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Effect of curcumin analogues on oxidation of haemoglobin and lysis of erythrocytes

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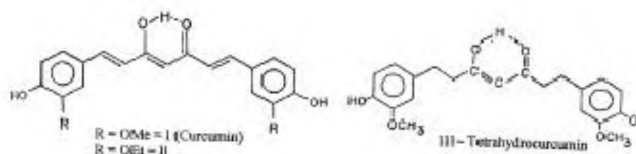
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A number of ring-substituted analogues of curcumin were studied for their ability to prevent nitrite-induced oxidation of haemoglobin and lysis of erythrocytes. Phenolic analogues were more active than their non-phenolic counterparts. Many of the active compounds were more potent than standard antioxidants such as tocopherol and trolox. Tetrahydrocurcumin showed higher activity than curcumin, suggesting that unsaturation in the central portion of curcumin may not be important for activity.

CURCUMINS from *Curcuma longa* (turmeric) are potent antioxidants^{1,2} and possess a number of therapeutic properties³. Many of the biological activities of curcumin are attributed to its antioxidant properties^{4–6}. While both phenolic groups and the 1,3-diketone system of curcumin are ascribed for its activity by some reports^{7,8}, several others suggest that the phenolic group may not be essential^{8,9}. In our earlier work¹⁰ to understand the importance

of the phenolic group and other substituents in the aromatic ring, several analogues of curcumin were synthesized and studied for their antioxidant activity in models such as inhibition of lipid peroxidation and scavenging of radicals such as 1,1'-diphenyl picryl hydrazyl (DPPH) and 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS⁺). It was observed that the phenolic group is important and ortho substitution with groups such as methoxy, methyl group enhances the activity. It was also observed that the unsaturation in the side chain was not essential for the activity.

Nitrite is an environmental pollutant causing harm to marine fauna, chiefly by oxidizing the haemoglobin to methaemoglobin. Nitrite (NO₂⁻) is formed when *Nitrosomonas* sp. bacteria oxidize ammonia produced by fish and decomposing organic matter¹¹. Prolonged exposure to low levels can lead to stress and is often associated with stress-related disease such as bacterial ulcers and fin-rot. At higher levels, it is actively transported across the gills and into the bloodstream of the fish where it oxidizes normal haemoglobin to methaemoglobin that cannot transport oxygen. This results in tissue hypoxia even in the presence of oxygen (functional hypoxia)¹².



Structure of curcumin and its analogues.

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Nitrite oxidizes haemoglobin in two stages, viz. a slow stage followed by a rapid autocatalytic stage¹³. This autocatalytic reaction is a result of several free radical species such as superoxide anion, peroxyxynitrite and nitrogen dioxide, which are generated during the course of nitrite-induced oxidation of haemoglobin¹⁴. Earlier observations have shown that curcumin, being a scavenger of both superoxide and nitrogen dioxide, successfully arrests the autocatalytic stage at micro molar concentrations¹⁴. Hence, as an extension of the structure-activity relationship studies, the compounds tested in our earlier study¹⁰ were evaluated for their potential in inhibiting nitrite-induced oxidation of haemoglobin.

Curcumin and analogues were synthesized as described by Pabon¹⁵. Tetrahydrocurcumin was a gift sample from Sami Chemicals and Extracts, Bangalore, India. All the compounds except III are known in the literature. Melting point determination, UV, IR and NMR spectra confirmed the structures. The purity of the compounds was tested by

TLC and elemental analysis (C, H) of the compounds showed less than 0.5% variation. The compounds used are shown in Figure 1. DEAE-sephadex, α -tocopherol and trolox were obtained from Sigma Chemical Co, USA. All other chemicals were of analytical grade. Human blood was collected from a blood bank at Kasturba Hospital, Manipal. Blood was drawn by vein puncture and stored in containers with acid-citrate-dextrose as anticoagulant.

The procedure described by Unnikrishnan and Rao¹⁴ was followed to study the effect of curcumin analogues on oxidation of haemoglobin present, either free in solution form or inside erythrocytes. Pure haemoglobin was isolated from haemolysate of erythrocytes by chromatography over a DEAE-sephadex column. Fractions containing pure haemoglobin were suitably diluted with phosphate-buffered saline (PBS) and used for studies. To the incubation mixture containing purified haemoglobin (56 μ M), test compounds dissolved in methanol

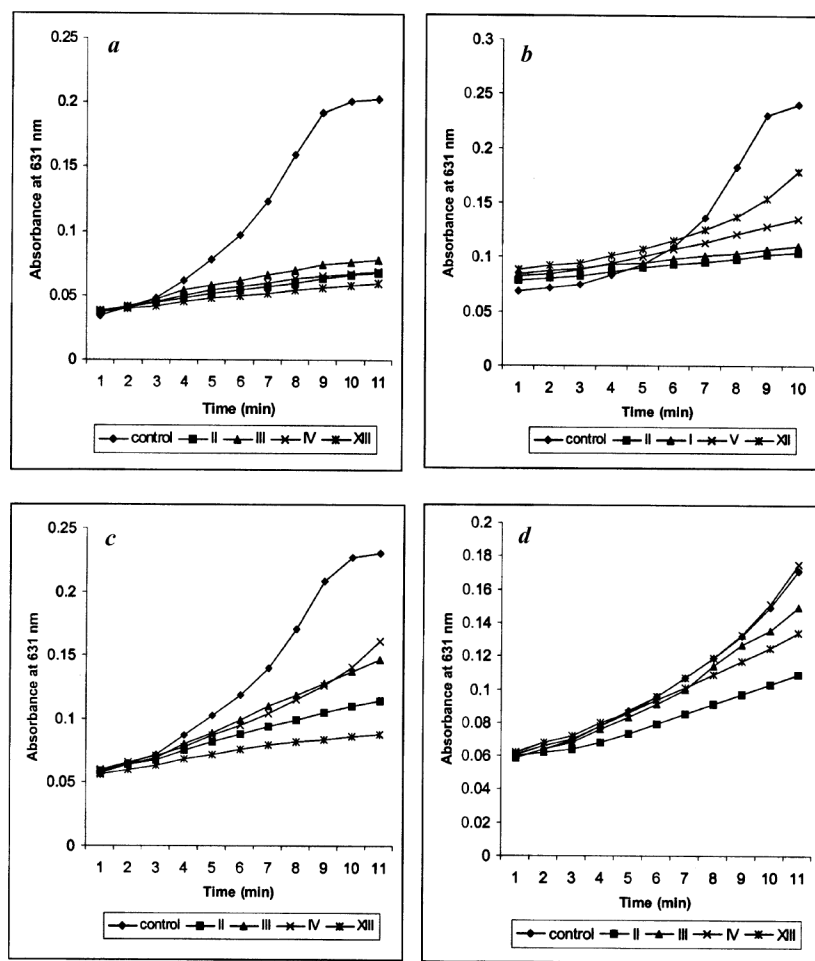


Figure 1. Time course of nitrite-induced methaemoglobin formation with and without curcumin analogues. Purified haemoglobin (56 μ M) was treated with nitrite (300 μ M) in the presence/absence of test compounds and absorbance was measured at 631nm at regular intervals. *a*, Compounds II, III, IV and XIII at a concentration of 5 μ M; *b*, Compounds I, II, V and XII at a concentration of 5 μ M; *c*, Compounds II, III, IV and XIII at a concentration of 1 μ M; and *d*, Compounds II, III, IV and XIII at a concentration of 0.25 μ M.

or dimethyl sulphoxide (final conc. 10%) were added followed by sodium nitrite (300 μM). The time course of methaemoglobin formation was recorded by monitoring the absorbance at 631 nm.

To study the effect of curcumin analogues on nitrite-induced methaemoglobin formation in intact erythrocytes, the washed erythrocyte suspension was incubated with test compounds for 30 min followed by addition of sodium nitrite (final conc., 1.8 mM) and incubation for further 90 min. The suspension was centrifuged at 2500 g for 20 min to remove excess test compounds and nitrite. The cells were washed thrice with PBS and lysed with phosphate buffer (20 mM, pH 7.4). The haemolysate was then centrifuged at 25,000 g for 60 min to remove the membrane, and the clear supernatant was removed and absorbance at 631 nm was measured. Control experiments were conducted without the test compounds.

Effect of the test compounds on haemolysis was studied, by incubating the washed erythrocyte suspension with test compounds for 30 min followed by addition of sodium nitrite (final conc., 40 μM) and incubation for further 90 min. The suspension was centrifuged at 2500 g for 20 min to remove excess test compounds and nitrite. The cells were washed thrice with PBS and lysed with phosphate buffer (20 mM, pH 7.4). The haemolysate was then centrifuged at 25,000 g for 60 min to remove the membrane, and the clear supernatant was removed and absorbance at 577 nm was measured. Control experiments were conducted without the test compounds.

Addition of nitrite resulted in rapid oxidation of haemoglobin to methaemoglobin. The oxidation was slow at the initial stage and became rapid later. In presence of curcumin (compound II) and its analogues the oxidation process was delayed in a dose-dependent manner (Figure 1). From these data, time taken to oxidize 50% of haemoglobin to methaemoglobin ($t_{1/2}$) was calculated (Table 1). In

the absence of the test compounds (control), $t_{1/2}$ was 7.5 ± 0.7 min. Compound XIII (5 μM) was the most active and was comparable to trolox ($t_{1/2} = 49.2$ min). Compound II (5 μM) was less active than compound XIII, and was comparable to α -tocopherol ($t_{1/2} = 37.2$ min).

Compounds III, IV and I also showed good activity at 5 μM . Nonphenolic substituted analogues were found to be inactive. Steric crowding of the phenolic group with methyl groups (compound IV) did not increase the activity, but the bulkier di-*t*-butyl group (compound V) eliminated the activity. The order of the activity was found to be XIII = trolox >> II = IV = α -tocopherol > III = I > V > XII.

None of the test compounds (0.04 to 90 μM) showed any significant protection against nitrite (10 to 90 μM)-induced methaemoglobin formation in intact erythrocytes. α -tocopherol and trolox also failed to exert significant activity at the tested concentrations (5 to 50 μM). Data are not shown since none of the compounds showed any activity.

During the above studies, at particular concentrations of nitrite, partial haemolysis was observed. The supernatant solution obtained after centrifuging the incubation mixture containing erythrocytes and nitrite (40 μM), showed threefold increase in haemoglobin compared to control where no nitrite was present. In the presence of curcumin analogues, the level of haemoglobin in nitrite-treated erythrocytes was significantly lower. Hence systematic studies were conducted. Here again, a number of phenolic analogues showed good protection against haemolysis (Table 2). At tested concentrations (5–40 μM), compound I showed highest protection followed by compound XIII. Di-ortho substituted analogues such as compounds IV (methyl groups substituted) and V (*tert*-butyl groups substituted) showed better protection than mono-ortho substituted analogues such as compounds II (methoxy) and III (ethoxy group substituted). Other compounds

Table 1. Effect of curcumin analogues on nitrite-induced oxidation of haemoglobin

Code	Compound added			Time to form 50% methaemoglobin ($t_{1/2}$); (min) ^a		
	R1	R2	R3	5 μM	1 μM	0.25 μM
Control				7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.7
I	H	OH	H	20.3 ± 0.1^b	8.1 ± 0.6	7.5 ± 0.7
II	OCH ₃	OH	H	39.7 ± 0.4^c	19.8 ± 1.3^b	12.7 ± 0.1
III	OEt	OH	H	25.6 ± 0.3^b	14.5 ± 2.2	9.1 ± 0.5
IV	CH ₃	OH	CH ₃	38.5 ± 0.4^c	13.2 ± 0.1	9.7 ± 2.0
V	<i>t</i> -C ₄ H ₉	OH	<i>t</i> -C ₄ H ₉	10.8 ± 3.8	4.1 ± 0.5	7.5 ± 0.7
VI	H	H	H	7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.7
VII	H	CH ₃	H	7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.7
VIII	H	SCH ₃	H	7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.7
IX	H	OCH ₃	H	7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.7
X	OCH ₃	OCH ₃	H	7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.7
XI	OCH ₃	OCH ₃	OCH ₃	7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.7
XII	OCH ₃	OCOCH ₃	H	9.8 ± 1.1	7.5 ± 0.7	7.5 ± 0.7
XIII	Tetrahydrocurcumin			54.6 ± 0.5^d	29.9 ± 1.1^c	9.6 ± 1.7
α -tocopherol				37.2 ± 1.3^c	17.4 ± 0.9^b	9.2 ± 1.3
Trolox				49.2 ± 0.9^d	32.2 ± 1.7^c	10.1 ± 1.6

^aAll values are mean \pm S.E. ($n = 3$); ^b $P < 0.05$; ^c $P < 0.01$; ^d $P < 0.001$ compared to control.

Table 2. Effect of curcumin analogues on nitrite-induced lysis of erythrocytes

Code	Compound added			Percentage prevention of lysis of RBC ^a		
	R1	R2	R3	40 μ M	20 μ M	5 μ M
I	H	OH	H	81.4 \pm 1.6 ^{d,g}	41.9 \pm 2.1 ^f	22.1 \pm 1.0
II	OCH ₃	OH	H	18.6 \pm 0.8	10.7 \pm 1.2	NA
III	OEt	OH	H	22.0 \pm 0.9	12.8 \pm 1.9	NA
IV	CH ₃	OH	CH ₃	64.5 \pm 2.9 ^{e,f}	38.7 \pm 2.4 ^f	12.4 \pm 2.2
V	<i>t</i> -C ₄ H ₉	OH	<i>t</i> -C ₄ H ₉	60.2 \pm 0.9 ^{e,f}	33.9 \pm 1.5 ^e	10.5 \pm 1.5
VI	H	H	H	NA	NA	NA
VII	H	CH ₃	H	NA	NA	NA
VIII	H	SCH ₃	H	NA	NA	NA
IX	H	OCH ₃	H	NA	NA	NA
X	OCH ₃	OCH ₃	H	NA	NA	NA
XI	OCH ₃	OCH ₃	OCH ₃	NA	NA	NA
XII	OCH ₃	OCOCH ₃	H	NA	NA	NA
XIII	Tetrahydrocurcumin			72.0 \pm 1.3 ^{d,f}	40.3 \pm 1.9 ^f	22.12 \pm 1.0
α -tocopherol				14.3 \pm 1.8	NA	NA
Trolox				25.1 \pm 2.1	10.3 \pm 0.8	NA

^aAll values are mean \pm S.E. ($n = 3$); ^b $P < 0.05$; ^c $P < 0.01$; ^d $P < 0.001$ compared to α -tocopherol; ^e $P < 0.05$; ^f $P < 0.01$; ^g $P < 0.001$ compared to trolox.

did not show any significant protection. The order of activity was found to be I, XIII > IV = V > trolox = III = II = α -tocopherol.

The behaviour of these thirteen compounds in the present study was more or less similar to that observed in our earlier study on inhibition of Fe³⁺/ascorbate-induced lipid peroxidation and scavenging of radicals such as DPPH and ABTS⁺ (ref. 10). Compounds with phenolic groups exerted good activity, and masking of phenolic group resulted in less activity. Many of the active compounds were more potent than the standard antioxidants, α -tocopherol and trolox. In scavenging the radicals in solution (DPPH and ABTS⁺), derivatives with less bulkier substituents at the position ortho to the phenolic group were optimal for the antioxidant activity¹⁰. Similarly, in preventing the nitrite-induced oxidation of haemoglobin in aqueous solution, compounds with smaller ortho substituents such as methyl, methoxy, ethoxy (compounds IV, III and II) showed better activity than the compounds with bulkier substituents such as *tert*-butyl groups (compound V). Among all, a saturated derivative of curcumin (i.e. tetrahydrocurcumin, compound XIII) showed higher activity and this may be due to increased solubility^{16,17} due to saturation at the side chain. In several *in vitro* and *ex vivo* antioxidant studies, the higher activity of tetrahydrocurcumin over curcumin has already been demonstrated¹⁸⁻²⁰.

Earlier observations have shown that curcumin, being a scavenger of both superoxide and nitrogen dioxide, successfully arrests the autocatalytic stage of nitrite-induced oxidation at micromolar concentrations¹⁴. Thus, the mechanism of action of other active curcumin analogues may be similar to that of curcumin.

Though a few curcumin analogues could prevent the nitrite-induced oxidation of haemoglobin in aqueous solution, none of them could do so when haemoglobin

was present in intact red blood cells. Several radical-scavenging antioxidants such as glutathione and uric acid could effectively reduce the chemiluminescence caused by *tert*-butyl hydroperoxide on haemolysate than erythrocytes. It was attributed to the fact that antioxidant reacts much more easily in the aqueous medium²¹. This may be the reason for the failure of curcumin analogues in preventing intracellular oxidation of haemoglobin by nitrite.

It has been shown that curcumin could prevent haemolysis caused by hydrogen peroxide²² and primaquine²³. Hence the compounds were tested for anti-haemolytic activity. Several phenolic analogues showed good anti-haemolytic activity. Compounds I (bisdesmethoxy curcumin) and XIII (tetrahydrocurcumin) showed higher activity than curcumin (compound II) and its ethoxy analogue (compound III). The activity lies in the effective localization of the compounds and how it presents the polar head at the membranous region to counter the incoming radicals from the solution²⁴. Compounds XIII and I were more active than compounds II and III; this may be due to better solubility and planarity of these molecules¹⁶. Also, di-ortho substituted compounds such as IV (methyl groups substituted) and V (*tert*-butyl groups substituted) showed potent activity; it was higher than that for compounds II and III. The activity of compounds IV and V may be due to their membrane-stabilizing property. Urano and coworkers²⁵ also observed that bulkier the group substituted ortho to the phenolic group of tocopherol, more effective is the compound in suppressing the haemolysis.

Thus the present study demonstrates the importance of the phenolic group for the activity of curcumin. The saturated analogue of curcumin, namely tetrahydrocurcumin (XIII) showed better activity than curcumin. The good antioxidant property²⁶ and pharmacological property^{27,28} of tetrahydrocurcumin increase the interest on the effi-

cacy of curcumin for its probable action through its active metabolite such as tetrahydrocurcumin even after its metabolism.

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Herbicide-resistant transgenics of bread wheat (*T. aestivum*) and emmer wheat (*T. dicoccum*) by particle bombardment and *Agrobacterium*-mediated approaches

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Hexaploid bread wheat (*Triticum aestivum*) and tetraploid emmer wheat (*T. dicoccum*) hold immense agricultural and economical importance as their end products have varied utilities depending upon the visco-elasticity and other properties of the flour. In the present study, highly regenerable basal segment calli have been employed as the target tissue for genetic transformation of Indian varieties of bread wheat (CPAN1676, PBW343) and emmer wheat (DDK1001). The *bar* gene conferring herbicide resistance was introduced in one-month-old calli employing both particle bombardment and *Agrobacterium*-mediated transformation strategies. Transgenic calli were selected on phosphinothricin-containing regeneration medium and putative transformants were raised to maturity. Though the plants exhibited reduced vigour in terms of height and tillering, nonetheless, seed set was normal. The presence of the transgene (*bar*) was confirmed by PCR and Southern hybridization. In general, the transformation efficiency was found better with *Agrobacterium*, even though the construct carried CaMV35S driven *bar* gene, whereas in the case of biolistics *Ubi1* (a monocot promoter)-driven *bar* gene was employed. Transformation efficiency in the range of 4% was obtained with particle bombardment, whereas it was 7.5% using *Agrobacterium*-mediated co-cultivation. The different varieties of bread and emmer wheat investigated did not show any marked difference in their transformation ability and could be attributed to a well-established regeneration system in these varieties.

AMONGST various cereal grains, wheat is an important ingredient of the human diet. Different varieties of wheat, depending upon their dough quality and other visco-elastic characteristics, are used for making a range of food products. Modern-day hexaploid wheat (*Triticum aestivum*) is used generally for bread-making, but lacks certain agronomically important features such as drought or disease resistance. Tetraploid emmer wheat (*Triticum dicoccum*), in contrast, shows high adaptability to dry, parched lands and exhibits drought and salinity resistance; but its poor visco-elastic properties and hardness

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