

# Effect of Curcumin Against Oxidation of Biomolecules by Hydroxyl Radicals

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## ABSTRACT

**Background:** Among various reactive oxygen species, hydroxyl radicals have the strongest chemical activity, which can damage a wide range of essential biomolecules such as lipids, proteins, and DNA.

**Objective:** The objective of this study was to investigate the beneficial effects of curcumin on prevention of oxidative damage of biomolecules by hydroxyl radicals generated in *in vitro* by a Fenton like reaction.

**Materials and Methods:** We have incubated the serum, plasma

and whole blood with  $H_2O_2/Cu_2^+$ /Ascorbic acid system for 4 hours at 37 °C and observed the oxidation of biomolecules like albumin, lipids, proteins and DNA.

**Results:** Curcumin at the concentrations of 50,100 and 200  $\mu$ moles, prevented the formation of ischemia modified albumin, MDA, protein carbonyls, oxidized DNA and increased the total antioxidant levels and GSH significantly.

**Conclusion:** These observations suggest the hydroxyl radical scavenging potentials of curcumin and protective actions to prevent the oxidation of biomolecules by hydroxyl radicals.

**Keywords:** Biomolecules, Curcumin, Fenton reaction, Hydroxyl radical, Oxidation

## INTRODUCTION

The aerobic organisms undergo complex interactions during normal metabolism which leads to the release of harmful mediators such as, superoxide anion radical ( $O_2^-$ ), singlet oxygen ( $^1O_2$ ), hydrogen peroxide ( $H_2O_2$ ) and highly reactive hydroxyl radical (OH). These mediators are referred as free radical because of its unpaired electron. In general, OH radical is considered to be a harmful by-product of oxidative metabolism, which can cause molecular damage in living system [1,2]. It shows an average lifetime of  $10^{-9}$  s and can react with nearly every biomolecule such as nuclear DNA, mitochondrial DNA, proteins and membrane lipids. OH can be artificially generated by the Fenton-reaction [3]. Copper can induce oxidative stress by catalysing the formation of ROS via a Fenton-like reaction [4,5]. The cupric ion (Cu (II)), in the presence of superoxide anion radical or biological reductants such as ascorbic acid or GSH, can be reduced to cuprous ion (Cu (I)) which is capable of catalyzing the formation of reactive hydroxyl radicals through the decomposition of hydrogen peroxide via the Fenton reaction [6,7].



The combination of ascorbate, copper, and hydrogen peroxide is an efficient hydroxyl radical generating system called "the Udenfriend system" [8]. Generation of OH radical can ultimately leads to the oxidative damage to biomolecules like proteins, lipids and DNA.

Curcumin (diferuloylmethane) is a naturally occurring yellow pigment isolated from ground rhizomes of the plant *Curcuma longa* L. (*Zingiberaceae*) found in south Asia [9]. Curcumin is a potent antioxidant agent and free radical scavenger [10]. Several animal studies have reported hypocholesterolemic, antiatherogenic [11–14], and antiplatelet activity [15], of this compound. Furthermore, *in vitro* studies of curcumin have shown the anti-inflammatory Action on human vascular cells. Curcumin attenuates inflammatory response of TNF- $\alpha$  stimulated human endothelial cells by interfering with NF- $\kappa$ B [16,17] and is capable of preventing platelet-derived growth factor (PDGF). It also stimulates the migration of vascular smooth muscle cells (VSMCs) by inhibiting MMP-9 activity and expression [18,19].

To our knowledge, there is very little evidence that shows the protective effects of curcumin on hydroxyl radical oxidative damage to biomolecules like proteins, lipids and DNA. Hence, this study is first of this kind, to evaluate the toxic effects of  $\cdot OH$  and the protective effects of curcumin, a potent antioxidant in food.

## MATERIALS AND METHODS

Fifty healthy subjects, both male and female within the age group of 18-25y were recruited for the present study, out of which 15 subjects agreed to participate. Blood samples from these subjects were obtained by venipuncture, and EDTA-plasma and serum were separated by centrifugation at 2000 rpm for 10min at 4°C. Plasma, serum and whole blood of all the 15 subjects were pooled and aliquots of serum, plasma and whole blood (three replicate experiments per group) were randomly selected for the following incubations for 4h at 37°C. This study consists of five groups, Group 1, Control: Serum, plasma and whole blood samples were incubated with phosphate buffer saline. Group 2, Oxidative stress induced group: In this group, serum, plasma and whole blood samples were incubated with Fenton's reagent ( $CuSO_4$  – 5mm, Ascorbic Acid – 8mm and  $H_2O_2$  - 3mm) separately. Under *in vitro* conditions,  $Cu^+$  is able to generate the hydroxyl radicals by dismutation of  $H_2O_2$  according to the Fenton-redox reaction [4,5]. Groups 3, 4 and 5 were curcumin treated groups. In these groups, serum, plasma and whole blood samples were pre incubated with curcumin for one hour at final concentrations of 50,100 and 200  $\mu$ moles respectively. Fenton's reagent was then added to these groups and incubated.

Curcumin was procured from Laila Pharmaceuticals Pvt. Ltd. (with 95% purity synthesized in Laila Impex R&D Centre), L-Ascorbic acid from Hi-Media (Mumbai, India), Dithiothreitol (DTT), 2,4,6-tripyridyl-striazine (TPTZ) and DTNB were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and chemicals were of analytical grade and of highest purity commercially available.

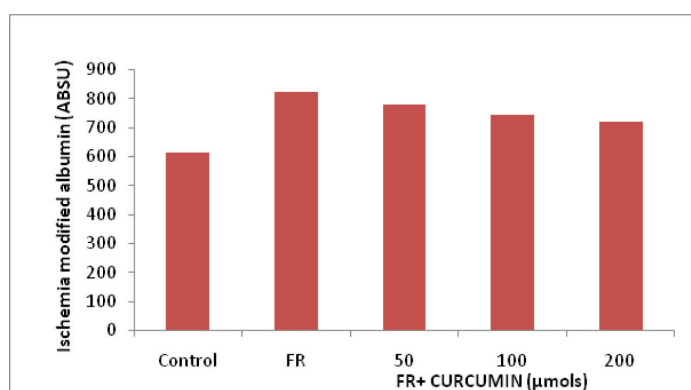
To determine the oxidative damage caused by hydroxyl radicals to biomolecules, the following tests were performed.

Under oxidative stress, the levels of Ischemia Modified albumin (IMA) elevates in the serum. The levels of IMA were determined by the rapid

Groups	MDA	PC	IMA	GSH	TAC
Control (Group I)	12.57 ± 0.95	49.38 ± 3.17	615.98 ± 29.52	0.62 ± 0.20	1579.27 ± 46.64
Fenton's Reagent (Group II)	20.67 ± 1.17 a***	111.46 ± 3.97 a***	824.02 ± 21.22 a***	0.40 ± 0.05 a**	1117.28 ± 58.75 a***
Fenton's Reagent + Cur (50µM) (Group III)	17.65 ± 1.14 a*	85.46 ± 4.06 a***, b***	781.15 ± 15.95 a***	0.46 ± 0.02 a*	1225.75 ± 37.84 a***
Fenton's Reagent + Cur (100µM) (Group IV)	15.55 ± 2.31 b**	76.52 ± 3.22 a***, b***	746.26 ± 30.32 a***, b**	0.49 ± 0.08	1300.02 ± 69.25 a***, b**
Fenton's Reagent + Cur (200µM) (Group V)	14.42 ± 1.82 b***	66.33 ± 2.10 a***, b***	721.58 ± 18.57 a***, b***	0.54 ± 0.08	1425.57 ± 33.13 a*, b***

**[Table/Fig-1]:** Effect of curcumin on hydroxyl radical induced oxidation of biomolecules

MDA: Malondialdehyde, PC: Protein carbonyls, IMA: ischemia modified albumin, GSH: Reduced glutathione and TAC: Total antioxidant capacity. Values presented are Mean ± S.D. of 3 nos. of samples in each treatment group. Post- Hoc multiple comparison was performed by Duncan's test. a - Group I compared to Group II, III, IV and V. b - Group II compared to Group III, IV and V. ns- not significant; \*\*\* p<0.001; \*\* p<0.01; \* p<0.05.

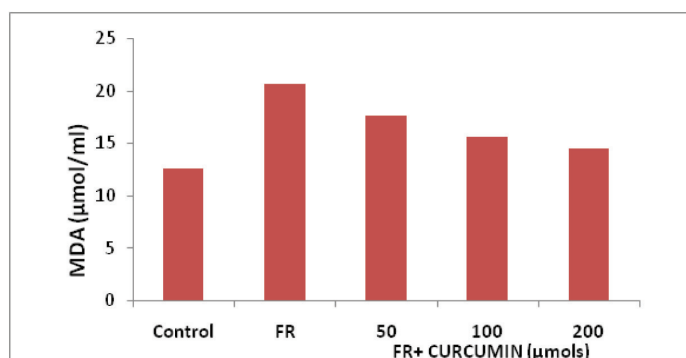


**[Table/Fig-2]:** Effect of curcumin on the content of reduced glutathione in Hydroxyl radical induced Erythrocytes. The data represent the mean±S.D of three replicates

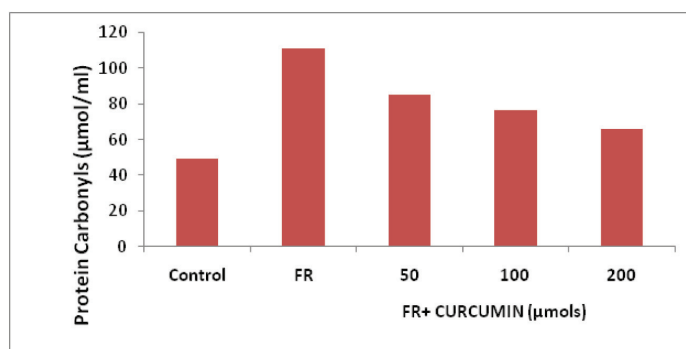
colorimetric method developed by Bar-Or et al., [20]. The values are expressed in absorbance units (ABSU). Formation of protein carbonyls are the indication of protein oxidation. Serum protein carbonyl (PC) content was spectrophotometrically determined by DNPH assay [21,22]. Lipid peroxidation because of oxidative damage was estimated by the method of Okhawa H, et al., [23]. The total antioxidant capacity of plasma was evaluated by applying the FRAP assay (ferric reducing antioxidant power or ferric reducing ability of plasma) according to the method of Benzie & Strain [24]. Reduced glutathione in blood was estimated by the method of Beutler et al., [25]. Reactive oxygen species can damage the DNA. DNA damage can be determined by the method of Lee et al., [26] with slight modifications. Genomic DNA was isolated by salting out method [27]. DNA was incubated with Fenton's reagent containing test sample and the final volume of the mixture was raised up to 20µl. The mixture was then incubated for 30min at 37°C and the DNA was analysed on a 1% agarose gel electrophoresis followed by ethidium bromide staining. Finally gel was analysed using uvitec gel document (Cambridge, UK).

## STATISTICAL ANALYSIS

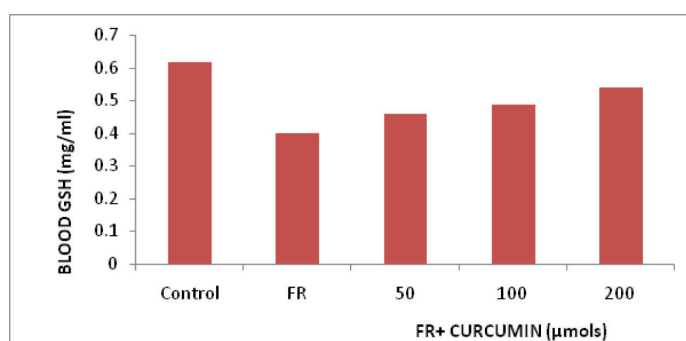
The data are expressed as the mean and standard deviation of three parallel measurements. The data were subjected to one-way-analysis of variance (ANOVA). Duncan's test was used to determine significant differences. p-values ≤ 0.05 were considered as level of significance. The statistical analysis was carried out using SPSS for Windows software (Version 13.0, SPSS, Chicago, IL, USA).



**[Table/Fig-3]:** Effect of curcumin on the formation of ischemia modified albumin in hydroxyl radical exposed serum. The data represent the mean±S.D of three replicates



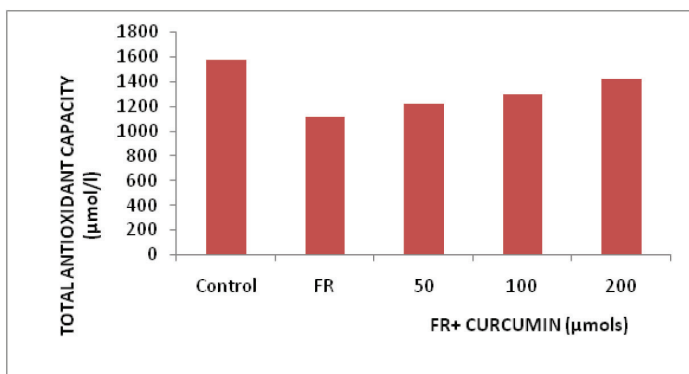
**[Table/Fig-4]:** Effect of curcumin on the protein carbonyls formation in hydroxyl radical exposed serum. The data represent the mean±S.D of three replicates



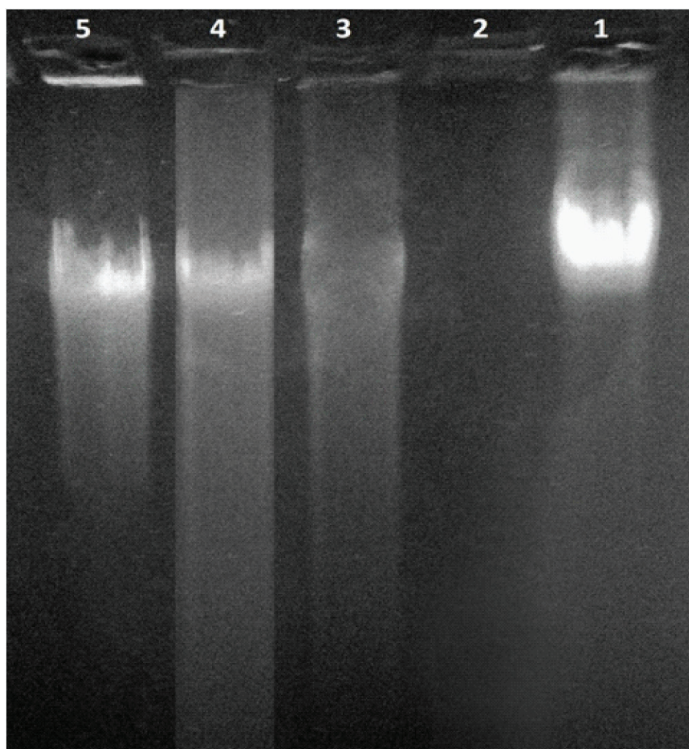
**[Table/Fig-5]:** Effect of curcumin on the malondialdehyde formation in hydroxyl radical exposed serum. The data represent the mean±S.D of three replicates

## RESULTS

The effect of curcumin on ischemia modified albumin formation is presented in [Table/Fig-1,2]. We have noted that, when serum was exposed to hydroxyl radicals the production of ischemia modified albumin was highly significant (p<0.001) compared to control samples. However, pre-treatment with curcumin effectively prevented the increase in IMA levels significantly. Exposure to •OH radicals increased the degree of MDA formation as compared to the control (p<0.001), pre-treatment with curcumin significantly prevented MDA production [Table/Fig-1,3]. After oxidative stress, the intracellular concentration of protein carbonyls increased when compared with that of the control (p<0.001). Curcumin significantly decreased protein carbonyls content [Table/Fig-1,4]. Hydroxyl radicals significantly decreased the levels of blood GSH and total antioxidant capacity of plasma compared with the control (p<0.001). Curcumin significantly increased the levels of both GSH levels and total antioxidant capacity of plasma [Table/Fig-1,5,6]. The extent of damage to DNA assessed by the relative electrophoretic mobility of the oxidized and curcumin-treated DNAs on 1% agarose gel as compared to native DNA. Lane-2 which is a Fenton's reagent treated sample showed a maximum fragmentation of DNA as compared to other lanes [Table/Fig-7]. The DNA bands in the 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> lanes corresponding to the band of native DNA with maximum intensity was considered in determining the protection offered by curcumin.



**[Table/Fig-6]:** Effects of curcumin on the total anti oxidant capacity of plasma exposed with hydroxyl radical. The data represent the mean±S.D of three replicates



**[Table/Fig-7]:** Effect of curcumin on hydroxyl radical induced DNA fragmentation. Lane 1: DNA. Lane 2: DNA + Fenton's reagent (DNA damage control). Lane 3: DNA + Fenton's reagent + Curcumin (50 µmole). Lane 4: DNA + Fenton's reagent + Curcumin (100 µmole) Lane 5: DNA + Fenton's reagent + Curcumin (200 µmole)

Curcumin substantially diminished the DNA damage as evidenced by the intensity determination, and displays almost an equal pattern of DNA protection with higher DNA protecting ability.

## DISCUSSION

Reactive oxygen species (ROS) can cause oxidative damage all types of biomolecules, lipids, proteins and DNA which could be deleterious and concomitant [28]. According to Marx G and Chevion M [29], albumin, the predominant protein in circulation, undergoes site-specific modification in N-terminus, especially of the N Asp-Ala-His-Lys sequence during overproduction of ROS resulting from conditions related to ischemia, hypoxia, acidosis, free radicals [30–33] is unable to bind transition metals like, cobalt [31]. This is the basic mechanism to detect the ischemia modified albumin. Though, the name of the test is ischemia modified albumin (IMA) test, apart from ischemia, levels of IMA elevates in generalized rather than organ- or tissue-specific state of oxidative stress [34]. Our study showed that •OH generation led a rise of IMA concentrations [Table/Fig-1,2]. This is in agreement with the study of Roy D et al., [33], who also found the increase of IMA concentrations with the rise of •OH radical. In our study, curcumin showed significant reduction in the formation of IMA with dose dependent manner [Table/Fig-1, 2].

Lipid peroxidation is a well-established mechanism of oxidative damage caused by ROS, and the measurement of the MDA provides a convenient index of lipid peroxidation [35]. This study provides evidence of an increase MDA by hydroxyl radical induced lipid peroxidation. There was a significant decrease in MDA concentration in the curcumin treated samples due to antioxidative effect of curcumin. Our study data is in accordance with the studies of Eybl V et al., [36], Mahfouz MM et al., [37], Manju M et al., [38] showing significant antioxidant effect of curcumin on lipid peroxidation.

The process of protein oxidation introduces new functional groups such as hydroxyls and carbonyls group, which were generated by many different mechanisms, this can indicate the degree of ROS mediated protein oxidation contribute to altered function and turnover. The outcomes of protein oxidation are fragmentation, cross-linking and unfolding. This may accelerate or hinder proteolytic and proteasome-mediated turnover, according to the severity of oxidative damage [39,40]. In the present study, protein oxidation was significantly inhibited by curcumin. This is in good agreement with the reports of Dkhar P et al., [41], who stated the effective antioxidant effect of curcumin on age induced oxidative stress in mice cerebral hemispheres and Kolodziejczyk J et al., [42], who illustrated the peroxynitrite (ONOO<sup>-</sup>) induced oxidative stress in human blood platelets and plasma.

ROS can rapidly be removed by non-enzymatic and enzymatic antioxidants that prevent their harmful effects and maintain a prooxidant/oxidant balance [43]. Glutathione (GSH) is a multifunctional intracellular non-enzymatic antioxidant. It is considered to be the major thiol-disulphide redox buffer of the cell. GSH protects cells against apoptosis, the protective role originates from multifactorial mechanisms that involve detoxification and modulation of cellular redox state and the subsequent redox-sensitive cell-signalling pathways and interaction with pro- and anti-apoptotic signals [44]. The current study demonstrated that curcumin maintained GSH content, which was depressed following hydroxyl radical exposure [Table/Fig-1,5]. This was in agreement with Biswas SK et al., [45], who stated that curcumin can induce glutathione synthesis and increase the level of GSH and glutamylcysteine ligase catalytic subunit mRNA expression in oxidant and TNF  $\alpha$  treated cells. The results in this study indicates that, curcumin may have profound effects on cellular redox control and similar to the studies of Eybl V et al., [36] and Sehgal A et al., [46].

In our study, the total anti oxidant capacity of plasma exposed to hydroxyl radicals was evaluated by FRAP Assay. Principally, FRAP assay treats the antioxidants in the sample as reductant in a redox linked colorimetric reaction. Antioxidant compounds which act as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus break the radical chain reaction [24]. The results showed the total anti-oxidant capacity of plasma exposed to hydroxyl radical increased when treated with curcumin in 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> groups [Table/Fig-1,6]. This is in accordance with the studies of Weber WM et al., [47] and Bhullar KS et al., [48], who also estimated the total anti oxidant capacity of curcumin and its analogs.

It is stated that, hydroxyl radicals generates as result of Fenton chemistry, cause oxidative damage which includes single and double strand breaks in DNA, base modification, cross linking of DNA with proteins and fragmentation of genomic DNA. So, tissue uncovered to hydroxyl radicals may lead to cancer, cardiovascular diseases, cataract and neurological disorders [49]. DNA fragmentation has been utilized as a characteristic feature of apoptosis [50]. H<sub>2</sub>O<sub>2</sub>/Cu<sub>2</sub><sup>+</sup>/ascorbic acid system produced very extensive base modification in DNA. Thus there is a greater ability of Cu<sub>2</sub><sup>+</sup> ions, as compared with Fe<sup>3+</sup> ions, to promote DNA damage via oxygen- derived species [51]. Cu<sub>2</sub><sup>+</sup> ions bind to the DNA and cause damage by generating OH in site-specific reactions [52]. In our study, curcumin prevented the fragmentation of DNA by quenching the hydroxyl radicals efficiently in 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> groups [Table/Fig-7]. Similar studies were

conducted by Ryu Ar et al., [53], Madkour NK [54] Zhu YG et al., [55], Chan WH et al., [56], who stated that curcumin can also inhibit the oxidative DNA damage by Chrysotile, Lambda cyhalothrin, tert-butyl hydroperoxide (t-BHP) and Methylglyoxal respectively.

Curcumin is one of the few anti-oxidants that possess both phenolic hydroxy and  $\beta$ -diketone groups in one molecule. Its unique conjugated structure includes two phenols and an enol form of a  $\beta$ -diketone. Antioxidant mechanisms of curcumin have been studied by laser flash photolysis and pulse radiolysis, and it was found that the keto-enol-enolate equilibrium of the heptadienone moiety of curcumin determined its physicochemical and antioxidant properties [57,58]. Hence overall antioxidant effect of curcumin on the oxidative stress induced by aerobic metabolism has proved to be effective.

## CONCLUSION

The present study suggested that curcumin has the ability to protect the biomolecules like proteins, lipids and DNA from hydroxyl radical oxidation. These results demonstrate that exposure to  $\bullet$ OH in serum and plasma is a good experimental model for studying ROS-induced toxicity and evaluating the protective effects of various agents. Since this study was done in invitro, in future in vivo studies are needed to establish the  $\bullet$ OH radical scavenging potential of curcumin.

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