

Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties

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Abstract

It is believed that anticancer and apoptosis inducing properties of green tea are mediated by its polyphenolic constituents particularly catechins. A number of reports have shown that green tea polyphenol (–)-epigallocatechin-3-gallate (EGCG) is among the most effective chemopreventive and apoptosis-inducing agents present in the beverage. Plant polyphenols are naturally occurring antioxidants but they also exhibit prooxidant properties. Over the last several years we have shown that various classes of plant polyphenols including flavonoids, curcuminoids and tannins are capable of catalyzing oxidative DNA cleavage particularly in the presence of transition metal ions such as copper and iron. With a view to understand the chemical basis of various pharmacological properties of green tea, in this paper we have compared the prooxidant properties of green tea polyphenols—EGCG and EC ((–)-epicatechin). The rate of oxidative DNA degradation as well as hydroxyl radical and superoxide anion formation was found to be greater in the case of EGCG as compared with EC. It was also shown that copper mediated oxidation of EC and EGCG possibly leads to the formation of polymerized polyphenols. Further, it was indicated that copper oxidized catechins were more efficient prooxidants as compared with their unoxidized forms. These results correlate with the observation by others that EGCG is the most effective apoptosis inducing polyphenol present in green tea. They are also in support of our hypothesis that prooxidant action of plant polyphenols may be an important mechanism of their anticancer properties. A model for binding of Cu(II) to EC has been presented where the formation of quinone and a quinone methide has been proposed.

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1. Introduction

There has been increasing realization in recent years that several plant derived polyphenolic compounds may possess anticancer and apoptosis-inducing properties (Mukhtar et al., 1998; Clement et al., 1998). Therefore, the role of plant-derived polyphenols in chemoprevention of cancer has emerged as an interesting area of research. The data in literature points to the possible role of green tea as a chemopreventive agent against different types of cancers (Picard, 1996; Sato, 1999; Sadzuka et al., 1998; Otsuka et al., 1998). Tea (*Camellia sinensis*) is the second most common beverage in the world next to water (Wei et al., 1999). Although both green and black teas are derived from *C. sinensis*, it is the

production process which differentiates the two types of teas. Green tea contains polyphenols which include flavanols, flavandiols, flavonoids and phenolic acids. Most of the green tea polyphenols are flavanols, commonly known as catechins. The primary catechins in green tea are (–)-epicatechin (EC), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC), (–)-epigallocatechin-3-gallate (EGCG), (+)-gallocatechin and (+)-catechin. It is believed that much of the anticancer effects of green tea are mediated by its polyphenolic constituents (Ahmad et al., 1998; Katiyar and Mukhtar, 1996). During the manufacture of black tea these polyphenols undergo polyphenol oxidase catalyzed oxidative polymerization giving rise to the formation of theaflavins and thearubigins in the process referred to as ‘tea fermentation’ (Wei et al., 1999). However, it is considered that black tea is not as effective in its chemopreventive properties. Other studies have shown that black tea polyphenols—theaflavins—exhibit

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stronger anticarcinogenic activity than EGCG. Thus, the molecular mechanisms of cancer chemopreventive effects of tea polyphenols are not completely understood (Lin and Liang, 2000).

Plant polyphenols are natural antioxidants and most of their pharmacological properties are considered to be due to their antioxidant action (Ames et al., 1995). This is generally considered to reflect their ability to scavenge endogenously generated oxygen radicals or those radicals formed by various xenobiotics, radiation etc. However, some data in the literature suggest that the antioxidant properties of the polyphenolic compounds may not fully account for their chemopreventive effects (Gali et al., 1992). Most plant polyphenols possess both antioxidant as well as prooxidant properties (Inoue et al., 1994) and we have earlier proposed that the prooxidant action of polyphenolics may be an important mechanism of their anticancer and apoptosis inducing-properties (Hadi et al., 2000).

In our laboratory we have confirmed that a number of plant polyphenols such as flavonoids, tannins and *trans*-stilbenes possess oxidative DNA cleavage properties either alone or in the presence of transition metal ions such as copper (known to be a normal component of chromatin) (Rahman et al., 1989; Khan and Hadi, 1998; Ahsan and Hadi, 1998; Ahmad et al., 2000). It is to be noted that a number of reports have shown the green tea polyphenol EGCG to be among the most effective apoptosis inducing agents present in green tea (Chen et al., 1998). With a view to understand the chemical basis of various pharmacological properties of green tea, in this paper we have compared the prooxidant properties of green tea polyphenols—EGCG and EC (Fig. 1). Our results indicate that of the two EGCG is more effective as a prooxidant. It is also the more efficient reducer of Cu(II) to Cu(I), a reaction which leads to the formation of reactive oxygen species such as

the hydroxyl radical (Rahman et al., 1990). A model of Cu(II) binding to epicatechin has also been proposed.

2. Materials and methods

Calf thymus DNA (sodium salt, average molecular weight 1×10^6), bathocuproine, S_1 nuclease, EC and EGCG were purchased from Sigma Chemical Company (St. Louis, MO). Supercoiled plasmid pBR322 DNA was prepared according to the standard methods (Maniatis et al., 1982). All other chemicals were of analytical grade.

2.1. Copper oxidation of green tea catechins

Catechins (EC & EGCG) and Cu(II) (4 mM each) were incubated overnight in a final volume of 200 μ l. 100 mg of chelex in 1 ml of 10 mM NaCl was centrifuged at 2500 rpm for 10 min. The supernatant was removed and 1 ml of distilled water was added to the pellet. 200 μ l of this suspension was added to the overnight incubated sample of catechin and Cu(II). After 10 min shaking the sample was centrifuged at 2500 rpm for 10 min. The supernatant containing oxidized catechins was collected.

2.2. Thin layer chromatography

Copper oxidized catechins were applied on to silica gel (25 μ m layer thickness) plates for thin layer chromatography (TLC) along with the standard compounds. A mixture of toluene–ethyl acetate (1:8) was used as the solvent (Wei et al., 1999). Polyphenols were detected by exposure to iodine as well as under UV (254 nm).

2.3. Reaction of catechins and copper oxidized catechins with calf thymus DNA and digestion with S_1 nuclease

Reaction mixtures (0.5 ml) contained 10 mM Tris–HCl (pH 7.5), 500 μ g DNA, cupric chloride and polyphenols as indicated. Incubation was performed at room temperature for 1 h. All solutions were sterilized before use. Single strand specific nuclease digestion was performed as described previously (Naseem and Hadi, 1987). Acid soluble deoxyribonucleotides were determined colorimetrically (Schneider, 1957).

2.4. Reaction with plasmid pBR322 DNA

Reaction mixtures (30 μ l) contained 10 mM Tris–HCl, pH 7.5, 0.5 μ g plasmid DNA and other components as described in the figure legend. Incubation at room temperature was carried out for 1 h. After the incubation 10 μ l of a solution containing 40 mM EDTA, 0.05% bromophenol blue tracking dye and 50% (v/v) glycerol was added and the solution was subjected to

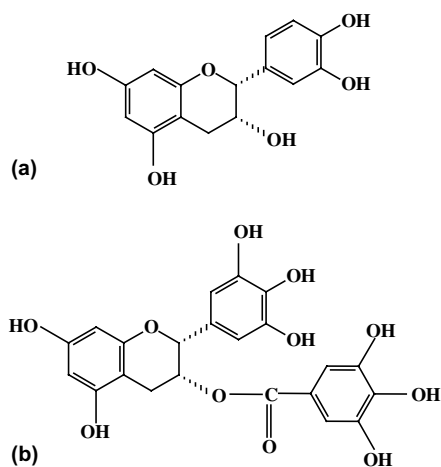


Fig. 1. Structures of (a) (–)-epicatechin; (b) (–)-epigallocatechin-3-gallate.

electrophoresis on 1% agarose gel. The gel was stained with ethidium bromide (0.5 mg/l), viewed and photographed on a UV transilluminator.

2.5. Stoichiometric titration of EC and EGCG

The concentration of Cu(I) produced in the EC/EGCC–Cu(II) reaction mixture was determined by titration with bathocuproine. Bathocuproine complexes with Cu(I) to form a $\text{Cu}(\text{bathocuproine})_2^+$ which has an absorption peak at 480 nm (Nebesar, 1964). EC and EGCG (10 μM each) in 10 mM Tris–HCl, (pH 7.5) was mixed with varying concentrations of CuCl_2 (2.5–50 μM) and 0.3 mM bathocuproine solution in a total volume of 3 ml. The bathocuproine–Cu(I) complex was determined by measuring at 480 nm.

2.6. Assay of active oxygen species

Superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayama et al. (1983). A typical assay mixture contained 50 mM potassium phosphate buffer (pH 7.8), 33 μM NBT, 0.1 mM EDTA and 0.06% Triton X-100 in a total volume of 3.0 ml. After mixing, absorbance was recorded at 560 nm against a blank, which did not contain the compound, at different time intervals.

In order to compare the hydroxyl radical production by increasing concentrations of EC and EGCG in the presence of 100 μM Cu(II), the method of Quinlan and Guttridge (Quinlan and Gutteridge, 1987) was followed. Calf thymus DNA (200 μg) was used as substrate and the malondialdehyde generated from deoxyribose radicals was assayed as described earlier (Singh et al., 1998).

3. Results

3.1. Interaction of Cu(II) with EC and EGCG

We have previously shown that plant polyphenols are able to reduce Cu(II) to Cu(I) (Bhatt and Hadi, 1994). To assess the relative efficacy of reduction of Cu(II) by EC and EGCG, the experiment shown in Fig. 3 was performed. Increasing concentrations of Cu(II) were added to a fixed concentration of catechins in the presence of Cu(I) specific sequestering agent bathocuproine. Job plots of equivalents of $\text{Cu(II)}/[\text{EC}]$ or $[\text{EGCG}]$ vs. absorption of bathocuproine–Cu(I) complex at 480 nm reveal that there is no clear stoichiometry of reduction of Cu(II) to Cu(I) in both cases as a clear plateau is not observed (Fig. 3). It would appear that both EC and EGCG can reduce more than 1 mole of Cu(II) per mole of EC/EGCG. It can however be inferred from the figure

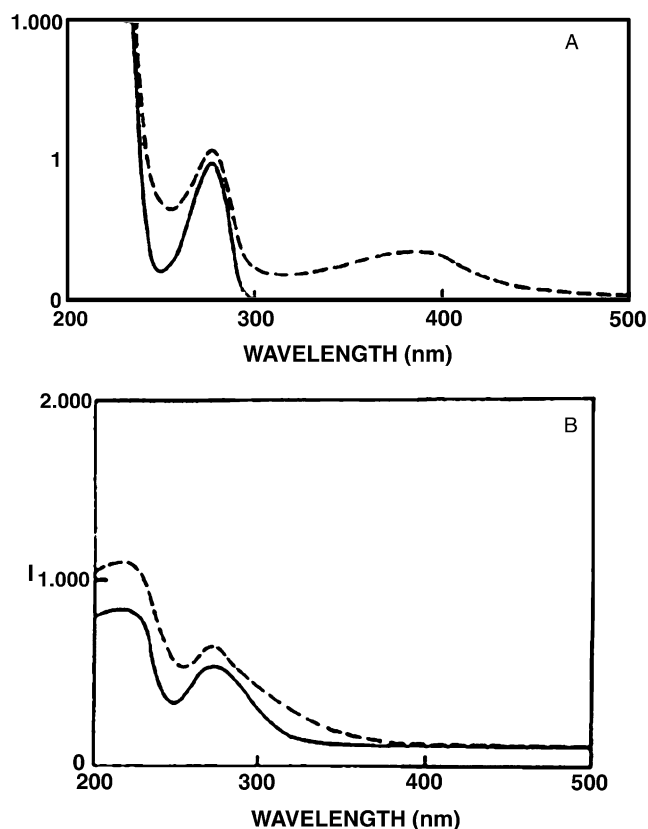


Fig. 2. Absorption spectra of EC and EGCG in the presence and absence of Cu(II). The concentration of (A) EC and (B) EGCG in a total volume of 2 ml reaction mixture containing 10 mM Tris–HCl (pH 7.5) was 200 μM . EC/EGCG alone (—); EC/EGCG in the presence of 200 μM Cu(II) (---).

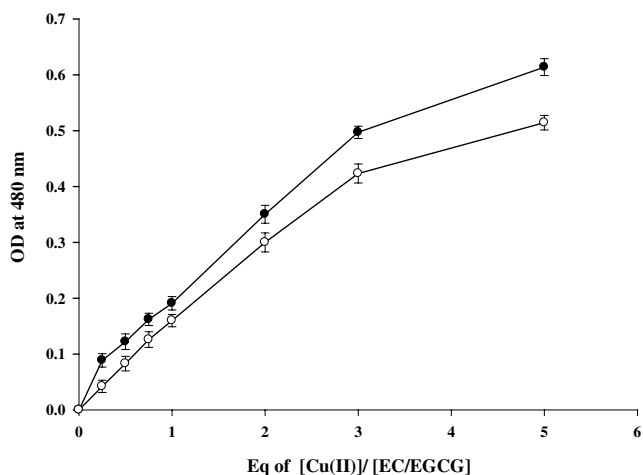


Fig. 3. Detection of stoichiometry of EC and EGCG. The concentration of EGCG (●) and EC (○) was 10 μM in the presence of 0.3 mM bathocuproine. The absorbance at 480 nm vs. equivalent of Cu(II) per molar equivalents of EC/EGCG is plotted.

that EGCG is a more effective reducer of Cu(II) as compared to EC.

As Cu(II) is reduced to Cu(I), it was therefore of interest to determine whether a complex with Cu(II) is formed. Fig. 2(A) and (B), shows the absorption spectra of catechins (EC & EGCG) alone and on the addition of Cu(II). The absorption maxima of EC and EGCG lie in the range of 200–280 nm. On addition of copper an enhancement in the absorption spectra of EC and EGCG is observed. As seen in Fig. 2(A) in the case of EC, a peak at 380 nm also appears which is indicative of the formation of a quinone (Harbone, 1973).

3.2. Comparison of prooxidant properties of EC and EGCG

It has been previously elucidated that during the reduction of Cu(II) to Cu(I), reactive oxygen species such as hydroxyl radicals are formed which serve as proximal DNA cleaving agent (Rahman et al., 1989). Therefore, the capacity of EC and EGCG to generate hydroxyl radicals in the presence of Cu(II) was compared (Fig. 4). This assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA (thiobarbituric acid) reactive material, which forms a colored adduct with TBA readable at 532 nm (Quinlan and Gutteridge, 1987). Increasing concentrations of both the compounds lead to a progressively increased formation of hydroxyl radicals. However, at all the concentrations tested, the formation of TBA reactive material was greater in the case of EGCG, indicating that it is relatively more efficient than EC in generating hydroxyl radical.

It is known that generation of the O_2^- anion may lead to the formation of H_2O_2 . The addition of a second electron to the O_2^- anion gives the peroxide ion ($2O_2^-$), which has no unpaired electron and is not a radical.

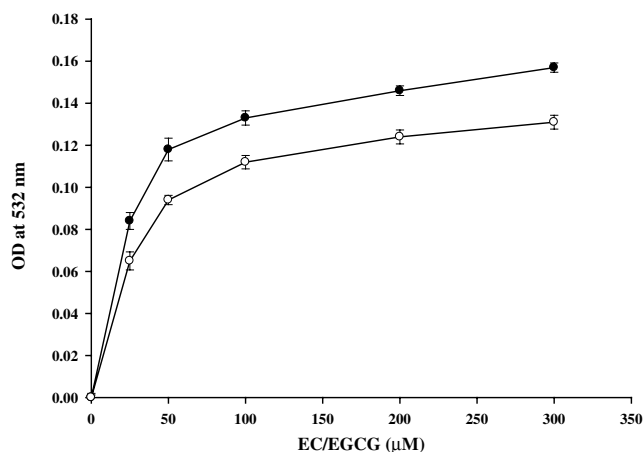


Fig. 4. Formation of hydroxyl radicals as a function of EC and EGCG concentration in the presence of Cu(II). The reaction mixtures were as described in 'Methods' containing 100 μ M Cu(II) and increasing concentrations of EGCG (●) and EC (○). The incubation was at 37 °C for 1 h.

However, at neutral pH the peroxide ion immediately protonates to give hydrogen peroxide (H_2O_2). Alternatively, in aqueous solution the superoxide anion undergoes dismutation to form H_2O_2 and O_2 (Halliwell and Gutteridge, 1984). Therefore, the rate of generation of superoxide anion by EC and EGCG was compared. The increase in absorption at 560 nm is observed on reduction of NBT by superoxide anion. From Fig. 5, it is evident that the production of superoxide radical increases with the increasing time of incubation. It is seen that EGCG is the more efficient producer of superoxide radical as compared to EC.

It is known from our previous results that polyphenols–Cu(II) system mediates DNA cleavage through the generation of reactive oxygen species such as the hydroxyl radicals (Rahman et al., 1989; Ahmad et al., 2000). Thus, the cleavage of supercoiled pBR322 plasmid DNA by EC/EGCG–Cu(II) systems was compared. Fig. 6 shows the ethidium bromide stained banding pattern of pBR322 DNA tested with EGCG or EC in the presence of Cu(II). As can be seen, the greater degrading effect is caused by EGCG where the complete conversion of supercoiled DNA to linear forms and progressively smaller heterogeneous sized fragments is seen (lane 2). In the case of EC the supercoiled DNA is converted to open circular and linear forms with some of the DNA also converted to smaller sized fragments (lane 3). These results correlate with the finding (Figs. 4 and 5), that EGCG is a more efficient producer of the reactive oxygen species.

3.3. TLC of catechins (EC and EGCG) and their copper mediated oxidized forms

EC and EGCG were oxidized by copper as given in 'Methods'. The unoxidized and copper oxidized cate-

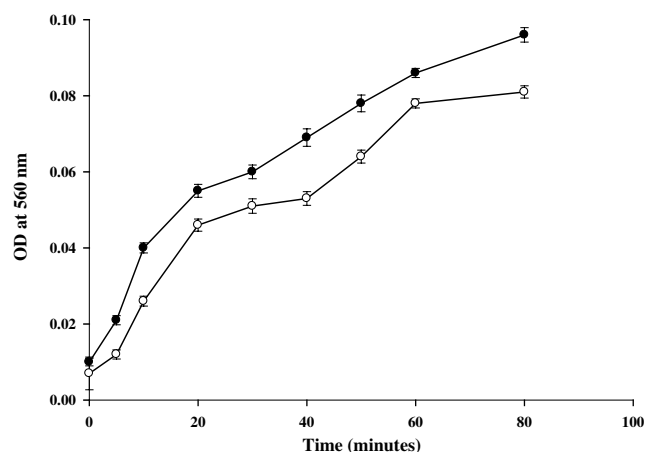


Fig. 5. Photogeneration of superoxide anion by EC and EGCG on illumination under fluorescent light. The concentration of EGCG (●) and EC (○) was 60 μ M. The two samples were placed 10 cm from the light source. Details are given in 'Methods'.

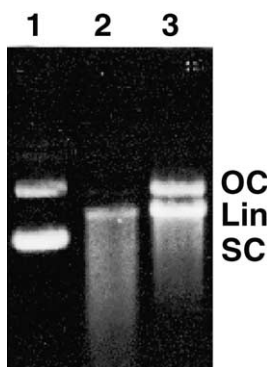


Fig. 6. Agarose electrophoretic pattern of ethidium bromide stained pBR322 DNA after the treatment with EC and EGCG in the presence of Cu(II). Reaction mixture containing EC/EGCG and Cu(II) (100 μ M each) was incubated for 1 h. Lanes (1) DNA alone; (2) DNA + EGCG + Cu(II); (3) DNA + EC + Cu(II). The positions of open circle (OC), linear (Lin) and supercoiled (SC) are indicated.

chins were applied on a silica gel plate for TLC. Fig. 7 shows the relative movement of the catechins on the plate in toluene–ethyl acetate (1:8) solvent. Results indicate that the unoxidized forms are readily mobile (lanes a&c) whereas a major portion of the copper oxidized catechins remains stationary (lanes b&d). These results are similar to those obtained when water extracts of green tea and black tea are subjected to TLC. The extract of green tea shows the presence of EC and EGCG whereas the polyphenols of black tea do not show any movement (results not shown). Thus copper mediated oxidation of EC and EGCG possibly leads to the formation of polymerized forms of catechins similar to those present in black tea.

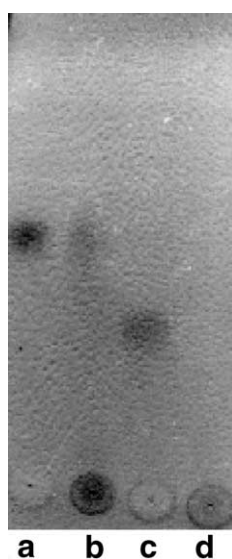


Fig. 7. TLC profile of catechins (EC and EGCG) and their copper mediated oxidized forms. Lane a: EC; lane b: copper oxidized EC; lane c: EGCG; lane d: copper oxidized EGCG.

3.4. Degradation of calf thymus DNA by EC/EGCG and their copper oxidized forms

To compare the extent of DNA degradation by catechins with their copper oxidized forms in the presence of Cu(II), double stranded calf thymus DNA was used as the substrate. The reaction was assessed by recording the proportion of double stranded DNA converted to acid soluble nucleotides by S_1 nuclease. Control experiments (data not shown) established that heat-denatured DNA underwent 100% hydrolysis following the treatment with S_1 nuclease, whereas only 9% of native form was hydrolyzed. Table 1 shows the extent of DNA degradation by the catechins under study in the absence and presence of Cu(II). It is seen from the results that the relative rates of DNA hydrolysis between EGCG, oxidized EGCG, EC and oxidized EC in the presence of Cu(II) are quite similar. However, in the absence of copper the oxidized EGCG and oxidized EC show considerably enhanced rate as compared to their unoxidized forms (11.2 vs. 7.68 and 10.93 vs. 2.26, respectively).

4. Discussion

The above studies lead to the following major conclusions: (i) Similar to several other classes of polyphenols both EC and EGCG exhibit prooxidant properties such as the generation of superoxide anion and the hydroxyl radical. Both the polyphenols lead to oxidative DNA cleavage in the presence of copper ions. (ii) Copper mediated oxidation of EC and EGCG possibly leads to the formation of polymerized polyphenols. It is indicated that copper oxidized catechins are better prooxidants as compared to their unoxidized forms.

In a previous study from this laboratory, reactivities of plant flavonoids with different hydroxyl substituents for the cleavage of DNA in the presence of copper ions

Table 1
 S_1 nuclease hydrolysis following damage to calf thymus DNA by EGCG/oxidized EGCG/EC and oxidized EC in the presence of copper ions

Compounds	% DNA hydrolysis ^a	
	With copper	Without copper
EGCG	17.97 \pm 0.77	7.68 \pm 0.091
Ox EGCG	19.87 \pm 0.93	11.20 \pm 0.085
EC	16.35 \pm 0.82	2.26 \pm 0.071
Ox EC	16.35 \pm 0.75	10.93 \pm 0.52

Reaction conditions were as described in Section 2. The concentrations of polyphenols and Cu(II) were 200 μ g/ml and 100 μ M, respectively. Ox EGCG—copper oxidized EGCG.

Ox EC—copper oxidized EC.

^a All values are expressed as Mean \pm SE for three different experiments.

was studied (Jain et al., 1999). It was concluded that the rate of DNA cleavage depended on the number of hydroxyl groups on the flavonoid molecule. Further, the presence of *orthodihydroxy* groups was particularly important in enhancing the activity of a compound. This was considered to be related to the relative efficacy of Cu(II) chelation at these positions. In the studies presented above EGCG has been shown to be a more efficient producer of hydroxyl radicals in the presence of Cu(II) leading to a greater rate of DNA cleavage as compared with EC. EGCG offers a number of possibilities of orthochelation of Cu(II) and is thus more efficient than EC as an oxidative DNA cleaving agent.

Green tea contains polyphenolic compounds such as EC, EGC and EGCG. During manufacture of black tea these gallo catechins undergo oxidation. The catechin quinones react in several complex manners. The quinones derived from a simple catechin or its gallate may react with a quinone derived from gallo catechin or its gallate to form seven membered ring compounds known as theaflavins (Wei et al., 1999). As shown above copper mediated formation of quinones is also indicated in the case of EC (Fig. 8). It is possible that such oxidation may also lead to the formation of theaflavins or similar

polymers. The hallmark of apoptosis is internucleosomal DNA fragmentation, which distinguishes it from necrosis. Most clinically used anticancer drugs can activate late events of apoptosis (DNA degradation and morphological changes) and essential signaling pathways differ between pharmacological cell death and physiological induction of programmed cell death (Smets, 1994). On the basis of our own observations and those of others a mechanism of DNA fragmentation involving mobilization of intracellular and extracellular copper has been proposed (Hadi et al., 2000). This is based on a number of observations including the facts that copper is an essential constituent of chromatin and that copper ion levels are elevated in a number of malignancies (Ebadi and Swanson, 1988; Yoshida et al., 1993). It would appear from the results of Table 1 that copper mediated oxidation of EC and EGCG transforms these compounds into even more potent prooxidant DNA cleaving agents that are active even in the absence of copper ions.

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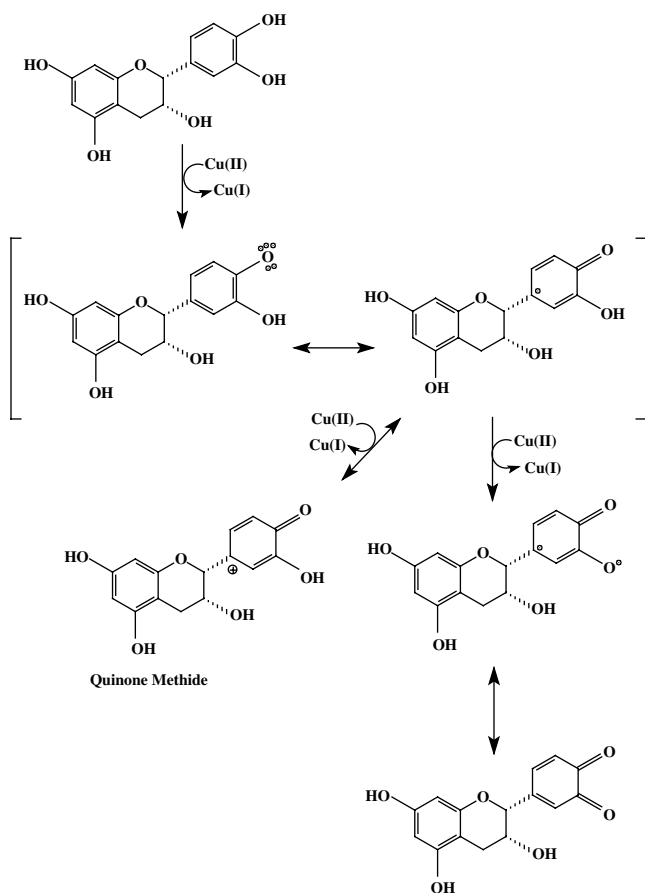


Fig. 8. Proposed model of binding of Cu(II) to (-)-epicatechin (EC).

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