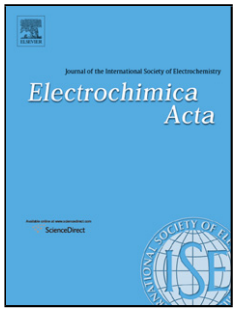

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Antioxidant activity and electrochemical elucidation of the enigmatic redox behavior of curcumin and its structurally modified analogues

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1 Equal Contribution

Graphical abstract

Highlights

- Structural analogues of curcumin have been synthesized.
- Confirmation of redox behaviour emanates from H- shift from central methylene group in curcumin.
- Mechanism of curcumin oxidation has been proposed.
- Correlation between redox behavior and antioxidant activity has been established.

Abstract

Here, we report studies on the antioxidant activity and redox behavior of curcumin and its structurally modified synthetic analogues. We have synthesized a number of analogues of curcumin which abrogate its keto-enol tautomerism or substitute the methylene group at the centre of its heptadione moiety implicated in the hydride transfer and studied their redox property. From cyclic voltammetric studies, it is demonstrated that H- atom transfer from CH_2

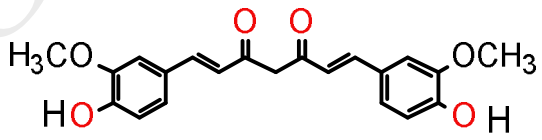
group at the center of the heptadione link also plays an important role in the antioxidant properties of curcumin along with that of its phenolic –OH group. In addition, we also show that the conversion of 1, 3- dicarbonyl moiety of curcumin to an isosteric heterocycle as in pyrazole curcumin, which decreases its rotational freedom, leads to an improvement of its redox properties as well as its antioxidant activity.

Keywords: Curcumin; Antioxidant; Free radical; Cyclic voltammetry; Alkoxyl radical; Hepatadione

1. Introduction

Curcumin, a yellow colored phenolic compound isolated from the roots and rhizomes of *Curcuma longa* has been used since centuries as a spice, dietary pigment and traditional medicine in India and China [1-3]. It displays a wide spectrum of medicinal properties ranging from anti – bacterial, anti-viral, anti-protozoal, anti-fungal and anti – inflammatory to anti-cancer activity [4-7]. The ability of curcumin to neutralize chemical carcinogens such as superoxide, peroxyl, hydroxyl radical and nitric oxide radical constitutes a major subject of interest. While mode of its action is still under exploration, it is proposed to act through a number of targets and metabolic pathways involving transcription, cell growth, apoptosis etc [6, 7]. Despite its invocation of a number of biologic targets it is practically non-cytotoxic to normal human cells but cytotoxic for cancer cells. It inhibits oncogenic cell proliferation by arresting cell cycle progression and induction of apoptosis [6, 7]. However curcumin's low solubility, high rate of metabolism and decomposition, poor bioavailability and pharmacokinetics are responsible for its limited efficacy [8-10].

Chemically curcumin is (1E, 6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-dienne-3,5-dione.



Curcumin

Curcumin is a multifunctional molecule and it has three functional groups that can contribute to its biologic activity, namely an aromatic *o*-methoxy phenolic group, α, β-unsaturated β-diketo moiety and a seven carbon linker. Its redox behavior is expectedly more complex and has led to conflicting views on the contribution of its key constituents [11-19].

The redox behavior of curcumin has led to proposal of two divergent mechanisms. Jovonoic et al, have proposed that its antioxidant mechanism involves H-atom abstraction from CH_2 group of the heptadione link and the H- atom donation from the phenol group makes merely 15% contribution towards it [13,14]. On the other hand, Barclay et al's proposal that it's a chain breaking antioxidant, donating H-atom from its phenolic group which discounts a role of CH_2 group of the heptadione link in its redox properties has received a much wider acceptance [15]. Litwinienko and Ingold later observed that curcumin donates hydrogen atom from the phenolic or enolic hydroxyl group via sequential proton loss electron transfer (SPLET) or hydrogen atom transfer (HAT) and hence, reconciled the earlier differing opinions [18,19]. Currently accepted mechanism of the antioxidant action of curcumin lays much of its emphasis on the chain breaking ability of its methoxyphenol group (s) that donates one H-atom and discounts a significant role of its methylene group in the process.

Here we have explored voltammetric analysis to understand the redox behavior and its correlation to phenolic O-H and methylene hydrogen attached to *β*-diketone of curcumin and its various synthesized analogues. These studies have investigated for the first time, systematically the role of the methylene radical at the center of curcumin's has crucial involvement in its redox behavior as its Knoevenagel condensate (*viz.* 4-(4-Hydroxy-3-methoxybenzylidene)) which lacks the ability of hydride transfer exhibits redox potential at a significantly higher potential (*viz.* decreased antioxidant ability) despite possessing an additional *ortho*-methoxy phenyl group. This in turn implies a significant role for the heptadione methylene group in manifesting the redox potential of its *ortho*-methoxy phenol group at a lower potential and higher intensity. That the redox behavior of curcumin emanates from an H-shift from its central methylene group was confirmed further by the diminution in the antioxidant abilities of butylidene and benzylidene derivatives of this methylene moiety.

2. Experimental

2.1. Chemicals

3-(3,4-Dihydroxyphenyl) prop-2-enoic acid (Caffeic acid) and (3-(3-hydroxy-4-methoxyphenyl) prop–2-enoic acid (ferulic acid), vanillin, isobutyraldehyde, benzaldehyde, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and others reagent were purchased from Sigma-Aldrich. All solvents used were of spectral grade or distilled prior to use.

2.2. General Procedures Used for the Synthesis and Characterization of Curcumin Analogues

Reaction progress was monitored by TLC using Merck silica gel 60 F_{254} with detection by UV. Column chromatography was performed using Merck silica gel 230–400 mesh. Melting points were determined in Pyrex capillary tube using Büchi Melting Point B-540 apparatus. ^{1}H NMR spectra was recorded on 300 and 400 MHz Bruker NMR spectrometers using tetramethylsilane as an internal standard and the chemical shifts are reported in (δ) units. Coupling constants are reported as a *J* value in Hertz (Hz).The sample concentration in each case was approximately 10 mg in chloroform-d/methanol-d_4/DMSO-d_6 (0.6 mL). Mass spectra were recorded on an Electrospray–MS (Bruker Daltonis) instrument.

2.3. General Procedure for the Preparation of Curcumin Pyrazole, N-(-Substituted) Phenyl Pyrazoles and Knoevengel Condensates of Curcumin

Curcumin pyrazole, and *N*-(substituted) phenyl pyrazoles derivatives of curcumin were prepared according to previously reported procedure by us and others with some modifications **(Scheme 1 & 2)** [1, 20-21]. Curcumin (1 mmol) was dissolved in glacial acetic acid (5 mL), hydrazine hydrate and the various phenyl substituted hydrazine hydrochlorides (1.2 mmol) were added to the solution. The solution was refluxed for 8 -24 hr, and then the solvent was removed in vacuum. Residue was dissolved in ethyl acetate and washed with water. Organic portion was collected, dried over sodium sulfate, and concentrated in vacuum. Crude product was purified by column chromatography.

Knoevenagel condensates of curcumin was prepared by treatment of curcumin with aromatic aldehyde (4-hydroxy-3-methoxybenzaldehyde; vanillin) in presence of piperidine (as catalyst) and anhydrous DMF (as solvent) to yield 4-(4-Hydroxy-3-methoxybenzylidene) curcumin. Likewise, butylidiene curcumin and benzylidiene curcumin were prepared in good yields using

the above procedure wherein isobutyraldehyde and benzyldehyde were used instead of (4-hydroxyl -3-methoxy benzaldehyde) (**Scheme 3**) [1, 22-25]. Reaction mixture was diluted with ethyl acetate and washed with water and saturated brine. The organic phase was dried over Na_2SO_4 concentrated under vacuum, and purified by chromatography with EtOAC/hexanes mixtures to provide pure compound.

2.4. Electrochemical Studies

Voltammetric experiments were performed with a potentionstat PGSTAT 30, Autolab (ECO CHEMIE Ltd., The Netherlands) driven with GPES software (Eco Chemie). A conventional three- electrode cell consisting of a gold electrode, a platinum wire as counter electrode and Ag/AgCl (saturated KCl) as a reference electrode were used. For characterization of gold electrode, we have used Ag/AgCl (saturated KCl) as a reference electrode. The gold (Au) electrode was carefully polished with 1.0, 0.3 and 0.05 μm Al_2O_3 slurry, and cleaned by brief ultrasonication. Cyclic voltammetry experiment was performed in 10 mM HEPES buffer, where Ag/AgCl has been used as a reference electrode. For electrochemical studies, a stock solution of 2×10^{-3} M curcumin was prepared in DMSO just before experiment and then these solutions were diluted with buffer to the convenient concentration after mixing with 10 mM HEPES as buffer supporting electrolyte. The 10 mM HEPES buffer solution has been made in 0.1 M KCl solution. The UV-vis absorption spectra of curcumin and its various analogues were collected after 15-20 min subsequent to its solubilization in buffer to estimate degradation of curcumin. Slight degradation of curcumin was noted at 15-20 min subsequent to dissolution. Pyrazole curcumin and its analog were stable at physiological pH and no significant degradation was observed even after 30 min subsequent to dissolution. Our observation is similar to finding of Griesser el.al. [26] and Soumyananda et.al [27]. The data were analyzed using origin 8 software for obtaining the value of current (I) and oxidation potential (V).

2.5. Evaluation of Antioxidant Activity

The antioxidant activities of curcumin analogues were determined in terms of radical scavenging activity using stable radical DPPH. Stock solution of each compound was diluted to variable concentration from 25-150 μM of each compound. 60 μM of freshly prepared methanolic solution DPPH solution (1 mM) was added to each sample solution to make final volume 1ml.

The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. In the radical form, $DPPH^{\cdot}$ shows a maximum absorbance at 517 nm, but on reduction by an antioxidant, the absorption disappears and the pale yellow non radical form is produced. The lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Methanol was used as a blank. The antioxidant effect was expressed in terms of the reduction percentage of the DPPH absorbance, refereed as scavenging effect was calculated using following equation **[**28]

$$\text{Scavenging Effect} = \frac{A_0 - A_T}{A_0} \times 100$$

Where A_0 is the absorbance of the control reaction and A_T is the absorbance in the presence of the sample.

The assay was carried out using curcumin and its various analogues as antioxidant and in triplicate. The absorbance was measured in spectrophometer UV/vis from JASCO V-530 spectrophotometer.

3. Results and Discussion

3.1. Synthesis and Characterization of Curcumin Analogues

Curcumin pyrazole, and *N*-(substituted) phenyl pyrazole derivatives of curcumin were prepared according to previously reported procedure by us and others [1, 20-21] with some modifications **(Schemes 1& 2)**. Curcumin (1 mmol) was dissolved in glacial acetic acid (5 mL), hydrazine hydrate and the various phenyl substituted hydrazine hydrochlorides (1.2 mmol) were then added it in solution. The solution was refluxed for 8 -24 hr, and then the solvent was removed in vacuum. Residue was dissolved in ethyl acetate and washed with water. Organic portion was collected, dried over sodium sulfate, and concentrated in vacuum. Crude product was purified by column chromatography. The ^{1}H NMR spectra showed singlet at δ 6.52–6.62 ppm corresponding to C*H*=C proton (pyrazole); multiplet at δ 6.81–7.83 ppm corresponding to aromatic protons; broad singlet at δ 9.23–9.80 ppm corresponding to OH and disappearance of peak of the methylene proton (δ 6.02 ppm) confirm the formation of curcumin pyrazole (**2**) and derivatives of curcumin pyrazole (**3**-**8**). Mass spectral studies of the compound **2** (ESI-MS) exhibited molecular ion at 365.0 $(M+H)^+$ corresponding to curcumin pyarazole. The derivatives

of curcumin pyrazole (**3-8**) were also further characterized by ESI-MS. [see Supporting Information MS spectral data Fig.S5-Fig.S16]. Knoevenagel condensates of curcumin was prepared by treatment of curcumin with aromatic aldehyde (4-hydroxy-3-methoxybenzaldehyde; vanillin) in presence of piperidine (as catalyst) and anhydrous DMF (as solvent) to yield 4-(4-Hydroxy-3-methoxybenzylidene) curcumin, likewise, butylidiene curcumin and benzylidiene curcumin were prepared in good yields using the above procedure wherein isobutyraldehyde and benzyldehyde were used instead of (4- hydroxyl -3-methoxy benzaldehyde) (**Scheme 3**) [1, 25-28] [see Supporting Information Experimental]. The formation of compounds **9-10** via Knoevengel's condensation was validated by the disappearance of aldehydic proton (δ 9.80-10.10 ppm) and appearance of singlet proton at 8.0 ppm and 7.85 ppm corresponds to benzylidene (=CH-Ar) ; indicates the structure of desired compounds **9** and **10.** Similiarly appearance of alkylidene proton (=CH-CH_2) at δ 7.83 showed the formation of compound **11.** Furthermore all the Knoevengel condensates of curcumin were characterized by ESI-MS also [Supporting Information MS spectral data Fig.S17-Fig.S22]. The structure of dimethyl curcumin (compound **12**) was confirmed by the presence of 12 protons peak at δ 3.74 ppm (singlet; 4 × OCH_3) in ^{1}H NMR spectrum and ESI-MS demonstrated molecular ion at 419.9 $(M+Na)^+$ validate compound **12** [Supporting Information MS spectral data Fig.S23-Fig.S24]. Chemical structures of curcumin and pyrazole derivative of curcumin are shown in Table 1.

3.2. Cyclic Voltammetry of Curcumin

Curcumin and its analogues belong to the class of polyphenols, their antioxidant activities are of great interest because of their widespread use as nutraceuticals, therapeutics and as components of industrial processes. How are the antioxidant activities of curcumin and its analogues manifested about diverse free radical forms? It is widely believed that much of the redox behavior of curcumin is a consequence of its two *o*-methoxy phenolic groups. In comparison the contribution of the methylene group located at the centre of its heptadione link has received little attention and its substituent have not been probed in the context of the overall anti-oxidant activity of curcumin. We report cyclic voltammetric studies of curcumin and its analogues to understand its redox behaviour comprehensively.

Fig. 1a shows representative cyclic voltammogram of a bare gold electrode in 0.5 M H_2SO_4. The formation of gold oxides on electrode surface is clearly reflected in the potential region of 1.1 V to 1.4 V. But when the potential scan is reversed then there is a sharp cathodic peak observed in

the range of 0.8 V to 1.0 V. vs. Ag/AgCl (saturated KCl), representing the reduction of gold oxide on the electrode surface. The peak current of gold oxide reduction is 55 µA. This is consistent with the formation of gold oxide and reduction of gold oxides on electrode surface which are the characteristic features of a well cleaned polycrystalline bare gold electrode [29]. The cyclic voltammetry of bare gold in 10 mM HEPES buffer solution, pH 7.4 at 25^0C at a scan rate of 20 mV s^{-1} shows an anodic peak at 0.23 V (Fig 1b) indicating that HEPES molecules are getting oxidized at the gold surface consistent with the property of this salt as a reductant [30,31]. Despite adsorption of HEPES on the gold surface observation of the redox peaks of gold demonstrates that the oxidation and reduction of gold has occurred in a reversible manner.

To understand the redox behavior of curcumin, we have performed a cyclic voltammetric (CV) study on a polycrystalline gold electrode in the absence and presence of various concentration of curcumin. It has been elucidated in Fig 2a. CV of curcumin at pH 7.4 showed one anodic peak at 0.66 V. There is an increasing trend of anodic peak intensity upto 8 µM concentration of curcumin whereas 10 µM onwards it does not follow linearity (Fig 2b). So, for further electrochemical understanding, we have chosen 8 µM concentration of curcumin and its modified analogues for these studies. Fig 2c illustrates the effect of scan rate on 8 µM curcumin in 10 mM HEPES buffer (pH 7.4, 25 °C) at gold electrode. The oxidation current of curcumin on the Au electrode increased linearly with the square root of the scan rate (Fig. 2d), in the scan range of 20 to 100 mV s^{-1}, which indicates the existence of a mass transfer controlled process. Since it does not emanate from the origin, it indicates curcumin electrooxidation is controlled by diffusion which can be preceded by a chemical reaction. The value of electron transfer coefficient for the reaction has been obtained from the following equation [32] which is valid for a totally irreversible-diffusion controlled process.

$$E_p = \left(\frac{R\,T}{2\,\alpha\,F}\right)\ln(\nu) + \mathit{constant}$$

Using the above equation a plot of anodic peak potential *vs.* ln (ν) yields a value of electron transfer coefficient (α) of 0.27 ± 0.01. These electrochemical studies are consistent with the involvement of two electrons per molecule of curcumin for its oxidation. The first one electron oxidation produces the phenoxy radical and the second correspond to the formation of quinone

[33]. Thus during the oxidation of curcumin electron transfer occurs in concert with proton transfer.

The diketo moiety in the heptadione link is known for its propensity for keto–enol tautomerism in a pH dependent manner. Hence, we have examined the redox behavior of 8 μM curcumin in 10 mM HEPES buffer at different pH values by cyclic voltammetry [Fig 2e]. The oxidation potential of curcumin is nearly the same (0.64 V-0.66 V) at various pH values examined while the oxidation peak intensity increases with increase in pH. The reduction peak in the potential range of 0.35 V to 0.55 V observed at acidic pH declines progressively as the pH is increased. However, a shoulder adjacent to 0.66 V peak or an additional peak in the higher potential region (~ 1.0 V) ascribed to that of the CH_2 group emanating from the centre of heptadione link is not observed [16]. This in turn suggests that the oxidation peak for this methylene group and the *o*-methoxy phenyl group overlap with each other in native curcumin. Hence, the result indicates that at pH > 7 deprotonation of hydroxyl moiety generating a quinone facilitates electron transfer boosting the antioxidant activity of curcumin at or above the physiological pH.

3.3. Redox Behavior of Diketone Anlogues of Curcumin

In the redox reaction of curcumin the alkoxy radical generated initially undergoes a rapid intramolecular H- shift to generate a phenoxy radical. Introduction of pyrazole group at the position of diketone moiety decreases the rotational freedom of the linker facilitating the intramolecular radical shift from alkoxyl radical at the methylene group at the centre of the heptadione link. This in turn leads to a substantial increase (60%) in intensity of redox potential with a slightly decreased potential value, 0.62 V for pyrazole curcumin. This is in good agreement with our previously reported results on the anti-oxidant properties of curcumin pyrazole [23]. A substituent in the aromatic ring attached to pyrazole is expected to influence the stability of the phenoxyl radical which in turn should perturb the redox behavior of the resulting compound. Hence, a few substituted pyrazole containing derivatives of curcumin were synthesized (Table 1, Ref 34) to understand the effect of introduction of a methoxy, halogen and a nitro group in the aromatic ring attached to pyrazole in the formation of the phenoxy radical as deduced from their electrochemical responses (Fig 3a – 3c and supporting information Fig S1). Phenyl pyrazole curcumin has an oxidation potential close to that of pyrazole curcumin with slight decrease in the intensity with respect to pyrazole curcumin [Table 1, Fig S1a]. A methoxy,

a moderate activating group substituted at *meta* position leads to a slight increase in the oxidation potential (0.66 V) as compared to that of pyrazole curcumin [Fig S1b]. The presence of methoxy at *meta* position of the phenyl ring decreases the resonance of the phenyl ring irrespective of its inductive and *ortho/para* effect increasing the oxidation potential as observed experimentally. Since halogens are weakly deactivating group, they perturb the electrochemical behaviour of pyrazole analogues of curcumin as evident from Fig 3a. Thus, a decrease in the oxidation potential of N-(3-fluorophenyl pyrazole) curcumin as compared to its pyrazole counterpart is attributed to the increase in the electron withdrawing nature of the fluorine atom at meta position. Since fluorine in ortho position of N-(2-Fluorophenyl pyrazole) can contribute to oxidation potential through both inductive and resonance effect, it marginally increases the oxidation potential with decrease in its intensity (supporting information Fig S1c**).** Fig. 3b clearly indicates that the introduction of a nitro group at the same position decreases the intensity of the oxidation potential as compared to its pyrazole counterpart and it is due to its *meta* directing nature. Whereas the $–CF_3$ group being a strong electron withdrawing group which increases the bond dissociation energy of the phenoxy O-H group, as a result it gives rise to a shift of its redox potential by +0.05 V with a 20% decrease in intensity for N-(3-Trifluoromethylphenyl) curcumin as compared to pyrazole curcumin (Fig. 3c).

3.4. Substitutions at the Methylene Group at the Centre of the Heptadione Link to Probe Its Role in the Redox Behaviour of Curcumin

There are several different interpretations on the mechanism of action of curcumin as an antioxidant, for example; it has been proposed that the hydrogen abstraction from its central methylene group is the major contributor of its redox properties [14, 15, 35]. But the theoretical study of Sun et al [35] concluded that the antioxidant action of curcumin involves H-abstraction from its phenolic groups, and not from its central CH_2 group in agreement with the conclusion of Barclay et al [15]. Later, Ingold et al. have proposed that in ionizing solvent curcumin undergoes sequential proton loss electron transfer process [18].

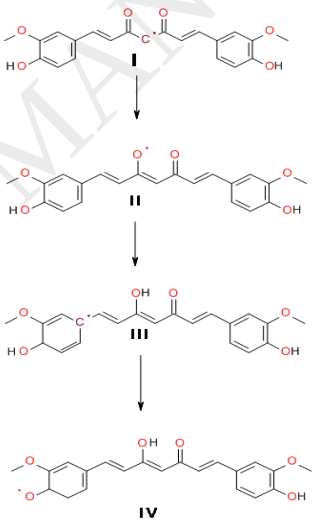
Thus despite a number of studies on the redox properties of curcumin and its derivatives in the past a systematic approach to delineate a role of the methylene moiety at the centre of its heptadione link has been lacking. We have addressed this issue by substituing it by butyldehyde or benzyldehyde or 3- methoxy–phenyl aldehyde and studied their redox behavior by cyclic

voltammetry. Fig. 4a shows a cyclic voltammogram of 8 μM of 4-(4-hydroxy-3-methoxy benzylidene) curcumin in HEPES buffer (pH 7.0; 25 °C) and scan rate 20 mVs^{-1}. It exhibits the oxidation potential at +0.80 V with low current intensity of 5.6 μA. This value can be assigned to the oxidation potential of the phenoxyl radical alone as probability of oxidation of methylene radical in it has been completely eliminated. From the data, it is apparent that the values of oxidation potential have become closer to those of phenol. It is evident from these results that the probability of intramolecular H- atom transfer through *β*-alkoxyl radical is completely prohibited in 4- (4-hydroxy-3-methoxybenzylidene) curcumin, which leads to increase in the oxidation potential with decrease in anodic peak intensity. Hence, 4-(4-hydroxy-3-methoxybenzylidene) curcumin has become poorer as an antioxidant as compared to curcumin. In other words, the inability to form the *β*-alkoxyl radical has hindered the efficacy of the formation of phenoxy radical. Further to rule out the contribution of the additional phenoxy group present in 4-methoxy phenyl benzylidene curcumin, we have synthesized 4-benzylidene curcumin and 4-butylidene curcumin which contain no additional –OH group at the central methylene. The oxidation potential for 4-benzylidene curcumin and 4-butylidene curcumin, respectively, are 0.84 V and 0.85 V (Fig 4b) which are again much higher than that observed for curcumin. Because of the substitution of the methylene group at the centre of curcumin these compound show a oxidation potential that is close to the oxidation potential values observed for a typical phenoxy radical (E_6 = 0.87 V) [15].

To understand the role of the phenolic –OH of curcumin, we have also studied the redox properties of half curcumin. 3-(3,4-dihydroxyphenyl) prop–2-enoic acids and 3-(3-hydroxy-4-methoxyphenyl) prop–2-enoic acid (Dehydrozingerone) which show the value of oxidation potential of +0.65 V and +0.79 V respectively (Fig 4c and 4d). The observation of the anodic peak at lower oxidation potential for 3-(3,4-dihydroxyphenyl) prop – 2-enoic acid i.e., at +0.65 V compared to that for 3-(3-hydroxy-4-methoxyphenyl) prop–2-enoic acid, 0.79 V, can be ascribed to *ortho*–hydroxyl phenoxy radical, which are more stable due to intramolecular hydrogen bonding interaction [36] which in turn will facilitate the further oxidization *ortho*-hydroxy phenoxy radical to form *ortho*-quinone.

In order to understand the contribution of the phenolic –OH in antioxidant activity in the curcumin, we have also studied oxidation potential of dimethyl curcumin where both the

phenolic –OH groups have been blocked. The dimethyl curcumin shows peak at 0.87 V with a peak intensity 4.4 µA (supporting information Fig S2). Thus the peak intensity in case of dimethyl derivative is much less as compared to curcumin and exhibits a higher potential for its oxidation. Since both the hydroxyl groups are blocked in dimethoxy curcumin, oxidation potential observed at +0.87 V can be assigned to CH_2 group at the centre of the hepatadione link. Taken together our studies demonstrate that the oxidation potential of native curcumin seen at +0.66 V is as a result of overlapping contributions from the central methylene group and o-methoxy phenyl group. It is, therefore, apparent that the lack of an intramolecular H- shift from the alkoxyl radical generated at the centre of dimethoxy curcumin to its termini shifts the oxidation potential of the methylene group to a higher potential i.e from a value +0.66 V for curcumin to +0.87 V in compound 12. It has been suggested earlier that *ortho*-methoxy phenol group can form intramolecular hydrogen bond with phenolic hydrogen, making H- abstraction from *ortho*-methoxy phenols easier [37,38].



Mechanism 1: Proposed mechanism of curcumin oxidation

Together, these data imply that the intramolecular H- atom transfer from *β*-alkoxyl radical plays a vital role in the antioxidant function of curcumin as shown in mechanism 1. It supports Jovanoic et al's conclusions that the alkoxyl radical generated at the methylene group at the centre of the heptadione link in curcumin undergoes an intramolecular H-shift towards the

phenoxy radical at its termini. Therefore, the presence of the $-CH_2$ group at the centre of curcumin and phenoxy hydroxyl groups at both of its termini are intrinsic and essential for the antioxidant function of curcumin.

3.5. DPPH Assay for Antioxidant Activity

To further confirm the anti-oxidant properties of substituted curcumin used for deducing its anti-oxidant mechanism, we have studied their free radical scavenging ability using DPPH radical which reacts with an electron or hydrogen donor to become a stable diamagnetic molecule *viz* hydrazine. The solution therefore loses colour depending on the number of electrons accepted. Substances capable of donating electrons /hydrogen atoms are able to convert the purple colour of DPPH to its non-radical yellow form; 1,1-diphenyl-2-picrylhydrazine, a reaction which can be followed spectrophotometrically. Curcumin exhibits better free radical scavenging than the 4-(4-hydroxy-3-methoxy benzylidene) curcumin (Fig 5). Structure- activity relationship showed that the decreased antioxidant active of 4-(4-hydroxy-3-methoxy benzylidene) curcumin could be attributed to absence of methylene hydrogen at the center of dicarbonyl moiety. In 4-benzylidene curcumin, the delocalization is larger with a higher degree of conjugation. Apparently, the small difference in delocalization has larger influence on antioxidant activity, as 4- benzylidene curcumin shows a higher antioxidant activity than less conjugated counterpart, 4-butylidene curcumin. These interpretations are strengthened further by the reduced free radical scavenging activity of 4-benzylidene and 4- butylidene curcumin as compared to the parent compound.

4. Conclusions

The oxidation of the curcumin involves H- atom transfer from *β*-alkoxyl radical generated at the centre of its heptadione link which undergoes molecular rearrangement to form the phenoxy radical. Thus the central methylene group of the heptadione link is also playing a role along with the hydroxyl group in the antioxidant activity of curcumin. A lower oxidation potential of curcumin than that observed for a typical phenoxy radical provide a rationale for explaining widely appreciated antioxidant properties of curcumin as compared to phenols. The pyrazole curcumin shows better antioxidant properties (*viz* lower oxidation potential) than curcumin due to the absence of keto–enol tautomerism and oxidation in the flexibility of its heptadione link. 4-(4-Hydroxy-3-methoxybenzylidene) curcumin and other analogues of the central methylene group eg. 4- butylidene and 4- benzylidene curcumin, fail to generate the *β*-alkoxy radical. Such

analogues of curcumin are therefore, poor as antioxidants. In other words, failure to generate β-alkoxyl radical in them compromises an efficient formation of phenoxy radical. Also, an oxidation potential that of alkyl peroxy radical (E_7 = 1.06V), and the superoxide radical (E_7 > 1.06 V) [39] makes curcumin an important nutritional antioxidant.

Acknowledgements

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Scheme 1 Synthesis of Pyrazole derivative of curcumin

Scheme 2 Synthesis of *N*-(substituted) phenylcurcumin pyrazole analogues

Scheme 3 Synthesis of Knoevenagel condensate of curcumin (4-(4-Hydroxy-3-methoxybenzylidene) curcumin (**10**), 4-Benzylidene curcumin (**11**) and 4-Butylidene curcumin (**12**).

Figure caption

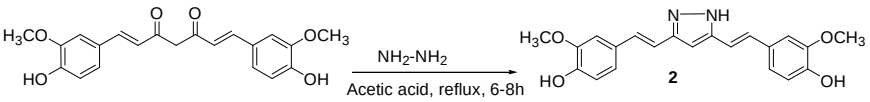
Fig. 1 Characteristic cyclic voltammogram of a cleaned polycrystalline gold electrode in (a) 0.5 M H_2SO_4. Scan rate is 100 mV s^{-1} (b) 10 mM HEPES buffer. Scan rate is 20 mV s^{-1}

Fig. 2 Cyclic voltammograms of (a) various concentrations of curcumin in 10 mM HEPES buffer (pH 7.4, 25 °C). Scan rate 20 mV s^{-1} (b) plot of anodic peak current versus concentration of curcumin, (c) various scan rate from 20 to 100 mV s^{-1} of 8 µM curcumin at pH 7.4, 25 °C (d) plot of anodic peak current versus ln (v) and plot of oxidation current versus the square root of the scan rate as inset (2d), and (e) 8 µM curcumin at various pH value. Scan rate is 20 mV s^{-1}.

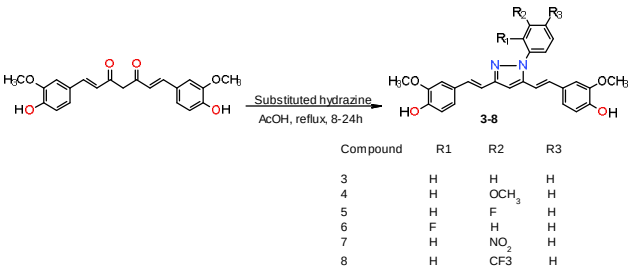
Fig. 3 Cyclic voltammograms of pyrazole curcumin **(-)** with (a) 3-fluorophenyl pyrazole curcumin(-), (b) 3-nitrophenylpyrazole curcumin(-) and (c) 3- trifluoromethyl phenyl pyrazole curcumin(-) , concentration 8µM, at a scan rate of 20 mV s^{-1}

Fig. 4 Cyclic voltammogram for 8 µM of (a) 4-(4-hydroxy-3-methoxy benzylidene) curcumin **(-)** (b) 4-benzylidene curcumin **(-)** and 4- butyldiene curcumin (-), (c) 3-(3,4-dihydroxyphenyl) prop–2-enoic acid(-) and (d) 3-(3-hydroxy-4-methoxyphenyl) prop–2-enoic acid (-), pH 7.4 at 25^0 C. Scan rate is 20 mV s-1

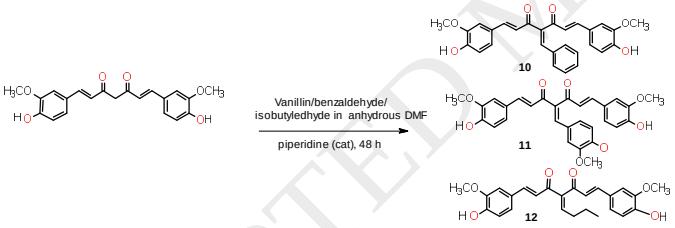
Fig. 5 Free radical scavenging activity of curcumin (▼), 2-methoxy 3-hydroxy benzyldiene curcumin (▲), 4-benzyldiene curcumin (●) and 4-butylidene curcumin (■) measured by DPPH.



Scheme 1



Scheme 2



Scheme 3

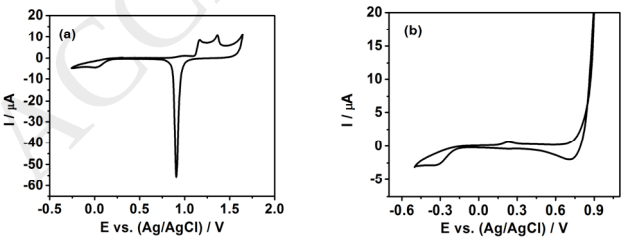


Fig. 1

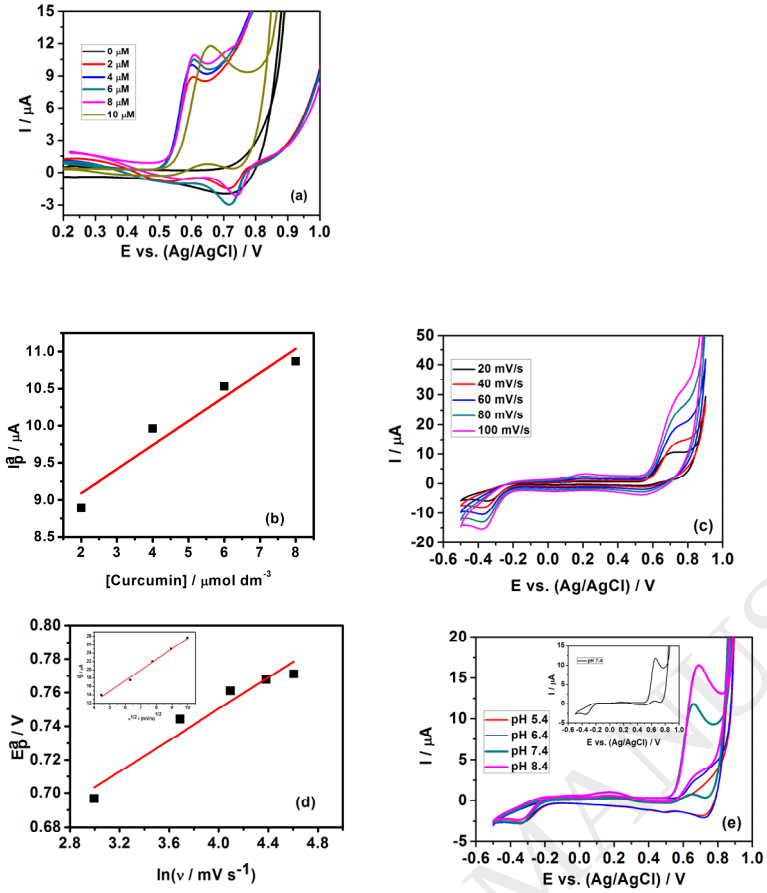


Fig. 2

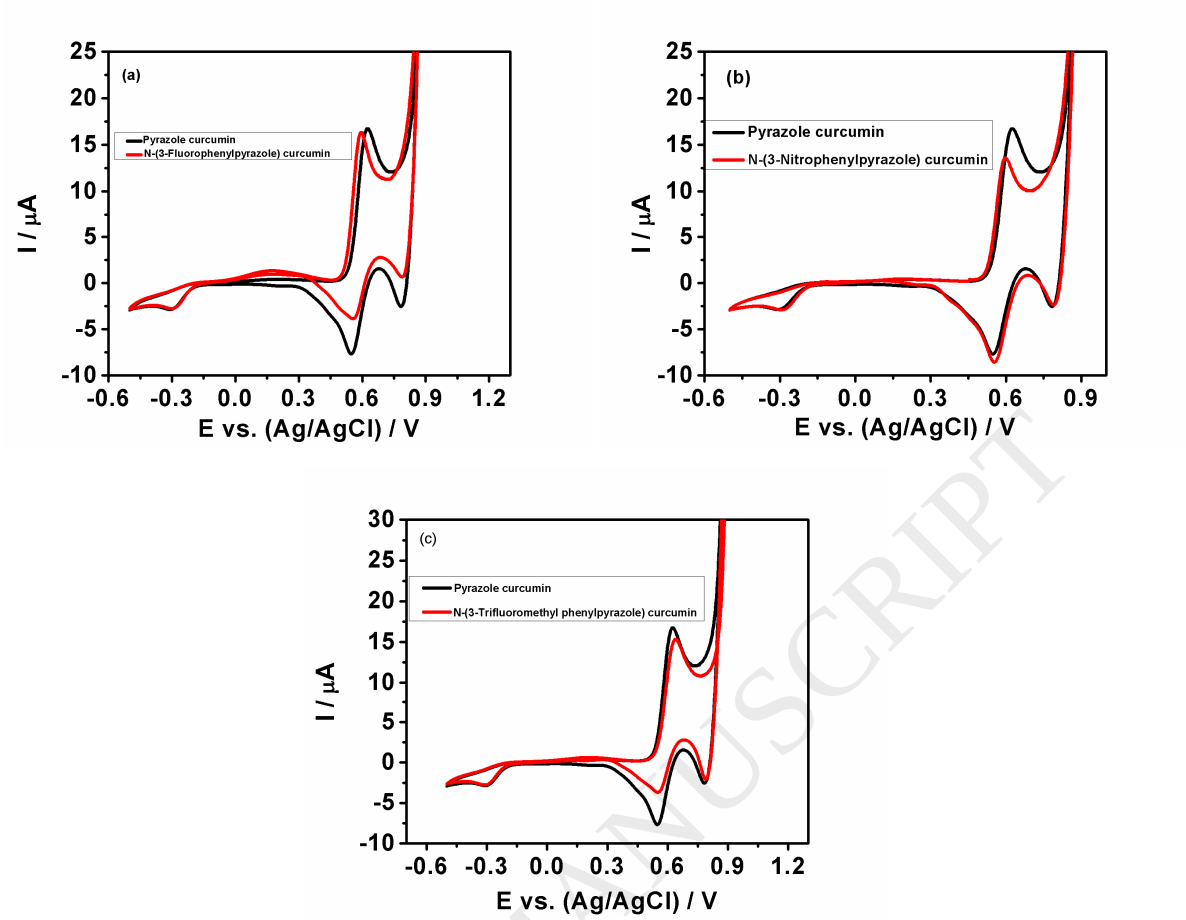


Fig. 3

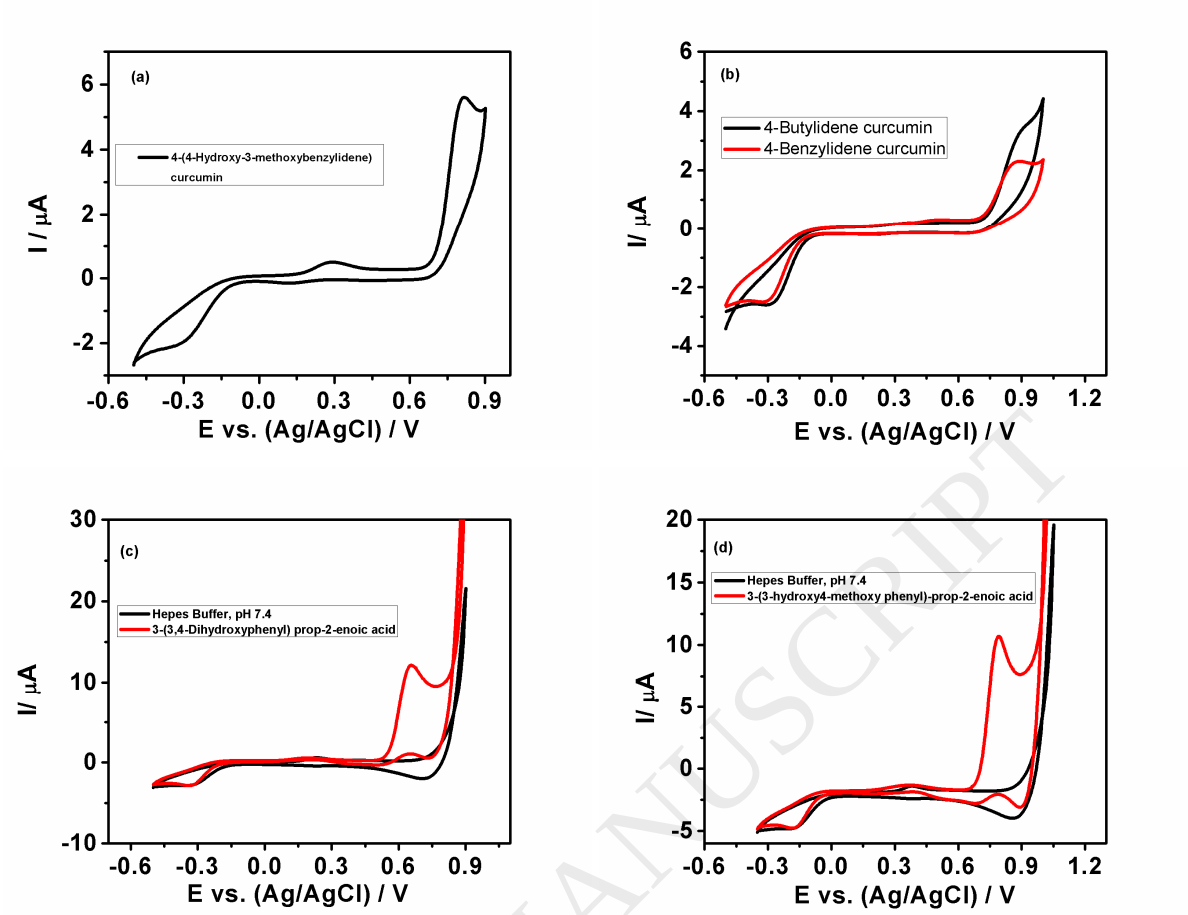


Fig. 4

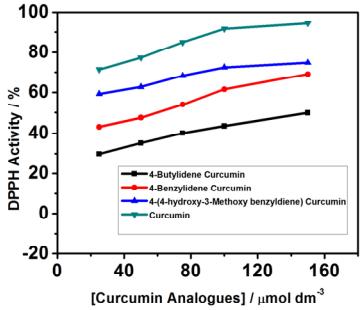
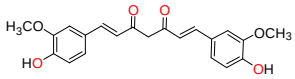
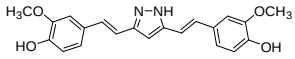
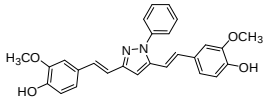
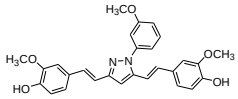
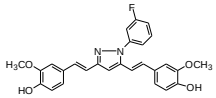
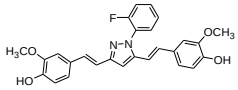
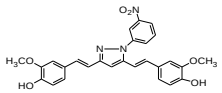
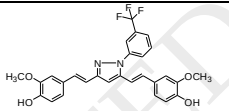
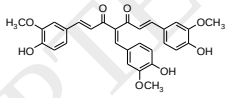
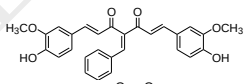
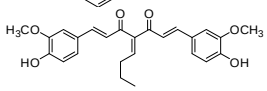
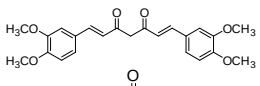
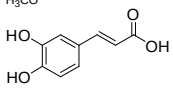
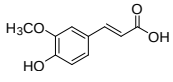


Fig. 5

Table 1

The oxidation potential of curcumin and its analogs at 8 μmol dm^{-3}, pH 7.4, 25^0 C.

Compound	Structures	Chemical Name	Electrochemical Oxidation Potential, E^0(V)	Current, I (µA)
1.		Curcumin	0.66	10.84
2.		Pyrazole curcumin	0.62	16.94
3.		N-(Phenyl pyrazole) curcumin	0.63	15.81
4.		N-(3-Methoxyphenyl pyrazole) curcumin	0.66	16.81
5.		N-(3-Fluorophenyl pyrazole) curcumin	0.59	16.11
6.		N-(2-Fluorophenyl pyrazole) curcumin	0.64	15.28
7.		N-(3-Nitrophenyl pyrazole) curcumin	0.59	13.59
8.		N-(3-trifluoromethyl pyrazole) curcumin	0.64	15.21
9.		4-(4-Hydroxy-3-methoxybenzylidene) curcumin	0.80	5.60
10.		4-Benzylidene curcumin	0.84	2.24
11.		4-Butylidene curcumin	0.85	3.32
12.		Dimethyl curcumin	0.87	4.40
13.		3-(3,4-dihydroxy phenyl) prop–2-enoic acid	0.65	12.31
14.		3-(3-hydroxy-4-methoxyphenyl) prop–2-enoic acid	0.78	10.76