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Reactions of reactive oxygen species (ROS) with curcumin analogues: Structure–activity relationship

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Abstract

Three curcumin analogues viz., bisdemethoxy curcumin, monodemethoxy curcumin, and dimethoxycurcumin that differ at the phenolic substitution were synthesized. These compounds have been subjected for free radical reactions with DPPH radicals, superoxide radicals ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$) and peroxyl radicals ($CCl_3O_2^{\bullet}$) and the bimolecular rate constants were determined. The DPPH radical reactions were followed by stopped-flow spectrometer, $^1O_2$ reactions by transient luminescence spectrometer, and $CCl_3O_2^{\bullet}$ reactions using pulse radiolysis technique. The rate constants indicate that the presence of *o*-methoxy phenolic OH increases its reactivity with DPPH and $CCl_3O_2^{\bullet}$, while for molecules lacking phenolic OH, this reaction is very sluggish. Reaction of $O_2^{\bullet-}$ and $^1O_2$ with curcumin analogues takes place preferably at β-diketone moiety. The studies thus suggested that both phenolic OH and the β-diketone moiety of curcumin are involved in neutralizing the free radicals and their relative scavenging ability depends on the nature of the free radicals.

Keywords: *Curcumin analogues, DPPH radical, superoxide radical, peroxyl radical, singlet oxygen*

Introduction

Curcumin, bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione, is a major yellow orange pigment found in turmeric, whose medicinal properties have been known since ancient times. Curcumin shows a number of pharmacological properties including antioxidant, anti-cancer, anti-inflammatory activities [1–7]. Curcumin is an excellent scavenger of reactive oxygen species (ROS) [8–13]. Although oxygen is essential for aerobic life, it acts as a source for the generation of different ROS. ROS are continuously produced during normal physiological events, when produced in excess, they are capable of damaging crucial biomolecules such as nucleic acids, lipids, proteins and carbohydrates leading to many diseased conditions [14]. Singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), peroxyl radical, are some of the important ROS generated inside the cells [14].

The molecular structure of curcumin has three important functional regions, a β-diketo group, an olefinic linker and ortho-methoxy phenolic group. It exhibits keto-enol tautomerism and in solution it exists predominantly in the enolic form. Curcumin has three ionizable protons and the enolic proton is more acidic than either of phenolic protons. Extensive structure–activity studies on curcumin have confirmed that the o-methoxy phenolic OH group is essential for the antioxidant activity and free radical reactions are initiated by both phenolic OH and enolic OH group [15–19]. The α,β unsaturated β-diketo group is key for the anti-cancer activity through the inactivation of NF-kβ by blocking the thiol via Michael addition reaction [20]. The β-diketo group is necessary for superoxide dismutase mimicking activity [13,21]. The length of the olefinic linker plays an important role in Alzheimer's disease mainly in preventing protein aggregation [22,23]. Taking into consideration of all these important findings, recent research is directed to the synthesis of new curcumin analogues by modifying the three functional groups [24–30].

Although turmeric contains curcumin as the major pigment, other curcuminoids like monodemethoxy

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curcumin (MDMC), bisdemethoxy curcumin (BDMC) and cyclic curcumin are present in minor quantities. MDMC and BDMC have also been examined for a variety of biological activities. MDMC was found to be more potent than curcumin or BDMC in inhibiting proliferation of MCF-7 breast cancer cells [31]. BDMC was more cytotoxic to human ovarian cancer cell lines compared to curcumin and MDMC [32,33]. Dimethoxy curcumin (DMC), a methylated synthetic curcumin analogue has been found to be more cytotoxic and exhibit promising anti-androgen activities in human prostate cancer cell lines [34,35].

With a view to understanding the role of different functional groups on reactivity with oxidizing free radicals, three curcumin analogues varying in aromatic substitution, viz., MDMC, BDMC and DMC have been tested for their reaction with ROS and DPPH radical and the results have been compared with those of curcumin. Attempts have also been made to correlate these changes with the aromatic substitution. The chemical structures of these analogues and their keto-enol tautomeric equilibrium are shown in Schemes 1 and 2, respectively.

Materials and methods

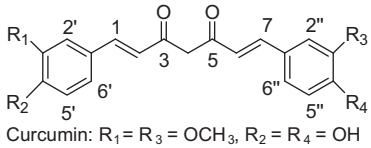
Commercially available curcumin mix purchased from Sigma/Aldrich Chemicals (St.Louis, MO, USA) contains ~70% curcumin along with MDMC and BDMC. Curcumin was separated and purified from the mix through conventional silica gel column using 95:5 chloroform:methanol as an eluent. 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azinobis (3-ethylbenthiazoline-6-sulphonate) (ABTS), xanthine, xanthine oxidase, cytochrome C, haematoporphyrin were purchased from Sigma/Aldrich Chemicals. All the other reagents used were of the highest purity available. Spectrograde solvents, acetonitrile, 2-propanol and carbon tetrachloride were procured from Spectrochem India ltd (Mumbai, India). Nanopure water from Millipore Elix 3/A-10 water polishing system was used for preparing the solutions and freshly prepared solutions were used for each experiment.

Synthesis of curcumin analogues

MDMC, BDMC and DMC were synthesized according to the procedure reported by Venkateshwarlu et al. [36]. In brief boric acid and acetyl acetone was allowed to react in dimethyl formamide (DMF) at 65°C to form an acetyl acetone–boric oxide complex. The borate complex was treated with different substituted aldehyde compounds in the presence of 1,2,3,4-tetrahydroquinoline as a catalyst to form the desired curcumin analogue. All the crude products were purified by passing through a conventional silica gel column, recrystallized in 10% aqueous methanol and were characterized by melting point, IR, NMR and mass spectroscopy. The purity was further confirmed by HPLC. The melting point and ^{1}H NMR data of curcumin and analogues were as follows. Curcumin: mp 168°C, ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.96 (6H, s, -OCH_3), δ = 5.8 (1H, s, H-4), δ = 6.46 (2H, d, J = 16.0 Hz, H-2,6), δ = 6.93 (2H, d, J = 8.0 Hz, H-5′,5″), δ = 7.06 (2H, d, J = 2.0 Hz, H-2′,2″), δ = 7.11 (2H, dd, J = 8.0, 2.0 Hz, H-6′,6″), δ = 7.57 (2H, d, J = 16.0 Hz, H-1,7). MDMC: mp 170°C, ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.84 (3H, s, -OCH_3), δ = 6.05 (1H, s, H-4), δ = 6.77 (1H, d, J = 15.8 Hz, H-2 or H- 6), δ = 6.71 (1H, d, J = 15.8 Hz, H-2 or H-6), δ = 6.82–6.84 (3H, m, H-5′,3″,5″), δ = 7.15 (1H, dd, J = 8.2, 1.8 Hz, H- 60), δ = 7.33 (1H, d, J = 1.8 Hz, H-2′), δ = 7.52–7.59 (4H, m, H-1,7, 2″,6″). BDMC: mp 220°C ^{1}H NMR (300 MHz, DMSO, d_6): δ = 6.02 (1H, s, H-4), δ = 6.68 (2H, d, J = 16 Hz, H-2,6), δ = 6.8 (4H, d, J = 7.2 Hz, H-3′,5′,3″,5″), δ = 7.5–7.56 (6H, br, H-1,7 and H-2′,6′,2″,6″). DMC: mp 128°C ^{1}H NMR (300 MHz, solvent: $CDCl_3$): δ = 3.92 (12H, br, –OCH_3), δ = 5.82 (1H, s, H-4), δ = 6.48 (2H, d, H-2), δ = 6.88 (2H, d, H-6′, 6″), δ = 7.08 (2H, s, H-2′, 2″), δ = 7.14 (2H, d, H-4′, 4″), δ = 7.60 (2H, d, H-1).

Kinetics of the reaction of DPPH radical with curcumin analogues was performed on a SX.18 MV stopped-flow spectrometer (Applied Photo Physics Ltd., London, UK), with absorption detector. The dead time of the instrument is 1.3 ms. It was used in single mixing mode where one syringe was filled with DPPH in acetonitrile and other syringe with different concentration of curcumin analogues in acetonitrile. The reaction was followed by monitoring the absorbance changes at 517 nm as a function of time, after mixing equal volumes of the two solutions in a stopped-flow cell. At least three independent runs were used to determine the rate constant at any concentration.

Kinetics of quenching of $^1O_2$ by curcumin analogues was followed by using transient luminescence spectrometer (TL900) obtained from Edinburgh Instrument Ltd. (Livingston, UK). $^1O_2$ was produced by photosensitization of haematoporphyrin (120 μM) in acetonitrile as shown in Scheme 3. A second harmonic (532 nm) of Continuum Minilite Nd:YAG Q-switched laser was used to excite the photosensitizer. The characteristic emission due to the decay of $^1O_2$ at 1270 nm was detected as a function of time by



Curcumin: $R_1 = R_3 = OCH_3$, $R_2 = R_4 = OH$
Monodemethoxy Curcumin (MDMC): $R_1 = H$, $R_2 = R_4 = OH$, $R_3 = OCH_3$
Bisdemethoxy Curcumin (BDMC): $R_1 = R_3 = H$, $R_2 = R_4 = OH$
DimethoxyCurcumin (DMC): $R_1 = R_2 = R_3 = R_4 = OCH_3$

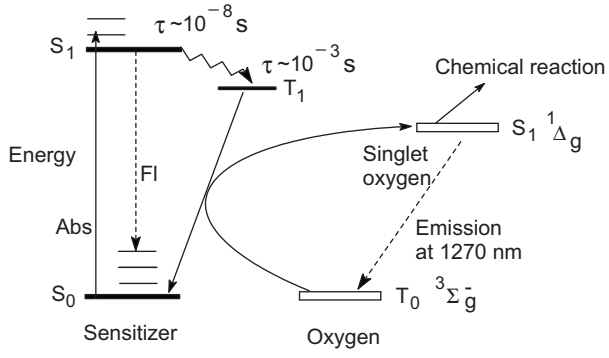
Scheme 1. Chemical structures of curcumin analogues.

Scheme 2. Keto-enol tautomerism in a curcuminoid.

liquid nitrogen cooled germanium detector. The output of the detector was fed to a Tektronix TDS3012B digital oscilloscope linked to an on-line PC for data transfer and analysis.

Kinetics of $O_2^{\bullet-}$ radical reaction with curcumin analogues was studied by generating $O_2^{\bullet-}$ radical by xanthine/xanthine oxidase method as reported in literature [37], employing 50 μM xanthine, xanthine oxidase (10 mU/ml) and 600 μM EDTA in phosphate buffer at pH 6.8. Its reactivity with curcuminoids was determined by competition kinetics monitoring the reduction of ferri cytochrome C to ferro cytochrome C, detected by absorption at 550 nm. The change in absorbance per unit time, ΔA/min, was monitored up to 300 s, where ΔA is the difference in absorbance at 550 nm. The concentration of xanthine oxidase was adjusted such that ΔA/min was ~ 0.025.

To follow the peroxyl radical scavenging ability of curcumin analogues, trichloromethyl peroxyl radicals ($CCl_3O_2^{\bullet}$), were employed. These radicals were generated by pulse radiolysis of aerated aqueous solution containing 48% of 2-propanol and 4% of CCl_4, with their radiation chemical yield of 0.64 μmol/J [38,39]. Pulse radiolysis experiments were carried out with high energy electron pulses (7 MeV, 500 ns) obtained from a linear electron accelerator and the transients were detected by kinetic spectrometry [40]. The absorbed dose was measured by using an aerated thiocyanate dosimeter by monitoring the $(SCN)_2^{\bullet-}$ species at 475 nm with Gε value of 2.59×10^{-4} m^2/J [41]. Here G denotes the radiation chemical yield in mol/J and ε the molar absorption coefficient in m^2/mol. Typical dose/pulse used for these studies was 20 Gy.



Scheme 3. Photosensitized generation and decay of singlet oxygen.

Results and discussion

DPPH radical scavenging activity

DPPH is not a biologically generated free radical, but it is often used to evaluate and compare the hydrogen donating ability of antioxidants. It is a stable free radical, purple in colour with strong absorption at 517 nm, but becomes colourless when its radical nature is neutralized. DPPH reaction kinetics with curcumin have been reported by many researchers [8,11,15,17–19]. Rate constants for the reaction of curcumin with DPPH in different solvents were reported and the role of phenolic OH vs keto-enol moiety in this reaction was extensively debated. It has now been concluded [17] that in protic and ionizable solvents, curcumin reacts with electrophillic radicals through the keto-enol moiety and the resultant radical loses a phenolic proton to yield phenoxyl radicals, whereas in non-polar and aprotic solvents, the reaction proceeds through hydrogen atom transfer from the phenolic OH. Studying the reactions of curcumin analogues differing in aromatic substitutions with DPPH would provide a method to validate this further. For these studies we employed acetonitrile, which is a polar and hydrogen bond acceptor solvent. In this solvent, the dissociation of phenolic and enolic protons would be expected to be very low due to higher values of pK_a in such medium. In spite of this, proton loss could still compete due to the stabilization of the deprotonated anions [42]. Thus the reactions of curcumin analogues with DPPH radical in acetonitrile would proceed through proton loss followed by electron transfer mechanisms.

To compare the DPPH radical neutralizing ability of the curcuminoids, 100 μM DPPH in acetonitrile was incubated with different concentrations (25–300 μM) of curcumin and its analogues for half an hour at room temperature and the absorbance at 517 nm was monitored. The scavenging efficacy was judged by the parameter IC_{50}, which is the concentration of curcumin or its analogue, required to reduce the absorbance of DPPH by 50% compared to the control DPPH. The estimated IC_{50} values were 31, 40, 181 and $>$ 250 μM for curcumin, MDMC, BDMC and DMC, respectively. The IC_{50} values can be used only for qualitative comparison, therefore for quantitative estimation, rate constants for the reaction of DPPH with curcumin analogues, were estimated by stopped flow spectrometer. The absorbance due to 50 μM

DPPH in acetonitrile at 517 nm decreased negligibly even after 1000 s in the absence of curcumin analogues, however in the presence of curcumin or its analogues the absorbance decayed. Representative absorption-time profiles of DPPH at 517 nm in the absence and in the presence of MDMC are given in Figure 1. This decay was monitored in the presence of varying concentrations of curcumin and its analogue in the concentration range of 50–300 μM. Since true pseudo-first order conditions could not be applied for these concentrations, the absorption-time profiles were fitted to second order integrated rate law [43], as given in equation (1).

$$In\left\{\frac{([B]_0 - [A]_0 + [A])[A]_0}{[B]_0[A]}\right\} = ([B]_0 - [A]_0)k_1 t \quad (1)$$

Here $[A]$ was the concentration of DPPH at any time point, $[A]_0$ and $[B]_0$ are the initial concentration of DPPH and curcuminoids, respectively, at zero time and k_1 is the 2nd order bimolecular rate constant. From the absorption vs time plot at 517, using $\varepsilon = 1.15 \times 10^4\ M^{-1}cm^{-1}$ for DPPH in acetonitrile [44], the concentration of DPPH at any time, $[A]$ can be obtained. This time-dependent DPPH concentration was fitted to a linear plot according to equation (1), as shown in the inset of Figure 1. From the slope, k_1 was estimated at a given initial concentration of DPPH and curcuminoids. This was repeated at different initial concentration of DPPH and curcuminoids and the average value is reported in Table I.

The bimolecular rate constant decreased in the order curcumin > MDMC > BDMC > DMC. The rate constant for *o*-methoxy phenols with DPPH are relatively faster as the electron donating property of the methoxy group reduces the phenolic O-H bond dissociation energy. Therefore, the analogues curcumin and MDMC having phenolic OH ortho to the methoxy group show higher rate constants with DPPH [15,18,19]. On the other hand, such lowering of bond dissociation energy may not be seen in the case of BDMC due to the absence of the methoxy group, showing lower rate constants compared to curcumin and MDMC. This further confirms that in acetonitrile DPPH reacts with curcuminoids by proton loss followed by electron transfer mechanism, and the reactivity is governed by the acidity of the phenolic OH group. Due to the absence of the phenolic OH group, DMC showed the lowest rate constant among the other curcumin analogues. Although slow, the observed reactivity of DPPH with DMC supports the earlier conclusion by Litwinienko and Ingold [17] that the H-atom abstraction from the methylenic group is definitely an important mode of reaction with DPPH radicals. This observation is also in line with the theoretical calculations, which showed that H-atom abstraction from the methylenic C-H bond in curcumin requires more energy than that from phenolic OH group [15,16,45]. This study further supports the earlier observation that dissociation of phenolic and enolic protons of curcuminoids followed by electron transfer plays an important role in scavenging the free radicals in biological models.

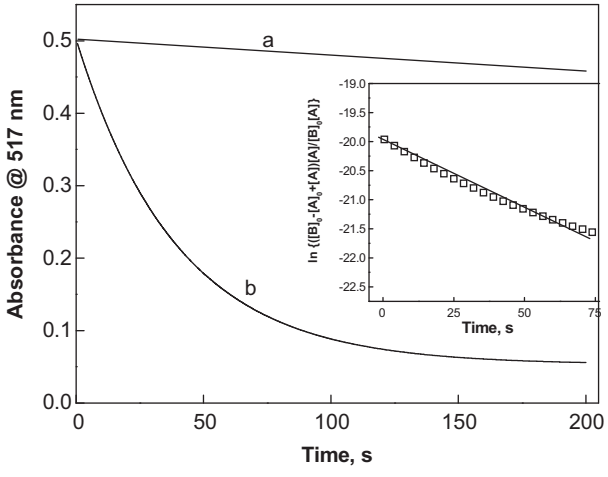


Figure 1. Typical decay signal of DPPH (50 μM) in acetonitrile at 517 nm (A) in the absence and (B) in the presence of MDMC (200 μM). Inset shows the data fitted to a line plot according to the inegrated rate equation (1).

Singlet oxygen scavenging activity

$^1O_2$ is the excited state of molecular oxygen (22.3 kcals/mol for $^1\Delta g$ state), where both the electrons are in anti-parallel configuration in the same orbital. When the excitation energy is smaller than the ground state dissociation energy, the isolated and unperturbed molecule can lose its excitation energy by radiative deactivation [46]. The excess energy in $^1O_2$ is deactivated through emission at 1270 nm in the absence of any external agents with characteristic lifetime τ_0. In the presence of molecules, capable of reacting with it, like for e.g. antioxidants, the lifetime decreases. The effectiveness of an antioxidant in deactivating $^1O_2$ damage can be determined from their quenching efficiency. Therefore, quenching of $^1O_2$ lifetime was followed by monitoring its lifetime in the absence (τ_0) and presence (τ) of different concentrations (0.25–10 mM) of curcumin or its analogue. Representative emission-time profiles of $^1O_2$ at 1270 nm in the absence and presence of MDMC are given in Figure 2, where $^1O_2$ was produced by photosensitization of haematoporphyrin (Scheme 3). The quenching rate constant (k_2) was estimated according to the Stern-Volmer relation [47] as given in equation (2) in the presence of different concentrations of curcumin or its analogues, as given in Figure 3.

$$\tau^{-1} = \tau_0^{-1} + k_2[Curcuminoid] \quad (2)$$

Table I. Bimolecular rate constant for the different radical scavenged by different analogues of curcumin.

Compounds	DPPH, $M^{-1}s^{-1}$	($^1O_2$), $M^{-1}s^{-1}$	($O_2^{\bullet-}$), $M^{-1}s^{-1}$	($CCl_3O_2^{\bullet}$), $M^{-1}s^{-1}$
Curcumin	1852 ± 180	(1.3 ± 0.2) × 10^6	(1.0 ± 0.1) × 10^5	(1.5 ± 0.1) × 10^8
MDMC	155 ± 15	(0.7 ± 0.1) × 10^6	(1.3 ± 0.1) × 10^5	(5.5 ± 0.3) × 10^7
BDMC	21 ± 2	(0.97 ± 0.1) × 10^6	(0.7 ± 0. 3) × 10^5	(3.2 ± 0.1) × 10^7
DMC	0.9 ± 0.2	(1.5 ± 0.1) × 10^6	(0.8 ± 0.1) × 10^5	ND

± refers to curve fitting error.

The k_2 values as obtained from the slope of the linear fit are listed in Table I. The rate constant for curcumin in acetonitrile is five times higher than that reported by Gorman et al. [48] in deuterated benzene, indicating that polar solvents favour this reaction more than that in non-polar solvents. Similar solvent effects on singlet oxygen quenching rate constants have been reported in mono- and disubstituted anthracene derivatives [49]. The order of the quenching rate constant only showed a marginal change with substitution in the order DMC > curcumin > BDMC > MDMC. From these observations, it can be concluded that the phenolic OH group does not have a significant role in modulating the reactivity of curcumin or its analogues with $^1O_2$ and the keto-enol moiety is the most preferred site for attack by $^1O_2$.

In general organic compounds can deactivate $^1O_2$ either by physical or by chemical quenching. In physical quenching, excited state charge transfer complex is formed, where $^1O_2$ acts as an electron acceptor. Then the charge transfer complex may decay by forming triplet oxygen and the respective phenolic type derivative through inter-system crossing [50]. In chemical quenching oxidation of the compounds takes place through hydroperoxides or epoxides, either by direct addition to double bonds or through proton transfer followed by addition. In curcuminoids $^1O_2$ would either add to the double bond at the α–β unsaturated keto-enol moiety, via cyclo-addition or ene type reaction [51]. Alternately it would pick up proton from the enol moiety followed by the addition to a carbon-centred radical. Methoxy groups in aromatic compounds are electron donating in nature and therefore increase the overall electron density on the α–β unsaturated bond, hence DMC shows highest quenching rate constant compared to the other curcumin analogues or curcumin. Summarizing these observations, it can be concluded that $^1O_2$ attacks the keto-enol moiety of curcumin and the electron donating methoxy groups on phenyl ring increase the reactivity with $^1O_2$.

Superoxide radical scavenging activity

$O_2^{\bullet-}$ is one of the important ROS. Although $O_2^{\bullet-}$ is not reactive, it acts as the source of powerful oxidants like hydroxyl radical and peroxynitrite, under some conditions [14]. $O_2^{\bullet-}$ absorbs at 245 nm (ε = 2350 $M^{-1}cm^{-1}$) [52] and earlier we reported the rate constant for the reaction of $O_2^{\bullet-}$ with curcumin by different methods, such as by direct monitoring by pulse radiolysis, employing KO_2-crown ether complex and by ferric cytochrome-C competition method [12,13,21,37]. Due to strong absorption of curcumin and its analogue at 245 nm, the indirect method of employing ferric cytochrome-C was found to be more suitable for the estimation of the bimolecular rate constants for the scavenging of $O_2^{\bullet-}$ by curcumin or

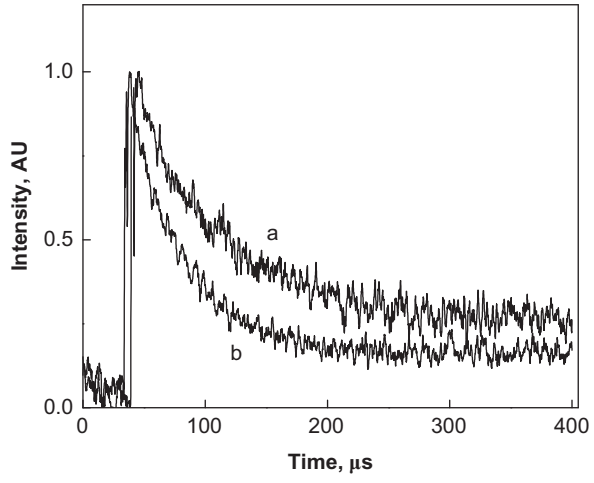


Figure 2. Typical trace showing the decay of $^1O_2$ at 1270 nm produced by photosensitization 120 μM heamatoporphyrine in acetonitrile (A) in the absence of MDMC and (B) in the presence of 10 mM MDMC.

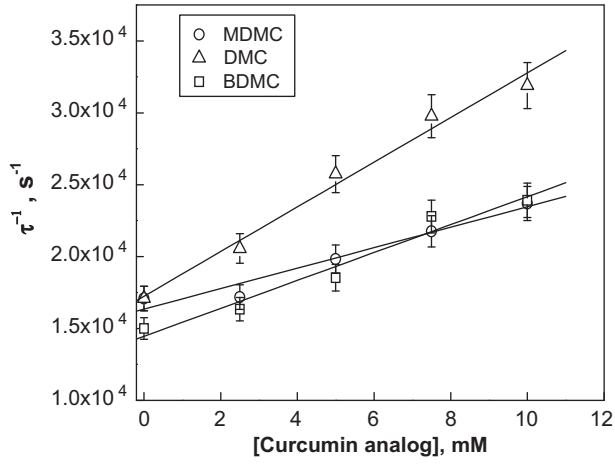


Figure 3. Linear plots for the inverse of $^1O_2$ lifetime at 1270 nm in the presence of different concentrations of MDMC, BDMC and DMC in acetonitrile solvent. Data presented as mean ± SEM, n = 3.

its analogues. The competing reactions are given in equations (3) and (4), and the bimolecular rate constant, k_4, was estimated by using equation (5):

$$\text{Cytochrome C}\,(Fe^{3+}) + O_2^{\bullet -} \xrightarrow{k_3} \text{Cytochrome C}\,(Fe^{2+}) \quad (3)$$

$$\text{Curcuminoid} + O_2^{\bullet -} \xrightarrow{k_4} \text{Product} \quad (4)$$

$$\frac{Abs_0}{Abs} = 1 + \frac{k_4}{k_3}\left(\frac{[Curcuminoid]}{[Cytochrome\ C]}\right) \quad (5)$$

Here, Abs_0 and Abs are the absorbances at 550 nm in the absence and presence of curcumin or its analogues, respectively. Slope of the linear plots as shown in Figure 4 for variation of (Abs_0/Abs) vs [Curcuminoid]/[Cytochrome C], gave k_4/k_3. Using the value of k_3 as 5.8×10^5 $M^{-1}s^{-1}$ for the reaction of $O_2^{\bullet -}$ radical with cytochrome C [53], k_4 was estimated and the values of k_4 are listed in Table I. From this table, it can be seen that the rate constants for the reaction between $O_2^{\bullet -}$ radical and curcuminoids did not vary much within experimental limits. Earlier we have shown that $O_2^{\bullet -}$ reaction with curcumin occurs mainly through nucleophilic attack at the β-diketo moiety with alternative reaction through oxidation at the phenolic OH [13,54]. Since there is not much change in the rate constant with these different curcumin analogues, it can be further concluded that in curcuminoids, the preferred reaction site for $O_2^{\bullet -}$ radical is the keto-enol functional group. There are several reports in the literature [55] indicating that the keto-enols react with superoxide by proton transfer followed by either electron transfer or by hydroperoxide addition to the double bond. Contrary to $^1O_2$ reaction, the rate constants with $O_2^{\bullet -}$ radicals did not increase significantly with methoxy substitution on the phenyl ring.

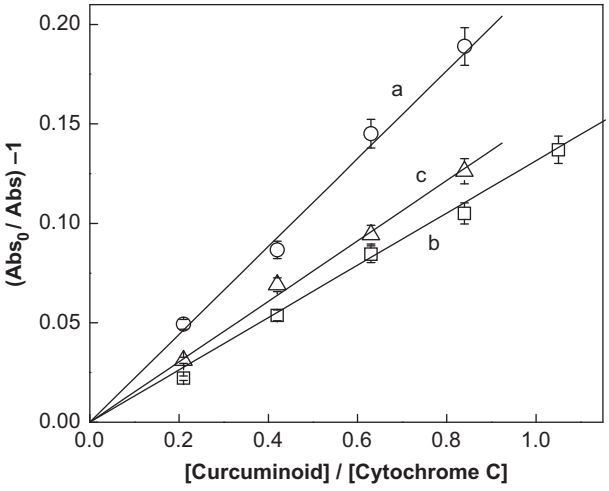


Figure 4. Change of absorbances of Cytochrome C at 550 nm in presence of different concentration of (A) MDMC, (B) BDMC and (C) DMC. Data presented as mean ± SEM, $n = 3$.

Peroxyl radical scavenging activity

Using pulse radiolysis technique, $CCl_3O_2^{\bullet}$ radicals are often used as model peroxyl radicals to understand reactivity with antioxidant molecules. Earlier it was reported that curcumin reacts with $CCl_3O_2^{\bullet}$ radicals by electron transfer followed by proton loss to yield phenoxyl radicals absorbing at 490 nm [12]. Similar transient with absorption maximum at ~ 490 nm was observed for the reaction of $CCl_3O_2^{\bullet}$ radicals with MDMC and BDMC, indicating formation of phenoxyl type radical. No such transient was observed with DMC, which has no phenolic OH group. Due to strong interference of the parent absorption, the bimolecular rate constant between these analogues with $CCl_3O_2^{\bullet}$ radical could not be estimated directly by monitoring the transient at 490 nm. Therefore the bimolecular rate constants were estimated by employing competition kinetic method using $ABTS^{2-}$ as a reference solute. $ABTS^{2-}$ is oxidized to $ABTS^{\bullet -}$ by $CCl_3O_2^{\bullet}$ radical, which has strong absorption at 645 nm. In the presence of curcumin and its analogues due to competition, the absorbance of $ABTS^{\bullet -}$ at 645 nm decreased, assuming no direct reaction between $ABTS^{\bullet -}$ and curcuminoids in the time scale employed in the study. The two competing reactions and the equation employed to estimate bimolecular rate constants are given in equations (6)–(8):

$$ABTS^{2-} + CCI_3O_2^{\bullet} \xrightarrow{k_5} ABTS^{\bullet -} \quad (6)$$

$$\text{Curcuminoid} + CCI_3O_2 \xrightarrow{k_6} \text{Product} \quad (7)$$

$$\frac{Abs_0}{Abs} = 1 + \frac{k_6}{k_5}\left(\frac{[Curcuminoid]}{[ABTS^{2-}]}\right) \quad (8)$$

where Abs_0 and Abs are the maximum absorbance values at 645 nm, respectively, in the absence and presence of curcumin or its analogues. Slope of the linear plot of Figure 5 for (Abs_0/Abs) vs [Curcuminoid]/[$ABTS^{2-}$] gives k_6/k_5. Using the value of k_5 as 1.9×10^9 $M^{-1}s^{-1}$ for the reaction of $CCl_3O_2^{\bullet}$ with $ABTS^{2-}$ [56], k_6 was estimated and the values are listed in Table I. From the table, it can be inferred that analogues with *o*-methoxy substituted phenolic –OH group show a higher rate constant with $CCl_3O_2^{\bullet}$ radical. DMC, which does not possess any phenolic –OH group, is unreactive towards the $CCl_3O_2^{\bullet}$ radical. The electron density on the phenolic OH group would be higher with compounds having ortho substituted methoxy group due to electron donating resonance effect. Also the resulting phenoxyl radical in these will be stabilized by the resonance effect. Hence, curcumin exhibits a higher bimolecular rate

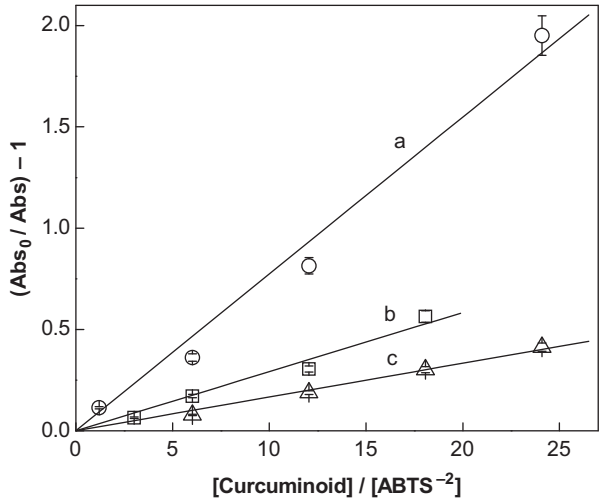
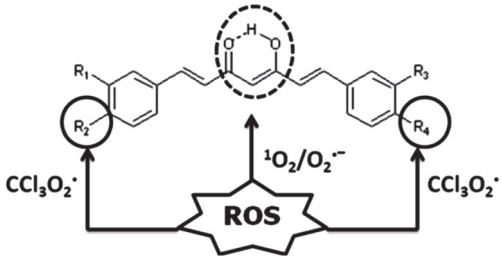


Figure 5. Change of absorbances of $ABTS^{\bullet-}$ at 645 nm in presence of different concentration of (A) curcumin, (B) MDMC and (C) BDMC. Data presented as mean ± SEM, $n = 3$.

constant. Therefore, analogues, MDMC and BDMC have comparatively lower reactivity than curcumin as the resulting phenoxyl radicals are less stabilized due to the absence of *o*-methoxy group.

Conclusions

The ROS scavenging mechanisms of curcumin analogues, varying mainly on the substituents present on the aromatic part, have been evaluated by different time resolved methods. The reaction rate constant for scavenging of DPPH radicals was found to solely depend on bond dissociation energy of phenolic OH group and the reactivity increased with the presence of *o*-methoxy group. $^1O_2$ and $O_2^{\bullet-}$ radicals react preferably at the keto-enol moiety and their scavenging efficiency is not influenced by the presence of phenolic OH group. DMC was found to be a very efficient $^1O_2$ quencher, probably due to the presence of four methoxy groups on the aromatic ring which increase the electron density on the β-diketo group. The reactions of $^1O_2$ and $O_2^{\bullet-}$ reactions at keto-enol moiety can sometimes lead to formation of radicals that would undergo fragmentation to smaller products that can act as pro-oxidants. However, in the case of curcumin the radicals derived from keto-enol moiety do not undergo fragmentation as they are converted to less reactive and resonance stabilized phenoxyl radicals, leading to antioxidant activity. Therefore, in DMC due to the absence of phenolic OH, fragmentation at the keto-enol moiety can lead to products that are more cytotoxic.



Scheme 4. Probable site of attack by ROS.

Like DPPH radical reaction, the reactivity with $CCl_3O_2^{\bullet}$ radical depends on the bond dissociation energy of phenolic OH group and the electron density on the aromatic part of the curcumin analogues. Formation of phenoxyl radical was the main route for the reaction of curcumin analogues with $CCl_3O_2^{\bullet}$ radicals. The most probable sites for the attack of different ROS on curcuminoids are represented in Scheme 4. From the studies it can be concluded that the reactivity of curcumin analogues not only depend on the substitution pattern in the aromatic ring but also on the nature of free radical and ROS.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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