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KINETIC AND MECHANISTIC STUDIES OF OXIDATION OF SOME BIOLOGICALLY IMPORTANT MOLECULES WITH PHOTOCHEMICALLY GENERATED TERTIARY BUTOXYL RADICALS

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Abstract

Reactive Oxygen species (ROS) are produced as byproduct of normal cellular processes, exposure to UV radiation and in the presence of transition metal ions. Metabolic degradation of endogenous and exogenous peroxides is thought to play a key role in the promotion of several diseases including cancer. Chemical, biochemical, clinical and epidemiological evidence had supported that antioxidants play an important role in the prevention of several chronic diseases including cardiovascular diseases, cancer, ageing and diabetes. In order to understand the mechanism of oxidation of antioxidants viz., α-tocopherol, β-carotene, folic acid and curcumin by photochemically generated *tert*-butoxyl radicals, a systematic kinetic study of these antioxidants with *tert*-butoxyl radicals is carried out. The oxidation of antioxidants by *t*-BuO$^{\cdot}$ radicals have been followed spectrophotometrically by measuring the absorbance of antioxidant at 294 nm, 451 nm, 284 nm and 428 nm for α-tocopherol, β-carotene, folic acid and curcumin respectively. The initial rates of oxidation of antioxidants were calculated from the plot of absorbance *vs* time. The initial rates of oxidation of antioxidants have been found to increase with increase in [antioxidant], [*t*-BuOOH] and light intensity in all cases. The quantum yields (ϕ) were calculated from the initial rates of oxidation of antioxidant and the measured light intensity at 254 nm, the wavelength at which the *tert*-butyl hydroperoxide (*t*-BuOOH) is activated to radicals. The quantum yields were found to depend on [antioxidant] and [*t*-BuOOH] while they were independent of light intensity. The order with respect to [antioxidant], [*t*-BuOOH] were found to be fractional whereas order with respect to intensity is one. The order of reactivity was found to be: α-tocopherol > curcumin > β-carotene > folic acid. The products were identified by Mass Spectrometric analysis. On the basis of kinetic results and product analysis, probable mechanisms were suggested.

Key words: antioxidants, *tert*-butoxyl radicals, α-tocopherol, β-carotene, folic acid and curcumin

Introduction

Organic peroxides form an important part of various chemical, pharmaceutical and cosmetic products. Reactive free radicals are continuously produced in living cells during respiration, metabolism and phagocytosis inducing multiple changes in lipids, proteins, etc., leading to cell death. DNA is one of the main molecular targets of toxic effects of free radicals formed in mammalian cells [1, 2]. These peroxides on photolysis produce alkoxyl and hydroxyl radicals. Although lethal effects of the hydroxyl radicals on DNA and its constituents have been studied extensively, relatively little is known about the biological effects of alkoxyl radicals and the key cellular targets for these species. *tert*-butyl

hydroperoxide (*t*-BuOOH) has been chosen as model peroxide to elucidate the mechanisms of damage caused by alkoxyl radicals to nucleic acid constituents because it is stable and available in pure form. Previous studies on the reactivity of tertiary butoxyl radicals suggest that these species might be expected to attack both the sugar and the base moieties of DNA. The experimental evidence indicates that base radicals also contribute to strand breaks by transfer of their radical sites from base moiety to sugar moiety.

Multitude of studies have implicated reactive free radicals and lipid peroxidation in aging and various diseases including cancer, arthritis, neurological damage, diabetic cataract, Parkinson's disease, etc [3, 4]. Antioxidants, in small quantities, significantly prevent the oxidation of biomolecules viz., proteins, nucleic acids, lipids, amino acids, etc., by interfering with the chain reaction of peroxidation and/or by scavenging reactive oxygen radicals thus minimizing the toxic effects of oxidative stress. The epidemiological studies have indicated that regular consumption of certain vegetables, fruits [5, 6] and spices are known to contain cancer chemopreventive agents. They are considered safe as they are derived from natural sources that humans use regularly as an integral part of their diet. The pharmacological and physiological functions of the dietary antioxidants have been demonstrated with regard to their ability to scavenge radicals by hydrogen atom donation/electron transfer, inhibition of oxidative enzymes and chelation of metal ions, protection of low density lipoprotein (LDL) from peroxidation as well as their effects on cell signaling pathways and gene expression [7]. In this context, search for novel, efficient and effective dietary antioxidants aimed at providing defense against free radical induced oxidative stress in diverse conditions has gained lot of significance. Our studies involving such antioxidants assumed importance due to their presence in many dietary phytochemicals in higher concentrations.

The objective of the present work was to find out suitable naturally occurring diet antioxidants and study the potency of these compounds towards scavenging of photochemically generated alkoxyl radicals from *t*-BuOOH. Such studies provide information about the mechanisms of oxidation antioxidants by alkoxyl radicals. Further, these studies can be extended to understand the protection and repair of nucleic acid constituents by diet antioxidants in presence of alkoxyl radicals. Keeping this

in view a systematic kinetic study of oxidation of few dietary antioxidants by photochemically generated *t*-BuO$^{\bullet}$ radicals has been undertaken under a variety of experimental conditions.

Materials and Methods

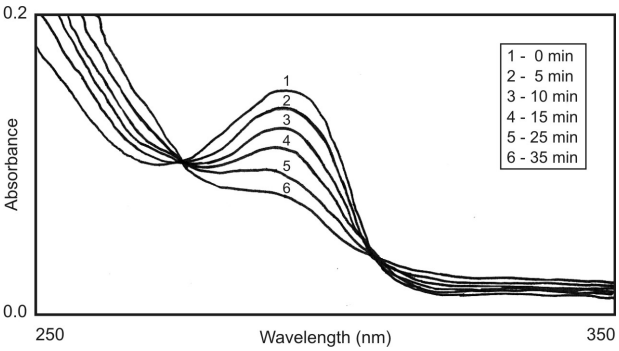
α-tocopherol, curcumin, β-carotene and folic acid were purchased from Sigma Chemical Co., St. Louis, USA and used as received. All solutions were prepared afresh using double-distilled water. *tert*-butyl hydroperoxide (*t*-BuOOH) was used as received from Merck-Schuchardt of Germany. There was no contamination of other peroxides in the assay of the sample. *t*-BuOOH was estimated by iodometric method [8].

The irradiations were carried out at room temperature in a quantum yield reactor model QYR-20 supplied by Photophysics, England, attached with 400 W medium pressure mercury lamp. The quartz cuvette containing the sample was irradiated and the irradiations were interrupted at definite intervals of time and the absorbance was noted. The light intensity corresponding to the irradiating wavelength (254 nm) was measured using peroxydisulphate chemical actinometry [9]. On photolysis, *t*-BuOOH was activated at 254 nm to generate $^{\bullet}$OH and *t*-BuO$^{\bullet}$ radicals by homolytic cleavage of –O-O-bond [10]. The $^{\bullet}$OH radicals produced were scavenged using sufficient concentration of *t*-BuOH [11].

In a typical kinetic run, the reaction mixture of antioxidant and *t*-BuOOH were taken in a specially designed 1 cm path length quartz cuvette, suitable for both irradiations and absorbance measurements. The reaction was followed by measuring the absorbance at the λ_{max} of antioxidant on a Chemito (model 2100) UV-Visible spectrophotometer (Fig. 1 - 4). The initial rates of photooxidation of antioxidant by *t*-BuOOH in presence of *t*-BuOH were calculated from the plots of absorbance of antioxidant *vs* time using Microcal origin (version 6.0) computer program on a personal computer. The quantum yields were calculated from initial rates of oxidation of antioxidant and measured light intensity at 254 nm, wavelength at which *t*-BuOOH was activated to give radicals.

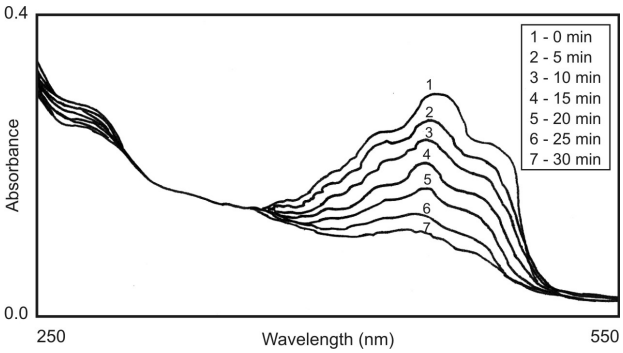
The reaction mixture containing antioxidant, *t*-BuOOH and *t*-BuOH in suitable concentrations in bulk (100 ml) was taken in a quartz RB flask. The solutions were irradiated in the photochemical reactor which was of Srinivasan Griffin Rayonet type, containing four 18 W

Fig. 1



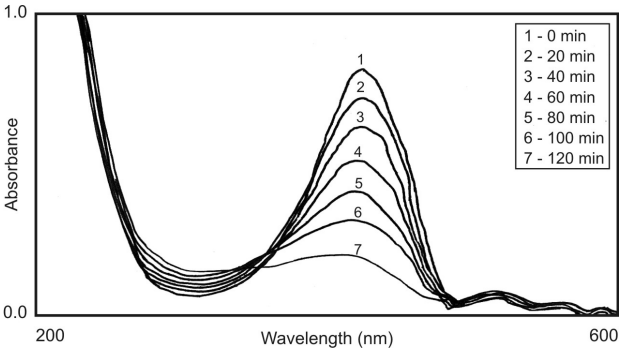
Absorption spectra of photooxidation of α-tocopherol in the presence of *tert*-butyl hydroperoxide at different irradiation times; [α-tocopherol] = 5×10^{-5} moldm^{-3}, [*t*-BuOOH] = 5×10^{-3} mol dm^{-3}, Light intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 294 nm, pH ~ 7.5, temperature = 298 K, [*t*-BuOH] = 1.0 M

Fig. 2



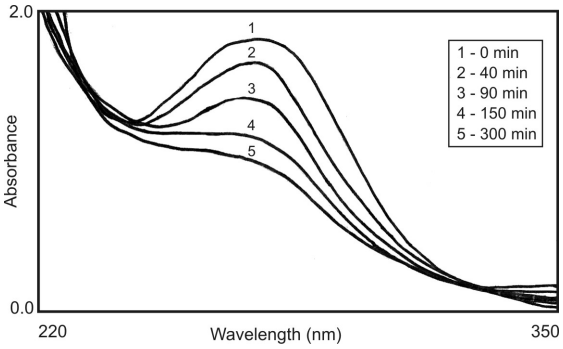
Absorption spectra of photooxidation of β-carotene in the presence of tert-butyl hydroperoxide at different irradiation times; [β-carotene] = 5×10^{-5} mol dm^{-3}, [*t*-BuOOH] = 5×10^{-3} mol dm^{-3}, Light Intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 451 nm, pH ~ 7.5, temperature = 298 K, [*t*-BuOH] = 1.0 M,

Fig. 3



Absorption spectra of photooxidation of curcumin in the presence of tert-butyl hydroperoxide at different irradiation times; [curcumin] = 1×10^{-5} mol dm^{-3}, [*t*-BuOOH] = 5×10^{-3} mol dm^{-3}, Light Intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 428 nm, pH ~ 7.5, temperature = 298 K, [*t*-BuOH] = 1.0 M

Fig. 4



Absorption spectra of photooxidation of folic acid in the presence of tert-butyl hydroperoxide at different irradiation times; [folic acid] = 2×10^{-5} mol dm^{-3}, [*t*-BuOOH] = 5×10^{-3} mol dm^{-3}, Light Intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 284 nm, pH ~ 7.5, temperature = 298 K, [*t*-BuOH] = 1.0 M

medium pressure mercury lamps arranged in a circular way was used specially for product analysis The RB Quartz flask containing the reaction mixture was placed in the middle of the well of the reactor using an external support and the flask was exposed to the light from all the sides making the irradiations uniform through out the bulk of the solution. After irradiation, the sample was subjected to Mass Spectrometric analysis in both positive ion and negative ion modes. Mass spectrum of each sample was recorded using EI mode at 70 eV in Shimadzu QP 1100EX EI

Quadrupole mass. Source, pulse and scanning temperature were 200 V, 100-300 ms and 25 ^{o}C respectively.

Results and Discussion

The photooxidation of antioxidant by *t*-BuO$^{\bullet}$ radicals were measured under different experimental conditions. The initial rates of oxidation of antioxidant by *t*-BuO$^{\bullet}$ were found to increase with increase in [antioxidant], [*t*-BuOOH] and light intensity (Tables 1 & 2).

Table 1: Effect of [*t*-BuOOH] and [Antioxidant] on the rates of oxidation of antioxidant by *t*-BuO$^{\cdot}$ in aqueous neutral medium

$10^3\times$ [*t*-BuOOH] mol dm^{-3}	$10^5\times$ [Antioxidant] mol dm^{-3}	Rate $\times 10^9$ dm^{3}mol^{-1}s^{-1}			
		α-tocopherol	β-carotene	Curcumin	Folic acid
5.0	0.2	-	-	0.322	0.094
5.0	0.4	-	-	0.481	0.184
5.0	0.5	0.687	0.316	-	-
5.0	0.6	-	-	0.585	0.236
5.0	0.8	1.656	0.478	0.841	-
5.0	1.0	3.673	0.532	0.994	0.403
5.0	2.0	11.627	1.057	-	0.848
5.0	5.0	33.691	2.500	-	1.659
5.0	8.0	63.357	4.836	-	-
5.0	10.0	69.840	6.790	-	-
10.0	1.0	-	-	2.338	0.851
10.0	5.0	39.130	5.825	-	3.318
15.0	1.0	-	-	3.557	1.559
15.0	5.0	48.750	8.024	-	6.238

[*t*-BuOH] = 1.0 mol dm^{-3}, light intensity = 2.7168 $\times$ 10^{15} quanta s^{-1}, pH ~ 7.5, Temperature = 298 K

The plots of log (rate) versus log[antioxidant] or log[t-BuOOH] were linear and the order with respect to [antioxidant] or [*t*-BuOOH] were found to be fractional and order with respect to light intensity was found to be one.

Table 2: Effect of Light intensity on the rates of oxidation of antioxidant by *t*-BuO$^{\bullet}$ in aqueous neutral medium

Light intensity $\times 10^{15}$ quanta s^{-1}	Rate $\times 10^{9}$ $dm^{3}mol^{-1}s^{-1}$			
	α-tocopherol	β-carotene	Curcumin	Folic acid
2.7168	33.691	2.500	0.9945	1.659
1.8317	20.498	0.267	0.6667	1.120
1.0623	11.927	0.112	0.3961	0.597

[*t*-BuOH] = 1.0 mol dm^{-3}, pH ~ 7.5, Temperature = 298 K, [*t*-BuOOH] = 5.0×10^{-3} mol dm^{-3}, [Antioxidant] = 5.0×10^{-5} mol dm^{-3}

Absorbance measurements have indicated that all the antioxidants absorbed substantially at 254 nm. Hence, in the present reaction system, antioxidant and *t*-BuOOH compete for the absorption of light at 254 nm. In the absence of *t*-BuOOH, antioxidant did not undergo any observable chemical change upon shining the light. The rates of oxidation of antioxidants by *t*-BuO$^{\bullet}$ were found to increase with increase in [antioxidant] (Table 1). These facts indicate that antioxidants act as photosensitizers transferring the absorbed energy to *t*-BuOOH in a fast step to activate it to generate t-BuO$^{\bullet}$ and $^{\bullet}$OH radicals. The quantum yields were calculated from the initial rates of oxidation of antioxidant and calculated light intensity absorbed by *t*-BuOOH at 254 nm. The quantum yields of oxidation of antioxidants were found to depend on [antioxidant] and [*t*-BuOOH] but independent of light intensity (Tables 3 & 4).

The dietary bioactive compounds such as α-tocopherol, β-carotene, folic acid and curcumin are of particular interest because of their potential biological properties as anti-inflammatory, antiallergic, antimicrobial, antiviral, anticarcinogenic, UV filter properties, etc [12-17]. The antioxidant activities of these compounds are related to their molecular structure, the presence of number of OH groups, bond dissociation energy between oxygen and a phenolic hydrogen, reduction potentials, pH, conjugation and resonance effects in particular. They scavenge the free radicals of foods by donating phenolic hydrogens to them. They produce relatively stable antioxidant radicals with low standard reduction potential less than 500mV due to resonance delocalization throughout the phenolic ring structure [18].

Table 3: Effect of [*t*-BuOOH] and [Antioxidant] on the quantum yields of oxidation of antioxidant by *t*-BuO$^{\cdot}$ in aqueous neutral medium

$10^3\times$ [*t*-BuOOH] mol dm^{-3}	$10^5\times$ [Antioxidant] mol dm^{-3}	Quantum yield (ϕ)			
		α-tocopherol	β-carotene	Curcumin	Folic acid
5.0	0.2	-	-	0.00021	0.00006
5.0	0.4	-	-	0.00032	0.00012
5.0	0.5	0.00045	0.00210	-	-
5.0	0.6	-	-	0.00039	0.00016
5.0	0.8	0.00110	0.00318	0.00056	-
5.0	1.0	0.00244	0.00354	0.00066	0.00027
5.0	2.0	0.00773	0.00703	-	0.00057
5.0	5.0	0.02240	0.01662	-	0.00110
5.0	8.0	0.04213	0.03216	-	-
5.0	10.0	0.04645	0.04516	-	-
10.0	1.0	-	-	0.00155	0.00056
10.0	5.0	0.02602	0.03874	-	0.00221
15.0	1.0	-	-	0.00236	0.00104
15.0	5.0	0.03242	0.05340	-	0.00415

[*t*-BuOH] = 1.0 mol dm^{-3}, Light intensity = 2.7168×10^{15} quanta s^{-1}, pH ~ 7.5, Temperature = 298 K

Table 4: Effect of Light Intensity on the quantum yields of oxidation of antioxidant by *t*-BuO$^{\cdot}$ in aqueous neutral medium

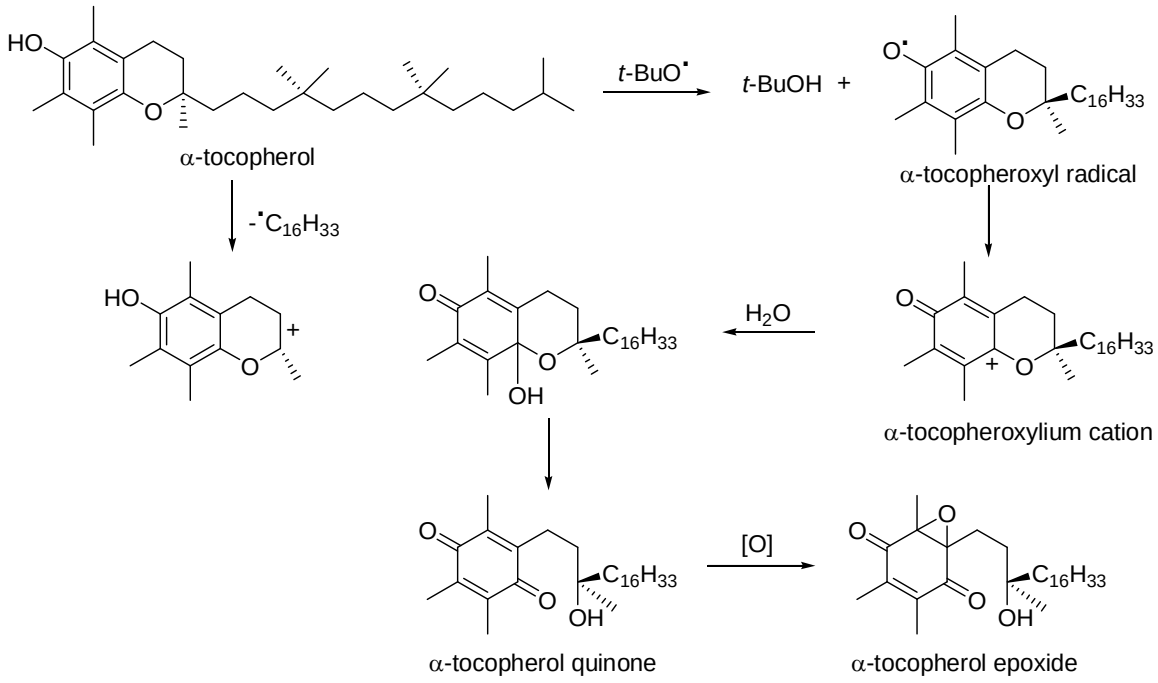
Light intensity $\times 10^{15}$ quanta s^{-1}	Quantum yield (ϕ)			
	α-tocopherol	β-carotene	Curcumin	Folic acid
2.7168	0.0224	0.0166	0.00066	0.00110
1.8317	0.0202	0.0177	0.00066	0.00112
1.0623	0.0203	0.0174	0.00067	0.00101

[*t*-BuOH] = 1.0 mol dm^{-3}, pH ~ 7.5, Temperature = 298 K, [*t*-BuOOH] = 5.0×10^{-3} mol dm^{-3}, [Antioxidant] = 5.0×10^5 mol dm^{-3}

α-tocopherol is one of the four structurally related compounds. It is a lipid soluble vitamin, which has the highest bioavailability. It is an important dietary nutrient for humans and animals synthesized only by plants. Structurally, α-tocopherol consists of a chroman head with two rings (phenolic and heterocyclic) and a phytyl tail. It can scavenge the reactive species and therefore is thought to have an important

function in prevention of degenerative diseases. The antioxidant activity of α-tocopherol [19] is due to its ability to donate the phenolic hydrogens to lipid free radicals. In the first step, a resonance stabilized phenoxyl radical is formed by donation of phenolic hydrogens to lipid peroxy radical, which scavenges another peroxy radical to give a non-radical stable product (Scheme 1).

Scheme 1



Carotenoid pigments are widely distributed in nature and play an important role in protecting cells and organisms against photosensitized oxidations. β-carotene is the major fat soluble carotenoid and play an important role in the oxidation of fats and oils. It is considered as a biological antioxidant and has antioxidant properties in vitro and in animal models. It is a polyenoic terpenoid (two cyclohexene type end groups) having conjugated trans double bonds. The antioxidant activity of carotenoids is based on their singlet oxygen quenching properties and their ability to scavenge peroxyl or alkoxyl radicals. The quenching ability of carotenoid mainly depends on the number of conjugated double bonds present in the molecule and is influenced to a lesser extent by carotenoid end groups (cyclic or acyclic) or the nature of substitutents in carotenoids containing cyclic end groups. β-carotene [20] was

found to interact with peroxyl radicals via an unstable β-carotene radical adduct which is highly resonance stabilized and are predicted to be relatively unreactive. They may further undergo decay to generate non-radical products and may terminate radical reactions by binding to the attacking free radicals. In this process, β-carotene molecules are destroyed.

Curcumin is isolated from the rhizomes of *Curcuma longa* shows wide range of pharmacological activity including anti-inflammatory and anticancer activity. It has a 1,3-diketone system (heptadiene-dione) with a styryl ketone with phenolic group at para position and methoxy group at ortho position to phenolic group.

Folic acid is a water soluble vitamin which shows protective effect on the pathogenesis of cardiovascular, hematological and neurological diseases and cancer. These beneficial effects have been associated with the antioxidant activity of folic acid. Joshi et al [17] gave the first evidence for the possible role of folic acid as an antioxidant. The presence of phenolic group on top of α, β-unsaturated functionality makes it an efficient antioxidant.

α-tocopherol was found to have the highest scavenging activity of *t*-BuO$^{\bullet}$ radicals of the bioactive molecules studied in the present work. α-tocopherol [19] is structurally expected to be more potent as a hydrogen donor than other antioxidants studied due to the presence of i) electron releasing methyl substituents in ortho and/or para position to the hydroxyl function and ii) stereoelectronic effects. The presence of methyl groups increases the electron density at the active centers facilitating the release of hydrogen atom of phenolic group thus increasing the reactivity towards the *t*-BuO$^{\bullet}$ radicals. Based on the stereoelectronic effects, the excellent antioxidant properties of α-tocopherol is due to the presence of fused heterocyclic ring in the chroman moiety with an oxygen atom para to the phenolic function. In this heterocyclic ring, the p-type lone electron pair of oxygen is perpendicular to aromatic plane. This p-type lone pair orbital overlaps with the semi-occupied molecular orbitals of the phenoxyl radicals by conjugative electron delocalization increasing the antioxidant activity of α-tocopherol.

Curcumin was found to have next highest scavenging activity of *t*-BuO$^{\bullet}$ radicals. Free radical scavenging activity of curcumin arises from the phenolic OH group or from the CH_2 group of the β-diketone moiety. A reactive free radical can undergo electron transfer or abstract H-atom from either of

these two sites. Jovanoic et al [21] suggested that the keto-enol-enolate equilibrium of the heptadienone moiety of curcumin determines its antioxidant properties. In acidic and neutral pH, the keto form dominates and curcumin acts as an extraordinarily potent H-atom donor. The H atom donation occurs from the central CH_2 group in the heptadienone link as C-H bonds in this group are very weak due to delocalization of the unpaired electrons on the adjacent oxygen. On the basis of experimental and theoretical studies on the structure and stability of curcumin, it was suggested that the phenolic OH group is responsible for the activity of curcumin. The free radical scavenging activity of curcumin involves radical trapping at the phenolic position as in the case of other phenolic antioxidants.

β-carotene which has lipid-soluble antioxidant activity was found to be less effective antioxidant than α-tocopherol in homogeneous lipid solutions, in membrane models and also in intact cells [22, 23]. In our system also, β-carotene is found to have less reactive towards *t*-$BuO^{\bullet}$ radicals than α-tocopherol. β-carotene may give electrons and then donate hydrogen to *t*-$BuO^{\bullet}$ radicals and produces carotene radical. Ease of electron donation of carotenoids depends on the nature of substitutents on the carotenoids. Reduction potentials of alkoxyl radical and carotene radical cation were reported to be 1600 mV and 780 mV respectively which indicates that carotene radical cation easily donate hydrogen to alkoxyl radicals [20]. Carotene radical is a fairly stable species due to delocalization of unpaired electrons in its conjugated polyene. These radicals have enough life time to react with *t*-$BuO^{\bullet}$ radicals even at low oxygen concentrations and forms carotene epoxides. The possible extent of resonance stabilization and presence of 11 conjugate bonds in β-carotene makes it less efficient antioxidant than α-tocopherol and curcumin.

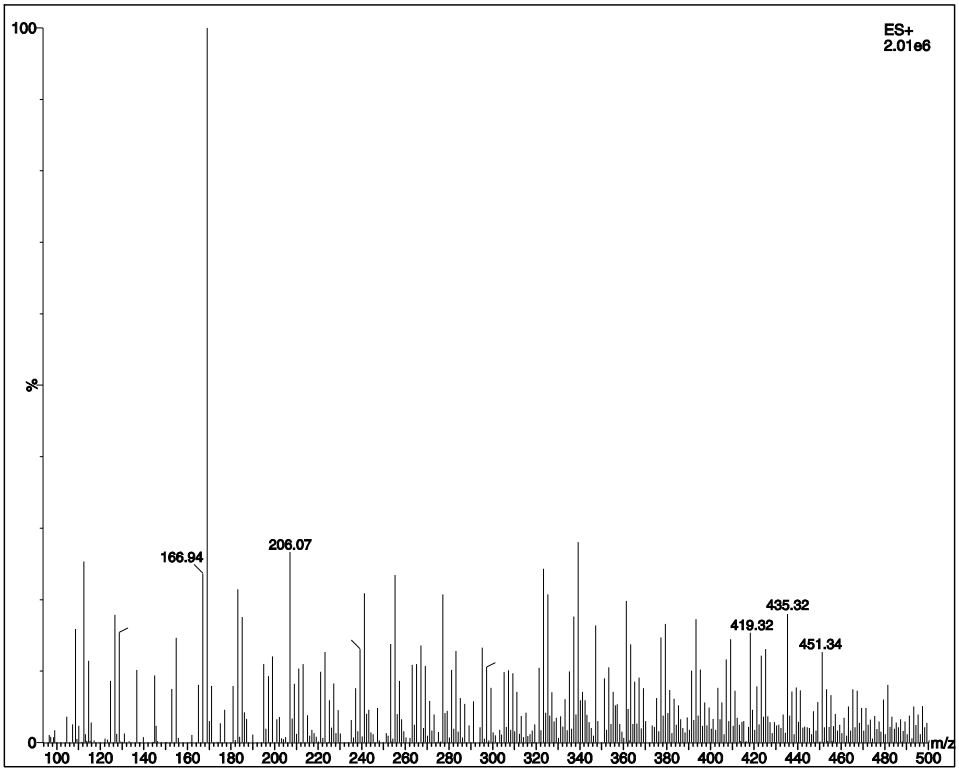
In our study, folic acid was found to have the least antioxidant activity compared to all the dietary antioxidants studied. This may due to fact that folic acid is a sensitive compound and is inactivated by UV light. pH of the solution was found to have interesting effect on folic acid in the presence of light. In the acidic medium (pH 2.0 – 4.0), folic acid [24] is present predominantly in protonated form and is found to undergo photolysis easily. At neutral pH, the molecule becomes deprotonated and a gradual decrease in photolytic action is folic acid is observed.

The neutral species was found to less susceptible to photolysis.

These antioxidants upon oxidation by *t*-BuO$^{\bullet}$ radicals produced different products based on their structure and substituents present. In the presence of *t*-BuO$^{\bullet}$ radicals, α-tocopherol is first oxidized to α-tocopheroxyl radical (chroman 6-oxyl radical) due to the donation of phenolic hydrogen to *t*-BuO$^{\bullet}$ radicals. Evidence for the formation of resonance stabilized α-tocopheroxyl radical is available from ESR and electron nuclear double resonance studies [25, 26]. α-tocopheroxyl radical then donates an electron to another radical to form the α-tocopheroxylium cation. It then reacts with either water to give α-tocopherol quinone or with oxygen to give epoxide as the final products [19] (Scheme 1). In our system, α-tocopherol quinone (m/z = 435.32) and epoxide (m/z = 419.29) were detected in the positive ion mode mass spectrum. A peak at m/z = 206.07 indicated the loss of phytyl tail from the α-tocopherol molecule (Fig. 5).

In case of curcumin, photochemically generated *t*-BuO$^{\bullet}$ radicals abstract phenolic hydrogen atom [27] to give oxygen centered radical which tautomerises to give carbon centered radicals having a quinone methide structure. In the keto form of curcumin, heptadienone linkage between the two methoxy phenol rings contains a highly activated carbon atom. It is obvious that the C-H bonds on this carbon should be very weak, due to delocalization of the unpaired electron on the adjacent oxygen, the radical formed by hydrogen atom donation may take the following resonance structures. The curcumin radical is formed as a transient by the hydrogen atom donation from the $-CH_2-$ group of curcumin to *t*-BuO$^{\bullet}$ radicals. The curcumin radicals further combines leading to the formation of dimer. The driving force for the formation of the dimer is presence of phenolic H-atom at para-position. The above proposed reaction scheme receives support from spectroscopic data. The UV-visible spectrum of the product dimer is similar to the one reported by Masuda *et al* [28]. Formation of curcumin dimer is further supported by mass spectral data with m/z value = 734.31 in the positive ion mode.

Fig. 5

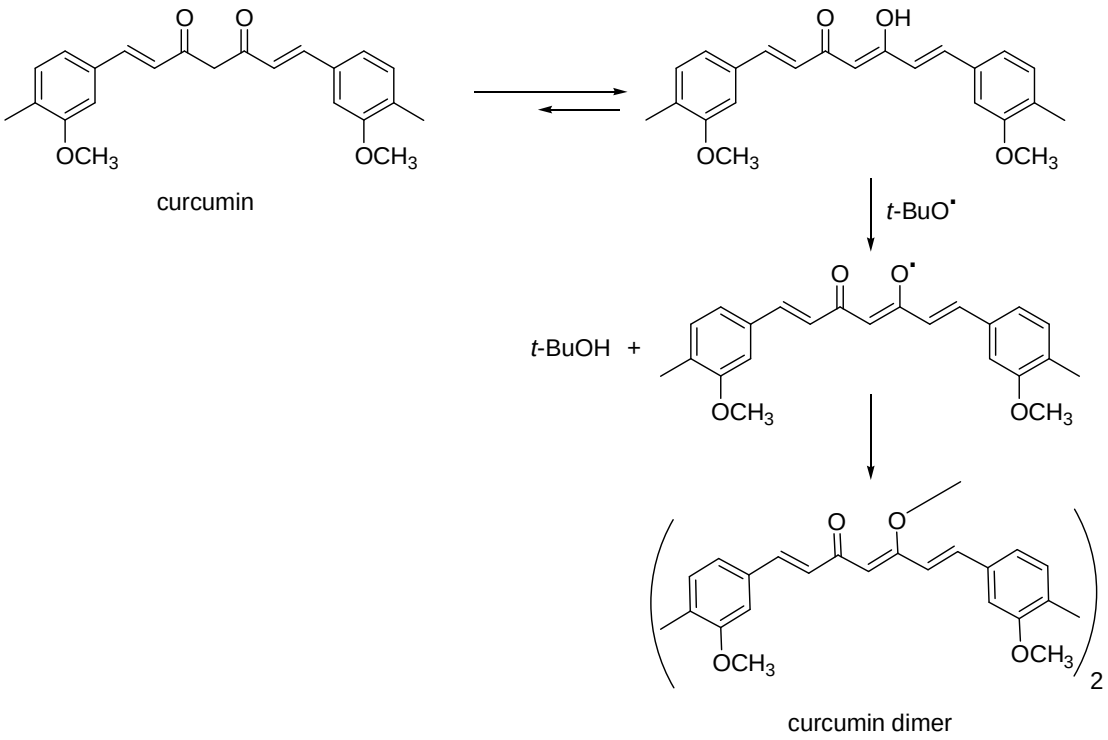


Mass Spectrum of product of α-tocopherol

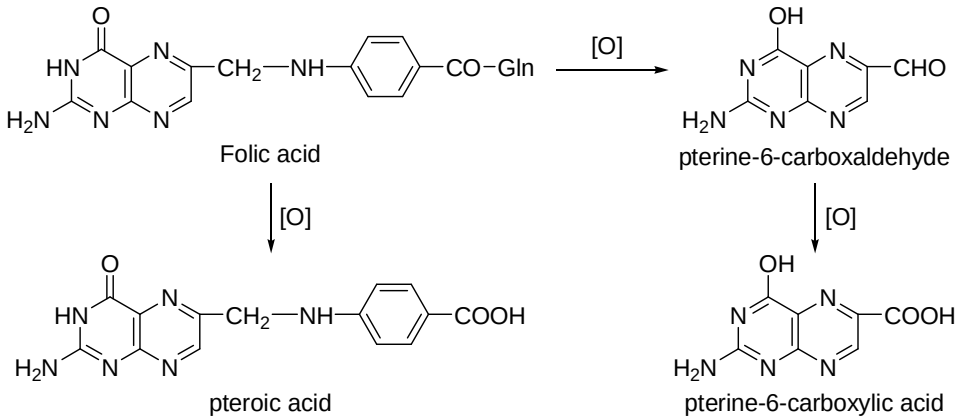
The oxidation products of β-carotene were identified as β-ionone, β-apo-14'-carotenal, β-apo-10'-carotenal, β-apo-8'-carotenal and β-carotene-5,8-endoperoxide [29]. In our system, upon oxidation with *t* -BuO$^{\bullet}$ radicals produced epoxide [29] via carotene radical cation and was detected in the positive ion mode mass spectrum with m/z = 552.89 with β-ionone as the major peak (m/z = 193.65.

In the presence of t-BuO$^{\bullet}$ radicals, folic acid was first converted to pterine-6-carboxaldehyde and a diazotisable p-aminobenzoyl-L-glutamic acid [30]. Further, it was converted to pterine-6-carboxylic acid and finally to pteroic acid. In the negative ion mode, oxidation products of folic acid were observed at pterine-6-carboxadehyde (m/z = 190.17), pterine-6-carboxylic acid (m/z = 190.17) and pteroic acid (m/z = 311.29) in the mass spectrum.

Scheme 2



Scheme 3



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