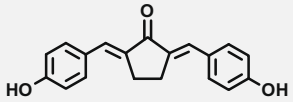
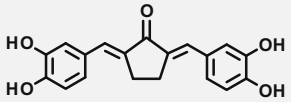
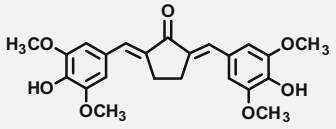
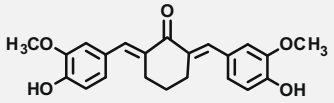
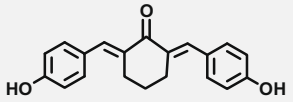
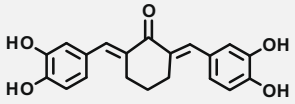
Fig. 3A illustrates the AAPH (50 mM)-induced RBC haemolysis and the inhibition effect by curcumin under an aerobic atmosphere. In the absence of AAPH the RBCs were stable and little haemolysis took place within 4 h (Data not shown). It can be seen from line a in Fig. 3A that haemolysis did not take place at once when RBCs were incubated with AAPH (50 mM). The endogenous antioxidants such as vitamin E and/or ubiquinol-10 present in RBC membrane, protected RBCs against AAPH-induced haemolysis (Deng et al., 2006) until they were exhausted completely, resulting in the inhibition time (t_{inh}). The inhibition time was 121 min when the concentration of AAPH was 50 mM.

Addition of curcumin into the 5% RBC suspension significantly increased the intrinsic inhibition time of the RBCs. The inhibition time produced by 20 μM curcumin was 165 min as shown line b in Fig. 3A. It corresponds to the additional or effective inhibition time, t_{eff}, produced by 20 μM curcumin being 44 min. Based on the steady state kinetic treatment for Eqs. (2)–(9), the t_{eff} can be expressed as Eq. (10) (Zhou et al., 2005). Here R_i is the apparent rate of chain initiation, and n is the stoichiometric factor that denotes the number of LOO· trapped by one molecule of antioxidant. Eq. (10) implies that n can be obtained if R_i is known. However, it is difficult to measure R_i directly. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, is always selected to be the reference antioxidant whose n is taken as 2 (Bowry & Stocker, 1993). Therefore, the R_i value can be determined from the inhibition time of trolox. We measured the R_i value (2.14×10^{-8} M s^{-1}) in 50 mM AAPH-induced RBC haemolysis by using trolox as reference antioxidant. Based on the determined R_i value and Eq. (10), the n value of curcumin is 2.82, implying that one molecule of curcumin can trap approroximately three LOO·. Fig. 3A–D shows the anti-haemolysis effect of curcumin-directed analogues. Obviously, the anti-haemolysis activity of curcumin analogues depended on the specific compound used (Fig. 3A–D). The t_{eff} and n values deduced from Fig. 3A–D are listed in Table 1.

$$t_{eff} = n[ArOH]/R_i \quad (10)$$

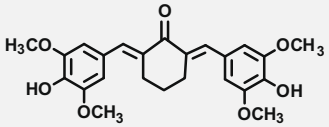
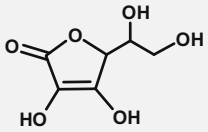
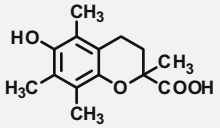
On the basis of the t_{eff} and n values, it was found that the anti-haemolysis activity of these compounds (**1c**, **1d**, **2c**, **2d, 3c, 3d, 4c** and **4d**) bearing *o*-diphenoxyl and *o*-dimethoxyphenoxyl groups is significantly higher than those bearing no such groups (**1a**, **1b**, **2a**, **2b, 3a, 3b, 4a** and **4b**). In addition, the compounds with a 7-carbon spacer between the two aryl rings (**1a–1d**) possess significantly higher activity than the corresponding compounds with a 5-carbon spacer between the two aryl rings (**2a–2d**). The results obtained from this experiment are similar to that obtained from

Table 1
DPPH·-scavenging and anti-haemolysis activities of curcumin and its enone analogues.

No.	Formula	IC_{50} (μM)[a]	t_{eff} (min)[b]	n[c]	CLogP
1a		29.99 ± 0.39	44	2.82	2.25
1b		>300	52	3.33	2.55
1c		6.73 ± 0.12	116	7.42	1.36
1d		22.63 ± 1.03	77	4.93	1.82
2a		45.65 ± 0.84	10	0.64	2.64
2b		>300	[d]	[d]	2.94
2c		9.63 ± 0.26	86	5.50	1.75
2d		38.07 ± 0.53	55	3.52	2.21
3a		101.8 ± 0.25	33	2.11	3.14
3b		>300	[d]	[d]	3.44
3c		9.52 ± 0.11	126	8.06	2.24
3d		15.8 ± 0.09	100	6.40	2.70
4a		68.83 ± 1.21	74	4.74	3.70
4b		>300	[d]	[d]	4.00
4c		13.11 ± 0.31	137	8.77	2.80

(*continued on next page*)

Table 1 (*continued*)

No.	Formula	IC_{50} (μM)[a]	t_{eff} (min)[b]	n[c]	CLogP
4d		53.66 ± 0.86	122	7.81	3.26
VC		23.52 ± 0.37			
Trolox			31	2	

[a] Data are expressed as the mean ± SD for three determinations.
[b] Data are the average of three determinations which were reproducible with deviation less than ±10%.
[c] Calculated from the equation $t_{eff} = n$ [ArOH]/R_i.
[d] No the additional or effective inhibition time (t_{eff}) was observed, but the rate of lipid peroxidation after the inhibition time (t_{inh}) decreased as shown in line b in Fig. 3B–D.

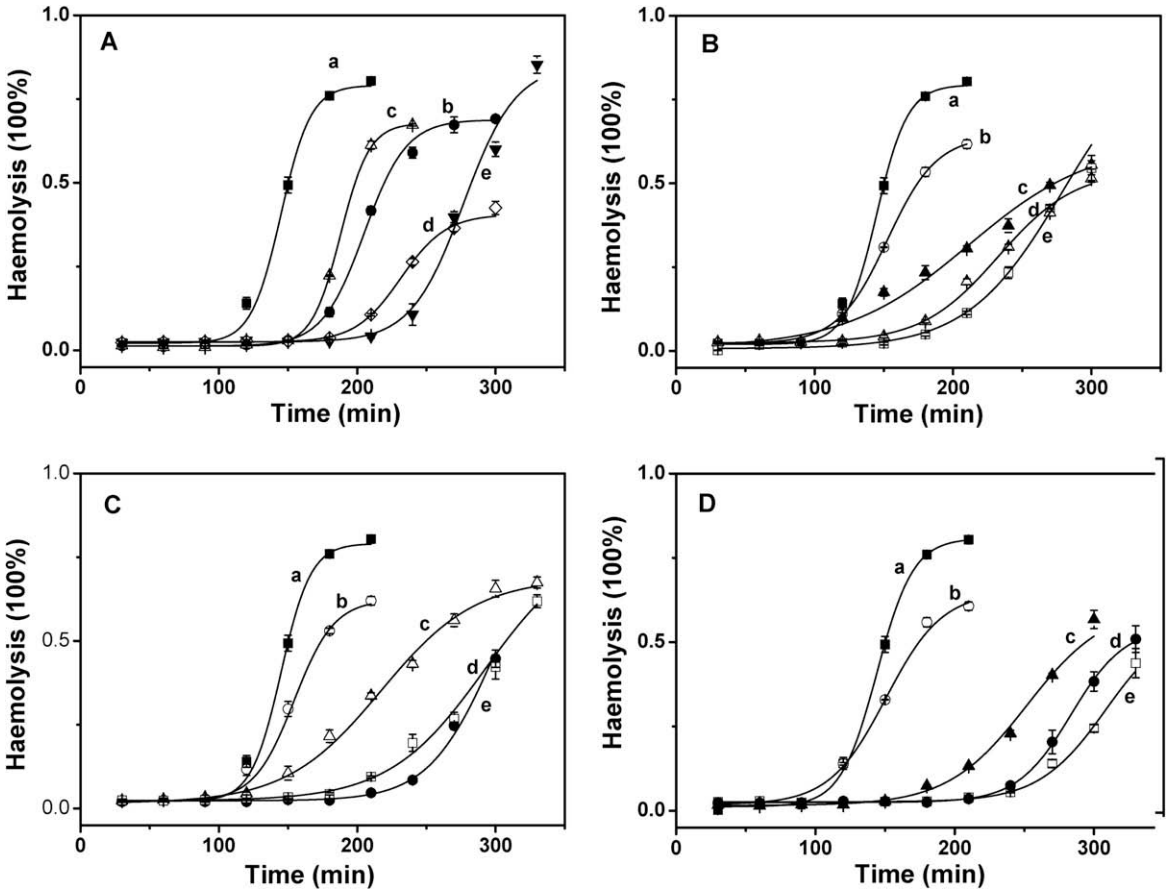


Fig. 3. Inhibition of AAPH (50 mM)-induced haemolysis of 5% human RBCs by the compounds in series 1 (A), 2 (B), 3 (C) and 4 (D). The initial concentration of the compounds was 20 μM. (a) native RBCs; (b) inhibited with **b**; (c) inhibited with **a**; (d) inhibited with **d**; (e) inhibited with **c**. Data are expressed as the mean ± SD of three determination.

DPPH·-scavenging experiment mentioned above. Notably, the antioxidation behavior of the compounds with the introduction of a ring (series 3 and 4) in the anti-haemolysis experiment is distinctly different from that in DPPH·-scavenging experiment. A comparison of t_{eff} values for the compounds **2a**–**2d**, **3a**–**3d** and **4a**–**4d** clearly indicates that the introduction of a ring remarkably increases the anti-haemolysis activity. For example, the effective inhibition time produced by **3c** and **4c** were 126 and 137 min respectively, which are much longer than that (86 min) produced by **2c**. Compounds **3c** and **4c** with a saturated ring and *o*-dihydroxyl groups are the most reactive ones among the examined curcumin analogues. On the contrary, the introduction of ring decreases the activity in the case of DPPH·. This indicates the dependence of the antioxidant activity upon the microenvironment of the

reaction media. In heterogeneous media (RBCs), the lipophilicity of the compound is an important factor in influencing the efficiency of the antioxidant. The lipophilicity should enable the compound to penetrate into the membrane, thus trapping LOO· within the membrane and resulting in the enhancement of t_{eff}. Therefore, we calculated the octanol–water coefficient logP (CLogP) of the compounds (Table 1) using Bio-Loom software (Hansch & Leo, 1995; Selassie et al., 2005). Intriguingly, the correlation was established between CLogP value and the anti-haemolysis activity. For example, molecules with higher lipophilic ability, that is, **3c** and **4c**, (CLogP 2.24 and 2.80, respectively) exhibit higher activity, while molecule (**2c**) with lower lipophilic ability (CLogP 1.75), is less active.

3.3. Structure–activity relationship and mechanism

Curcumin is a well-known natural antioxidant and cancer chemopreventive agent which has attracted much interest in the past decade. Effective effort has, therefore, been continuously devoted to the synthesis of new curcumin analogues, aiming at finding more effective antioxidants and cancer chemopreventive agents (Anand et al., 2008). The present study identified the structure–activity relationship in curcumin-directed analogues, which underscores the important chemical features for this class of molecules to increase antioxidant activity.

In the present work, the compounds (**c** and **d**) bearing *o*-diphenoxyl and *o*-dimethoxyphenoxyl groups in four series exhibited significantly higher DPPH·-scavenging and anti-haemolysis activities than the corresponding compounds (**a** and **b**) bearing no such groups. It is well known that O–H bond dissociation enthalpy (BDE) regulates the antioxidant potency in phenolic compounds (Wright, Johnson, & Dilabio, 2001). Therefore, enhancement in the antioxidant activity of the compounds (**c** and **d**) in four series can be explained by the fact that the introduction of electron-donating groups (methoxy and hydroxyl) in the *o*-position of 4-OH, reduces the BDE of O–H.

The compound (**c**) bearing *o*-diphenoxyl groups is the most active one in each series. It can also be understood because the oxidative intermediate, the *o*-hydroxyphenoxyl radical, is more stable due to the intramolecular hydrogen bonding interaction, as evidenced recently from experiments by spectrophotometric measurement (Foti & Ruberto, 2001) and theoretical calculations (Wright et al., 2001). The theoretical calculation showed that the hydrogen bond in the *o*-hydroxyphenoxyl radical is approximately 4 kcal/mol stronger than that in the parent catechol, and that the BDE of catechol is 9.1 kcal/mol lower than that of phenol and 8.8 kcal/mol lower than that of resorcinol (Wright et al., 2001). In addition, it should be easier to further oxidise the *o*-hydroxyphenoxyl radical to form the final *o*-quinone (Qian et al., 2009). We have recently found the significantly higher antioxidant activity of molecules bearing *o*-diphenoxyl groups in flavonols (Zhou et al., 2005) and resveratrol analogues (Fang & Zhou, 2008; Qian et al., 2009).

On the other hand, the decrease of the length of the spacer between the two aryl rings (series 2) reduces the DPPH·-scavenging activity. Because the DPPH· assay reflects the hydrogen donating capacity of phenolic compounds (Goupy et al., 2003), the reduction of the DPPH·-scavenging activity could come from the increase of phenolic O–H BDE. That is, the decrease of the length of the spacer which is adverse to electron delocalization in phenoxyl radicals increases O–H BDE and hence reduce the activity. It is also noticeable that the introduction of a ring decreases the DPPH·-scavenging activity, but does increase anti-haemolysis activity. The antioxidant activity of the compounds with a saturated ring has been reported previously (Lee et al., 2009; Sardjiman et al., 1997), and our data are in accordance with previous observations. This indicates that in addition to O–H BDE, the lipophilicity of the compound is the other important factor to influence the efficiency of antioxidant in heterogeneous media (RBCs). Moreover, the introduction of a ring might influence their biological properties such as receptor binding. Compound **4a** has been demonstrated to bind to nuclear type II sites on breast cancer cells with high affinity (K_d 1–7 nM) and to inhibit cell proliferation (Markaverich et al., 1992).

In conclusion, we have examined the antioxidative ability and SAR of four series curcumin analogues. We demonstrated enhanced DPPH·-scavenging and anti-haemolysis activities, rendering the compounds bearing *o*-diphenoxyl and *o*-dimethoxyphenoxyl groups potentially valuable for the development of curcumin-directed drugs. In addition, the observation that the introduction of a ring increases the lipophilicity and hence enhances anti-haemolysis activity gives us useful information for antioxidant drug design.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Nos. 20972063 and 20621091), the 111 Project, and Program for New Century Excellent Talents in University (NCET-06-0906).

References

Aggarwal, B. B., Kumar, A., & Bharti, A. C. (2003). Anticancer potential of curcumin: Preclinical and clinical studies. *Anticancer Research, 23*, 363–398.

Anand, P., Kunnumakkara, A. B., Newman, R. B., & Aggarwal, B. B. (2007). Bioavailability of curcumin: Problems and promises. *Molecular Pharmaceutics, 4*, 807–818.

Anand, P., Thomas, S. G., Kunnumakkara, A. B., Stundaram, C., Harikumar, K. B., Sung, B., et al. (2008). Biological activities of curcumin and its analogues (congeners) made by man and mother nature. *Biochemical Pharmacology, 76*, 1590–1611.

Barclay, L. R. C., Vinqvist, M. R., Mukai, K., Goto, H., Hashimoto, Y., Tokuanga, A., et al. (2000). On the antioxidant mechanism of curcumin: Classical methods are needed to determine antioxidant mechanism and activity. *Organic Letters, 2*, 2841–2843.

Bowry, V. W., & Stocker, R. (1993). Tocopherol-mediated peroxiation. The prooxidation effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *Journal of the American Chemical Society, 115*, 6029–6044.

Chen, W.-F., Deng, S.-L., Zhou, B., Yang, L., & Liu, Z.-L. (2006). Curcumin and its analogues as potent inhibitors of low density lipoprotein oxidation: H-atom abstraction from the phenolic groups and possible involvement of the 4-hydroxy-3-methoxyphenyl groups. *Free Radical Biology and Medicine, 40*, 526–535.

Daniel, S., Limson, J. L., Dairam, A., Watkins, G. M., & Daya, S. (2004). Through metal binding, curcumin protects against lead- and cadmium-induced lipid peroxidation in rat brain homogenates and against lead-induced tissue damage in rat brain. *Journal of Inorganic Biochemistry, 98*, 266–275.

Deng, S.-L., Chen, W.-F., Zhou, B., Yang, L., & Liu, Z.-L. (2006). Protective effects of curcumin and its analogues against free radical-induced oxidative haemolysis of human red blood cells. *Food Chemistry, 98*, 112–119.

Dinkova-Kostova, A. T., & Talalay, P. (2008). Direct and indirect antioxidant properties of inducers of cytoprotective proteins. *Molecular Nutrition and Food Research, 52*, S128–S138.

Du, Z.-Y., Bao, Y.-D., Liu, Z., Qiao, W., Ma, L., Huang, Z.-S., et al. (2006). Curcumin analogs as potent aldose reductase inhibitors. *Archiv der Pharmazie Chemistry in Life Science, 339*, 123–128.

Du, Z.-Y., Liu, R.-R., Shao, W.-Y., Mao, X.-P., Ma, L., Gu, L.-Q., et al. (2006). α-Glucosidase inhibition of natural curcuminoids and curcumin analogs. *European Journal of Medicinal Chemistry, 41*, 213–218.

Fang, J.-G., & Zhou, B. (2008). Structure–activity relationship and mechanism of the tocopherol-regenerating activity of resveratrol and its analogues. *Journal of Agricultural and Food Chemistry, 56*, 11458–11463.

Foti, M., & Ruberto, G. (2001). Kinetic solvent effects on phenolic antioxidant determined by spectrophotometric measurements. *Journal of Agricultural and Food Chemistry, 49*, 342–3488.

Goel, A., Kunnumakkara, A. B., & Aggarwal, B. B. (2008). Curcumin as "curecumin": From kitchen to clinic. *Biochemical Pharmacology, 75*, 787–809.

Goupy, P., Dufour, C., Loonis, M., & Dangles, O. (2003). Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *Journal of Agricultural and Food Chemistry, 51*, 615–622.

Hansch, C., & Leo, A. (1995). *Exploring QSAR: Fundaments and applications in chemistry and biology*. American Chemical Society: Washington, DC.

Jovanovic, S. V., Boone, C. W., Steenken, S., Trinoga, M., & Kaskey, R. B. (2001). How curcumin works preferentially with water soluble antioxidants. *Journal of the American Chemical Society, 123*, 3064–3068.

Kuang, Z. H., Wang, P. F., Zheng, R. L., Liu, Z.-L., & Liu, Y.-C. (1994). Making vitamin C lipo-soluble enhances its protective effect against radical induced hemolysis of erythrocytes. *Chemistry and Physics of Lipids, 71*, 95–97.

Lee, K., Ab. Aziz, F. H., Syahida, A., Abas, F., Shaari, K., Israf, D. A., et al. (2009). Synthesis and biological evaluation of curcumin-like diarylpentanoid analogues for anti-inflammatory, antioxidant and anti-tyrosinase activities. *European Journal of Medicinal Chemistry, 44*, 3195–3200.

Litwinienko, G., & Ingold, K. U. (2004). Abnormal solvent effects on hydrogen atom abstraction. 2. Resolution of the curcumin antioxidant controversy. The role of sequential proton loss electron transfer. *Journal of Organic Chemistry, 69*, 5888–5896.

Markaverich, B. M., Schauweker, T. H., Gregory, R. R., Varma, M., Kittrell, F. S., Medina, D., et al. (1992). Nuclear type II sites and malignant cell proliferation: Inhibition by 2,6-bis-benzylidenecyclohexanones. *Cancer Research, 52*, 2482–2488.

Mazumder, A., Neamati, N., Sunder, S., Schulz, J., Pertz, H., Eich, E., et al. (1997). Curcumin analogs with altered potencies against HIV-1 integrase as probes for biochemical mechanism of drug action. *Journal of Medicinal Chemistry, 40*, 3057–3063.

Naidu, K. A., & Thippeswamy, N. B. (2002). Inhibition of human low density lipoprotein oxidation by active principle from spices. *Molecular and Cellular Biochemistry, 229*, 19–23.

Nurfina, A. N., Reksohadiprodjo, M. S., Timmerman, H., Jenie, U. A., Sugiyant, D., & van der Goot, H. (1997). Synthesis of some symmetrical curcumin derivatives and their antiinflammatory activity. *European Journal of Medicinal Chemistry, 32*, 321–328.

Patro, B. S., Rele, S., Chintalwar, G. J., Chattopadhyay, S., Adhikari, S., & Mukherjee, T. (2002). Protective activities of some phenolic 1, 3-diketones against lipid peroxidation: Possible involvement of the 1, 3-diketone moiety. *ChemBioChem, 3*, 364–370.

Priyadarsini, K. I., Maity, D. K., Naik, G. H., Kumar, M. S., Unnikrishnan, M. K., Satav, J. G., et al. (2003). Role of phenolic O–H and methylene hydrogen on the free radical reaction and antioxidant activity of curcumin. *Free Radical Biology and Medicine, 35*, 475–484.

Pryor, W. A., Strickland, T., & Church, D. F. (1988). Comparison of the efficiencies of several natural and synthetic antioxidants in aqueous SDS [sodium dodecyl sulfate] micelle solutions. *Journal of the American Chemical Society, 110*, 2224–2229.

Qian, Y.-P., Cai, Y.-J., Fan, G.-J., Wei, Q.-Y., Yang, J., Zheng, L.-F., et al. (2009). Antioxidant-based lead discovery for cancer chemoprevention: The case of resveratrol. *Journal of Medicinal Chemistry, 52*, 1963–1974.

Roughley, P. J., & Whiting, D. A. (1993). Experiments in biosythesis of curcumin. *Journal of the Chemical Society, Perkin Transactions, 1*, 2379–2388.

Sardjiman, S. S., Reksohadiprodjo, M. S., Hakim, L., van deer Goot, H., & Timmerman, H. (1997). 1, 5-Diphenyl-1, 4-pentadiene-3-ones and cyclic analogues as antioxidative agents. Synthesis and structure–activity relationship. *European Journal of Medicinal Chemistry, 32*, 625–630.

Selassie, C. D., Kapur, S., Verma, R. P., & Rosario, M. (2005). Cellular apoptosis and cytotoxicity of phenolic compounds: A quantitative structure–activity relationship study. *Journal of Medicinal Chemistry, 48*, 7234–7242.

Sharma, R. A., Gescher, A. J., & Steward, W. P. (2005). Curcumin: The story so far. *European Journal of Cancer, 41*, 1955–1968.

Sun, Y.-M., Zhang, H.-Y., Chen, D.-Z., & Liu, C.-B. Theoretical elucidation on the antioxidant mechanism of curcumin: A DFT study. *Organic Letters, 4*, 2909–2911.

Venkatesan, P., & Rao, M. N. A. (2000). Structure–activity relationships for the inhibition of lipid peroxidation and the scavenging of free radicals by synthetic symmetrical curcumin analogues. *Journal of Pharmacy and Pharmacology, 52*, 1123–1128.

Weber, W. M., Hunsaker, L. A., Abcouwer, S. F., Deck, L. M., & Vander Jagt, D. L. (2005). Anti-oxidant activities of curcumin and related enones. *Bioorganic and Medicinal Chemistry, 13*, 3811–3820.

Wei, Q.-Y., Chen, W.-F., Zhou, B., Yang, L., & Liu, Z.-L. (2006). Inhibition of lipid peroxidation and protein oxidation in rat liver mitochondria by curcumin and its analogues. *Biochimica et Biophysica Acta, General Subjects, 1760*, 70–77.

Wright, J. S. (2002). Predicting the antioxidant activity of curcumin and curcuminoids. *Journal of Molecular Structure (Theochem), 591*, 207–217.

Wright, J. S., Johnson, E. R., & Dilabio, G. A. (2001). Predicting the activity of phenolic antioxidants: Theoretical method, analysis of substituent effects, and application to major families of antioxidant. *Journal of the American Chemical Society, 123*, 1173–1183.

Zhou, B., Miao, Q., Yang, L., & Liu, Z.-L. (2005). Antioxidative effects of flavonols and their glycosides against the free-radical-induced peroxidation of linoleic acid in solution and in micelles. *Chemistry-A European Journal, 11*, 680–691.