Indian Journal of Biochemistry & Biophysics
Vol. 46, October 2009, pp. 389-394

Kinetics of oxidation of adenosine by *tert*-butoxyl radicals: Protection and repair by chlorogenic acid

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Received 16 July 2008; revised 10 August 2009

The rates of oxidation of adenosine and chlorogenic acid by *tert*-butoxyl radicals (*t*-BuO$^{\cdot}$) were studied by measuring the absorbance of adenosine at 260 nm and chlorogenic acid at 328 nm spectrophotometrically. *t*-BuO$^{\cdot}$ radicals were generated by the photolysis of *tert*-butyl hydroperoxide (*t*-BuOOH) in presence of tert-butyl alcohol to scavenge OH$^{\cdot}$ radicals. The rates and the quantum yields (ϕ) of oxidation of chlorogenic acid by *t*-BuO$^{\cdot}$radicals were determined in the absence and presence of varying concentrations of adenosine. An increase in the concentration of adenosine was found to decrease the rate of oxidation of chlorogenic acid, suggesting that adenosine and chlorogenic acid competed for *t*-BuO$^{\cdot}$ radicals. From competition kinetics, the rate constant of chlorogenic acid reaction with *t*-BuO$^{\cdot}$ was calculated to be 3.20×10^9 dm^3 mol^{-1} s^{-1}. The quantum yields (ϕ_{expt}) were calculated from the experimentally determined rates of oxidation of chlorogenic acid under different experimental conditions. Assuming that chlorogenic acid acts as a scavenger of *t*-BuO$^{\cdot}$ radicals only, the quantum yields (ϕ_{cal}) were theoretically calculated. ϕ_{expt} and ϕ_{cal} values suggested that chlorogenic acid not only protected adenosine from *t*-BuO$^{\cdot}$ radicals, but also repaired adenosine radicals, formed by the reaction of adenosine with *t*-BuO$^{\cdot}$ radicals.

Keywords: Chlorogenic acid, Adenosine repair, *t*-BuO$^{\cdot}$ radicals, Oxidation

Organic peroxides form an important part of various chemical, pharmaceutical and cosmetic products. These peroxides on photolysis produce alkoxyl and hydroxyl radicals. DNA is one of the main molecular targets of toxic effects of free radicals formed in mammalian cells during respiration, metabolism and phagocytosis. Although lethal effects of the hydroxyl radicals on DNA and its constituents have been studied[1] extensively, relatively little is known about the biological effects of alkoxyl radicals and the key cellular targets for these species. Protein[2], lipid[3], amino acid[4] and pyrimidines[5] hydroperoxides in DNA are rapidly decomposed when treated with light, heat and transition metal ions, resulting in formation of alkoxyl radicals.

Recent studies have demonstrated that the exposure of cultured cells to alkoxyl radicals results in the generation of DNA strand breaks[6-8], though the mechanism of damage has not been elucidated. Organic oxygen radicals, particularly alkoxyl radicals participate in metabolic and pathological processes[9]. 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[10]. The experimental evidence indicates that base radicals also contribute to strand breaks by transfer of their radical sites from base moiety to sugar moiety. Strand breaks are considered to be a very serious kind of damage to DNA[11,12].

Antioxidants in small quantities prevent the oxidation of cellular organelles by minimizing the toxic effects of oxidative stress. Antioxidants, such as phenolics are widely distributed in the plant kingdom and are, therefore, integral parts of the diet, with significant amounts being reported in fruits, vegetables and beverages[13]. The pharmacological actions of phenolic antioxidants stem mainly from their free radical scavenging and metal chelating properties as well as their effects on cell signaling pathways and gene expression[14]. From our laboratory, caffeic acid has been reported[15,16] to repair adenosine radicals, in addition to efficiently scavenging of $SO_4^{\cdot-}$ and *tert*-butoxyl (*t*-BuO$^{\cdot}$) radicals. In this context, studies involving chlorogenic acid assume importance, due to its presence in many dietary phytochemicals in higher concentrations.

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The *t*-BuO$^{\cdot}$ radicals have been generated by steady-state photolysis of *tert*-butyl hydroperoxide in the presence of *t*-BuOH to scavenge the hydroxyl radicals in aqueous solution[17]. In the present paper, the reactions of *t*-BuO$^{\cdot}$ radicals with adenosine have been studied in the presence of chlorogenic acid to assess the protection by chlorogenic acid towards oxidation of adenosine by *t*-BuO$^{\cdot}$ radicals and also repair, if any offered by chlorogenic acid towards adenosine radicals. Adenosine is used as a model for DNA to understand the protection and repair by chlorogenic acid from *t*-BuO$^{\cdot}$.

Materials and Methods

Adenosine and chlorogenic 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 is no contamination of other peroxides in the assay of the sample. *t*-BuOOH was estimated by iodometric method[18].

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[19]. On photolysis, *t*-BuOOH was activated at 254 nm to generate $^{\cdot}$OH and *t*-BuO$^{\cdot}$ radicals by homolytic cleavage of –O-O-bond[20]. The $^{\cdot}$OH radicals produced were scavenged using sufficient concentration of *t*-BuOH[17]. In a typical kinetic run, the aqueous reaction mixture of adenosine, *t*-BuOOH and *t*-BuOH was taken in a specially designed 1 cm path length quartz cuvette, suitable for both irradiations and absorbance measurements. The absorbance measurements were made at the λ_{max} of adenosine (260 nm) on a Chemito UV-Visible spectrophotometer (model 2100).

The photochemical reaction of chlorogenic acid in the presence of *t*-BuOOH and other additives viz., *t*-BuOH and adenosine was followed by measuring the absorbance of chlorogenic acid at 328 nm at which adenosine was totally transparent.

Results and Discussion

The reported[16] initial rates and quantum yields of oxidation of adenosine by *t*-BuO$^{\cdot}$ are presented in Table 1. The initial rates of photooxidation of chlorogenic acid by *t*-BuOOH in presence of *t*-BuOH were calculated from the plots of absorbance of chlorogenic acid at 328 nm *vs* time using microcal origin computer program on a personal computer (Table 2). UV-visible absorption spectra of chlorogenic acid in presence of *t*-BuOOH and *t*-BuOH at different irradiation times were recorded (Fig. 1).

The substrates used in the present work viz., chlorogenic acid and adenosine have strong absorption at 254 nm at which *t*-BuOOH is activated to give *t*-BuO$^{\cdot}$ and $^{\cdot}$OH radicals. But, in the absence of *t*-BuOOH, chlorogenic acid or adenosine did not undergo any observable chemical change on shining the light. Even though a small fraction of the total light intensity was absorbed by *t*-BuOOH directly in

Table 1—Effect of [*t*-BuOOH] and [adenosine] on the rate and quantum yield of photooxidation of adenosine by *t*-BuOOH in the presence of light in aqueous neutral medium

$10^5 \times$ [Adenosine] (mol dm^{-3})	$10^3 \times$ [*t*-BuOOH] (mol dm^{-3})	$10^{10} \times$ Rate (mol dm^{-3} s^{-1})	ϕ_{expt}
1.00	5.00	2.2183	0.000147
2.00	5.00	2.5866	0.000172
4.00	5.00	3.4362	0.000228
5.00	5.00	4.1222	0.000274
5.00	8.00	5.3467	0.000356
5.00	10.0	6.5324	0.000434

Light intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 260 nm, pH ~ 7.5, Temperature = 298 K, [*t*-BuOH] = 1.0 mol dm^{-3}

Table 2—Effect of [*t*-BuOOH] and [chlorogenic acid] on the rate and quantum yield of photooxidation of chlorogenic acid by *t*-BuOOH in the presence of light in *t*-BuOH-water 1:4 (v/v) medium

$10^6 \times$ [Chlorogenic acid] (mol dm^{-3})	$10^3 \times$ [*t*-BuOOH] (mol dm^{-3})	$10^9 \times$ Rate (mol dm^{-3} s^{-1})	ϕ_{expt}
20.0	5.00	9.6908	0.006445
10.0	5.00	7.0008	0.004656
8.00	5.00	5.2798	0.003511
5.00	5.00	2.7845	0.001852
2.00	5.00	2.2974	0.001528
20.0	10.0	11.2030	0.007451
20.0	15.0	13.1571	0.008750

Light intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 328 nm, pH ~ 7.5, Temperature = 298 K

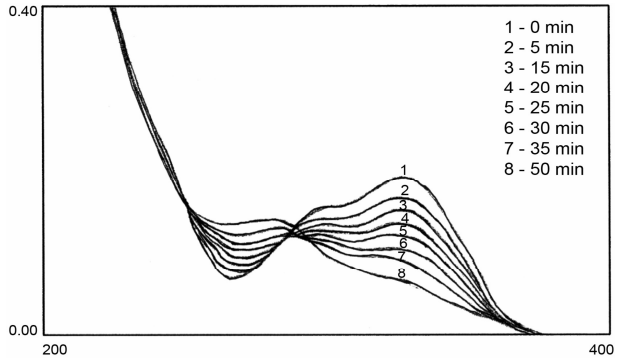


Fig. 1—Absorption spectra of photooxidation of chlorogenic acid in the presence of *t*-BuOOH at different irradiation times; [chlorogenic acid] = 1×10^{-5} mol dm^{-3}, [*t*-BuOOH] = 5×10^{-3} mol dm^{-3}, Light intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 328 nm, pH ~ 7.5, temperature = 298 K

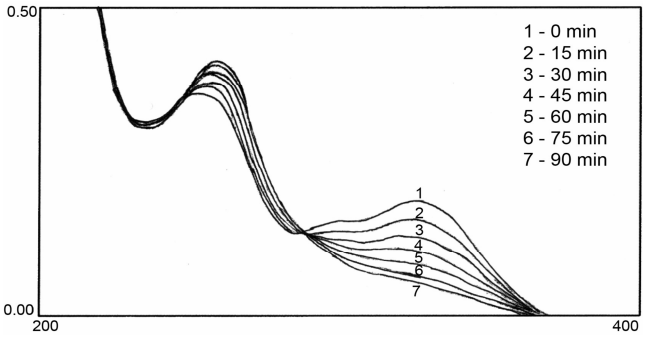


Fig. 2—Absorption spectra of photooxidation of chlorogenic acid in the presence of *t*- BuOOH and adenosine at different irradiation times; [chlorogenic acid] = 1×10^{-5} mol dm^{-3}, [t-BuOOH] = 5×10^{-3} mol dm^{-3}, [adenosine] = 5×10^{-5} mol dm^{-3}, Light intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 328 nm, pH ~ 7.5, temperature = 298 K

the presence of adenosine and/or chlorogenic acid, a considerable chemical change was observed with adenosine as well as chlorogenic acid (Tables 1 and 2). If adenosine and chlorogenic acid acted as only inner filters, the rates of the reaction of adenosine or chlorogenic acid with *t*-BuO$^{\cdot}$ would have been decreased with increase in the concentration of adenosine or chlorogenic acid. But, the results in Tables 1 and 2 were contrary to this. Another fact against the inner filter concept was that the rate of oxidation of chlorogenic acid in the presence of adenosine would have been much less than the experimentally observed values (Table 3). Hence, we proposed that the excited states of chlorogenic acid and adenosine acted as sensitizers to transfer energy to *t*-BuOOH to produce radical species. This type of sensitizing effect was proposed in similar systems earlier[15]. Therefore, the light intensity at 254 nm was used to calculate the quantum yields of oxidation of adenosine as well as chlorogenic acid under different experimental conditions.

In order to find the protection offered to adenosine by chlorogenic acid towards oxidation by *t*-BuO$^{\cdot}$, the reaction mixture containing known concentrations of adenosine, *t*-BuOOH and *t*-BuOH was irradiated in presence of varying concentrations of chlorogenic acid. The reactions were followed by measuring the absorbance of chlorogenic acid at 328 nm (Fig. 2) at which adenosine was transparent and the rate data are presented in Table 3. The photooxidation of chlorogenic acid by *t*-BuO$^{\cdot}$ at different concentrations of adenosine was also studied (Fig. 3) and the data are presented in Table 4.

Table 3—Effect of varying [chlorogenic acid] on the rate and quantum yield of photooxidation of chlorogenic acid by *t*-BuOOH in the absence and presence of adenosine in *t*-BuOH-water 1:4 (v/v) neutral medium

$10^6 \times$ [Chlorogenic acid] (mol dm^{-3})	$10^3 \times$ [Adenosine] (mol dm^{-3})	$10^9 \times$ Rate (mol dm^{-3} s^{-1})	ϕ_{expt}
20.0	0.00	9.6908	0.006445
10.0	0.00	7.0008	0.004656
8.00	0.00	5.2798	0.003511
5.00	0.00	2.7845	0.001852
2.00	0.00	2.2974	0.001528
20.0	5.00	4.0100	0.002667
10.0	5.00	2.7402	0.001822
8.00	5.00	2.6399	0.001756
5.00	5.00	1.9382	0.001289
2.00	5.00	0.7936	0.000528

[*t*-BuOOH] = 5×10^{-3} mol dm^{-3}, Light intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 328 nm, pH ~ 7.5, Temperature = 298 K

The oxidation rate of adenosine in the presence of *t*-BuOH refers exclusively to the reaction of *t*-BuO$^{\cdot}$ with adenosine[16]. These rates were found to increase with increase in concentration of adenosine as well as *t*-BuOOH. The quantum yield values were also increased with increase in [adenosine] as well as [*t*-BuOOH] (Table 1).

The rate of oxidation of chlorogenic acid increased with increase in concentration of chlorogenic acid (Table 2). The quantum yields of oxidation of chlorogenic acid were calculated from the initial rates and the light intensity at 254 nm. These values were also increased with increase in concentration of chlorogenic acid (Table 2). Having known the rates of *t*-BuO$^{\cdot}$ radical reactions with adenosine as well as chlorogenic acid under varying experimental conditions, both adenosine and chlorogenic acid were

introduced for the competitive studies with *t*-$BuO^{\cdot}$ radical. Aqueous solutions of reaction mixture containing chlorogenic acid, *t*-BuOOH and *t*-BuOH were irradiated in presence of varying concentrations of adenosine (Fig. 3). The initial rates and quantum yields of oxidation of chlorogenic acid by *t*-$BuO^{\cdot}$ radicals were found to decrease with increase in concentration of adenosine (Table 4). Comparison of the initial rates and quantum yields of oxidation of chlorogenic acid in presence and absence of adenosine clearly indicated that the initial rates and quantum yields of oxidation of chlorogenic acid were substantially decreased in presence of adenosine (Table 4). These observations clearly demonstrated that adenosine and chlorogenic acid were in competition for *t*-$BuO^{\cdot}$ radicals.

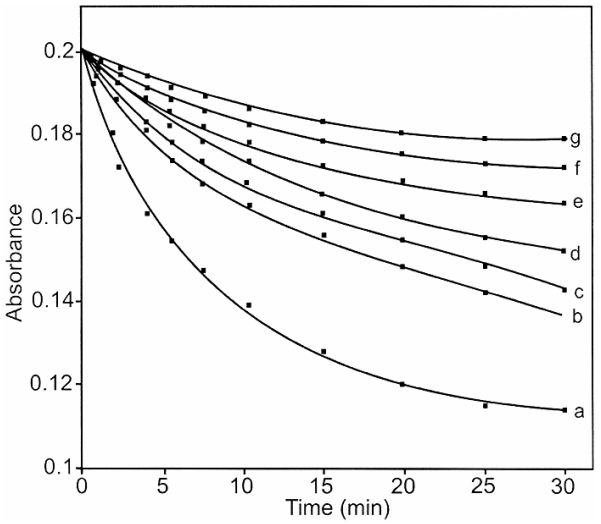


Fig. 3—Effect of varying concentrations of adenosine on the photooxidation of chlorogenic acid (1.0×10^{-5} mol dm^{-3}) in the presence of *t*-BuOOH (5×10^{-3} mol dm^{-3}) at 298 K. [adenosine] = (a) 0.0, (b) 5×10^{-5} mol dm^{-3}, (c) 8×10^{-5} mol dm^{-3}, (d) 1×10^{-4} mol dm^{-3}, (e) 5×10^{-4} mol dm^{-3}, (f) 8×10^{-4} mol dm^{-3}, (g) 1×10^{-3} mol dm^{-3}

The rate constant of the reaction of (*t*-$BuO^{\cdot}$) with adenosine has been reported[10] to be 1.40×10^{8} dm^{3} mol^{-1} s^{-1} under similar experimental conditions of the present work. The rate constant for the reaction of *t*-$BuO^{\cdot}$ with chlorogenic acid was calculated by the adenosine competition method, which was very similar to the method[21] used to determine the rate constant for the reaction of $^{\cdot}OH$ radicals with polyhydric alcohols in competition with KSCN. In the present study, solutions containing chlorogenic acid and varying amounts of adenosine in presence of *t*-BuOOH and *t*-BuOH were irradiated for 2 min and the decrease in absorbance of chlorogenic acid was measured. The decrease in absorbance of chlorogenic acid reflected the amount of *t*-$BuO^{\cdot}$ radicals that had reacted with chlorogenic acid. From the known rate constant of the reaction of adenosine with *t*-$BuO^{\cdot}$ radical under similar experimental conditions of the present work ($k_{\text{adenosine}} = 1.40 \times 10^{8}$ dm^{3} mol^{-1} s^{-1}), the rate constant of *t*-$BuO^{\cdot}$ radical reaction with chlorogenic acid ($k_{\text{chlorogenic acid}}$) can be calculated using the following equation:

$$\frac{[\text{Absorbance of chlorogenic acid}]_{o}}{[\text{Absorbance of chlorogenic acid}]_{\text{adenosine}}} = 1 + \frac{k_{\text{adenosine}[\text{adenosine}]}}{k_{\text{chlorogenic acid}}\,[\text{chlorogenic acid}]} \quad \ldots (1)$$

In Eq. (1), $[\text{Absorbance of chlorogenic acid}]_{o}$ and $[\text{Absorbance of chlorogenic acid}]_{\text{adenosine}}$ are the absorbance values of chlorogenic acid in the absence and presence of adenosine, respectively at the same interval of time. Experiments of this kind can be carried out with great accuracy. Using Eq. (1), the rate constant for the reaction of *t*-$BuO^{\cdot}$ radical with

Table 4—Effect of varying [adenosine] on the rate and quantum yield of photooxidation of chlorogenic acid in the presence of *t*-BuOOH in *t*-BuOH-water 1:4 (v/v) neutral medium

$10^{5} \times$ Adenosine (mol dm^{-3})	$10^{9} \times$ Rate (mol dm^{-3} s^{-1})	ϕ_{expt}	ϕ_{cal}	p	ϕ'	% Scavenging	% Repair
0.00	7.00	0.004656	0.004656	1.0000	0.004656	100.0	0.00
5.00	6.14	0.004084	0.003779	0.8118	0.005031	81.18	8.04
8.00	5.78	0.003845	0.003373	0.7244	0.005307	72.44	14.0
10.0	5.66	0.003767	0.003181	0.6832	0.005514	68.32	18.4
50.0	2.74	0.001822	0.001403	0.3013	0.006048	30.13	29.9
80.0	2.29	0.001528	0.000988	0.2123	0.007197	21.23	54.6
100.0	2.20	0.001462	0.000826	0.1774	0.008268	17.74	77.6

[Chlorogenic acid] = 1.0×10^{-5} mol dm^{-3}, [*t*-BuOOH] = 5.0×10^{-3} mol dm^{-3}, Light intensity = 2.7168×10^{15} quanta s^{-1}, λ_{max} = 328 nm, pH ~ 7.5, Temperature = 298 K

chlorogenic acid ($k_{\text{chlorogenic acid}}$) was calculated at different concentrations of chlorogenic acid and adenosine and the average of these was found to be 3.20×10^9 dm^3 mol^{-1} s^{-1}.

As chlorogenic acid had strong absorption at 260 nm, it is not possible for the direct measurement of protection and repair offered to adenosine by chlorogenic acid at this wavelength. However, one could calculate indirectly the extent of protection offered to adenosine by chlorogenic acid from competition kinetic studies measured at 328 nm, λ_{max} of chlorogenic acid. The method was as follows:

When the system containing adenosine, chlorogenic acid and *t*-BuOOH in the presence of *t*-BuOH was irradiated, the probability of *t*-BuO$^{\cdot}$ radicals reacting with chlorogenic acid

{ $p_{(t\text{-BuO}^{\cdot} + \text{ chlorogenic acid})}$ } was calculated using the following equation:

$$p_{(t-BuO^{\cdot}+\text{chlorogenic acid})} = \frac{k_{\text{chlorogenic acid}}\,[\text{chlorogenic acid}]}{k_{\text{adenosine}}\,[\text{adenosine}] + k_{\text{chlorogenic acid}}\,[\text{chlorogenic acid}]} \quad \ldots (2)$$

If chlorogenic acid scavenged only (*t*-BuO$^{\cdot}$) radicals and did not give rise to any other reaction (e.g. reaction with adenosine radicals), the quantum yield of oxidation of chlorogenic acid (ϕ_{cal}) at each concentration of adenosine may be given by equation:

$$\phi_{cal} = \phi^0_{expt} \times p \quad \ldots (3)$$

where ϕ^o_{expt} is the quantum yield of oxidation of chlorogenic acid in the absence of adenosine, and p is the probability given by Eq. (2).

The calculated quantum yield (ϕ_{cal}) values at different adenosine concentrations are presented in Table 4. The data showed that the ϕ_{cal} values were lower than the experimentally measured quantum yield (ϕ_{expt}) values. This indicated that more number of chlorogenic acid molecules were consumed in the system than expected and the most likely route for this was H atom donation by chlorogenic acid to adenosine radicals. In Table 4, are presented the fraction of (*t*-BuO$^{\cdot}$) radicals scavenged by chlorogenic acid at different concentrations of adenosine. These values referred to the measure of protection offered by adenosine, due to scavenging of *t*-BuO$^{\cdot}$ radicals by chlorogenic acid. Using the ϕ_{exptl} values, a set of values, viz., ϕ' values were calculated from Eq. (4) and are presented in Table 4.

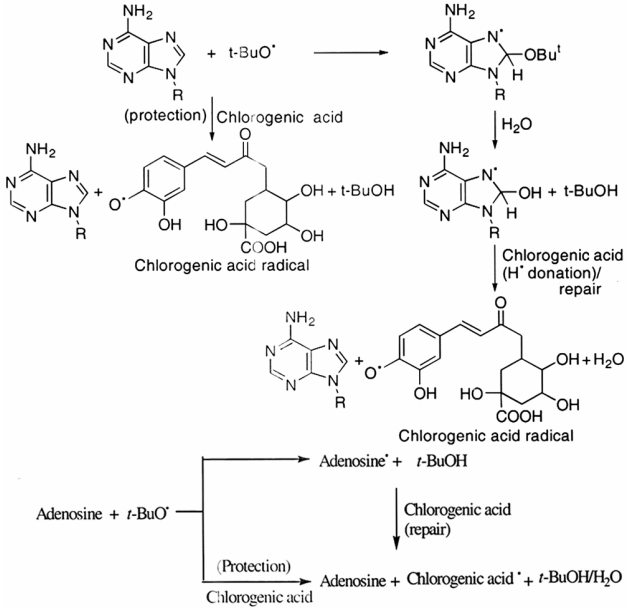
$$\phi' = \frac{\phi_{expt}}{p} \quad \ldots (4)$$

where ϕ's represent the experimentally found quantum yield values, if no scavenging of adenosine radicals by chlorogenic acid occurs. In the absence of any "repair" of adenosine radicals by chlorogenic acid, the ϕ' values should all be equal to ϕ^o_{expt}. The observed increase in the ϕ' with increasing adenosine concentration (Table 4) clearly indicated the repair of adenosine radicals. The extent of repair may be quantified by the following equation:

$$\%\ \text{Repair} = \frac{(\phi' - \phi^0_{expt})}{\phi^0_{expt}} \times 100 \quad \ldots (5)$$

The data on percentage repair is presented in Table 4. The experimentally determined quantum yield (ϕ_{expt}) values were higher than the quantum yield (ϕ_{cal}) values calculated using Eq. (3) under the assumption that chlorogenic acid acts only as a *t*-BuO$^{\cdot}$ radical scavenger. This showed that chlorogenic acid acted not only as an efficient scavenger of (*t*-BuO$^{\cdot}$) radicals, but also as an agent for the repair of adenosine radicals. The repair reaction of chlorogenic acid is explained in terms of the H donation as shown in **Scheme 1**.

The results obtained in the present study (Table 4) indicated that adenosine radicals were efficiently



Scheme 1

repaired by chlorogenic acid to the extent of ~77% at about 10 μM of chlorogenic acid concentration. This type of repair reactions by caffeic acid was reported in the oxidation of adenosine by *t*-$BuO^{\cdot}$ radicals[16]. The percentage repair found in the present study (Table 4) was similar to the one reported with caffeic acid[16]. Thus, these results justified two aspects. One was that the oxidation of adenosine by *t*-$BuO^{\cdot}$ radicals was via oxidizing transient radicals of adenosine as suggested earlier[16]. The electron density calculations showed that C_8 in adenosine was more electron rich compared to C_4 or C_5[22]. The bulkiness of the *t*-$BuO^{\cdot}$ radical was another reason that it preferred C_8 position, where no steric hindrance was present due to other groups. The attack of *t* - $BuO^{\cdot}$ radical at C_8 leads to the formation of N_7-centered radical, the nature of which has been reported to be oxidizing in nature. The percentage repair obtained in the present study further supported our contention that *t*-$BuO^{\cdot}$ radicals preferentially reacted at C_8 position, leading to the formation of N_7-centered oxidizing radicals to the extent of ~77%. The second aspect was that chlorogenic acid was also found to repair the transient oxidizing radicals of adenosine very efficiently, similar to caffeic acid[16]. The protection of adenosine and repair ofadenosine radicals are summarized in the **Scheme I.**

The chlorogenic acid radicals were generated in the process of protection of adenosine and repair of adenosine radicals. These radicals were reported[23,24] to have short lifetime, extremely unstable and rapidly converted to unknown compounds at physiological pH[25]. If chlorogenic acid radicals reacted with adenosine, then ϕ_{expt} would have been less than ϕ_{cal} values. Contrary to this, the ϕ_{cal} values were lower than the experimentally found quantum yield values (ϕ_{expt}) (Table 4). This supported our contentionthat the chlorogenic acid radicals might not beinvolved in oxidative stress in our experimental conditions.

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