Results
&
Discussion
Part -I
RESULTS (Part - I)

Constituents of the water extract of green and black teas

The major green tea catechins are (-)-epigallocatechin – 3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin – 3-gallate (ECG), (-)-epicatechin (EC), (+)-gallocatechin and (+)-catechin (Yang and Wang, 1993). Figure 3 shows thin layer chromatography separation with standard compounds of epicatechin (EC), epigallocatechin (EGC) and epigallocatechin – 3-gallate (EGCG) in green tea extract prepared as described in ‘Methods’. The results indicate that these three polyphenols are present in the green tea extract and are absent from black tea.

Breakage of calf thymus DNA by green and black tea extracts

The reaction was assessed by recording the proportion of double-stranded DNA converted to acid soluble nucleotides by S1 nuclease. Control experiments (not shown) established that heat denatured DNA underwent 100% hydrolysis following treatment with S1 nuclease, where as only 8% of native DNA was hydrolyzed. Figure 4 gives the acid soluble material produced from calf thymus DNA with increasing concentrations of green and black tea extracts at a fixed concentration of Cu(II). The rate of DNA hydrolysis and the maximum hydrolysis achieved was greater with green tea extract than with black tea. These results are confirmed by another assay, which uses strand cleavage in
Figure 3: Thin layer chromatography profile of green and black tea extracts with standard compounds.

Lane a: green tea extract
Lane b: black tea extract
Lane c: epicatechin
Lane d: epigallocatechin-3-gallate
Lane e: epigallocatechin
Figure 4: Degradation of calf thymus DNA as a function of increasing concentrations of green tea extract and black tea extract in the presence of Cu(II) as measured by the degree of $S_1$ nuclease digestion.

DNA was incubated with increasing concentrations (50-800µg/ml) of green tea extract (●) and black tea extract (○) in the presence of 0.1mM Cu(II) at 37°C for 1 hour. All points represent triplicate samples and mean values have been plotted.
supercoiled plasmid DNA as the substrate. This is a sensitive test for DNA breakage as a single nick per molecule of DNA leads to the conversion of supercoiled to the open circular form. Further strand breakage gives rise to the linear form and heterogeneous sized smaller molecules. As seen in Figure 5 the generation of smaller sized molecules is considerably greater in the case of green tea, which is indicated by a trail of smear. Figure 6 gives the kinetics of DNA degradation by the tea extracts and similar relative rates of hydrolysis are seen.

**Rate of Cu(II) reduction by tea extracts**

In order to examine the involvement of Cu(I) in the reaction I used neocuproine as an agent that sequesters Cu(I) selectively. Green and black tea extracts reduce Cu(II) to generate Cu(I) as evidenced by the formation of a complex absorbing at 450nm (results not shown) (Khan and Hadi, 1998). The reaction possibly leads to the formation of the 'oxidized species' of the components of tea extracts as is the case with other polyphenolic compounds such as flavonoids (Rahman *et al.*, 1989; Utaka and Takeda, 1985). Stoichiometry of the production of Cu(I) by tea extracts was studied (Figure 7) but not clear maximum where absorption plateaued (Khan and Hadi, 1998) was obtained, however the rate of Cu(II) reduction by green tea extract was found to be greater than black tea. The greater rate of Cu(II) reduction by green tea is possibly accounted for by the presence of epicatechin,
Figure 5: Agarose gel electrophoretic pattern of ethidium bromide stained pBR 322 DNA after treatment with green tea extract and black tea extract in the presence of Cu(II).

Reaction mixture in a volume of 30 μl containing 0.8 μg plasmid pBR322 DNA, 0.1 mM Cu(II) and 5 μg tea extracts were incubated for 1 hour at 37°C.  
Lane a: DNA alone  
Lane b: DNA + green tea extract + Cu(II)  
Lane c: DNA + black tea extract + Cu(II)
Figure 6: Effect of time of incubation on calf thymus DNA degradation by the tea extracts.

DNA was incubated with green tea extract (●) and black tea extract (○) in the presence of Cu(II) for the indicated time periods and then subjected to S1 nuclease digestion. The concentration of tea extracts and Cu (II) were 800μg/ml and 0.1mM respectively. All points represent triplicate samples and mean values have been plotted.
Figure 7: Rate of reduction of Cu(II) by green tea extract and black tea extract (formation of Cu(I)) using neocuproine.

The concentrations of neocuproine and tea extracts were 400 µM and 6.6 µg/ml respectively. Other details of the procedure are given in ‘Methods’.

(●) green tea extract
(○) black tea extract

All points represent triplicate samples and mean values have been plotted.
epigallocatechin and epigallocatechin-3-gallate. However, it is not excluded that other constituents of tea extracts might act as reductants for Cu(II) as well. Further, the results suggest a possible redox cycling of copper ions in the reaction. It would thus appear that the ‘oxidized species’ of the components of green and black tea are also able to catalyze the reduction of recycled copper ions [Cu(II)]. In such a case increased copper concentrations at a fixed concentration of tea extract should result in increased rate of DNA hydrolysis. As shown in Figure 8 this is indeed found to be the case. When calf thymus DNA was treated with increasing concentrations of Cu(II) at a fixed concentration of green tea extract concomitant enhancement in the rate of DNA hydrolysis was observed.

**Effects of alternative metal ions**

Of the several metal ions tested, only Cu(II) and Fe(II) complemented the tea extracts in the DNA breakage reaction to a significant extent (Table I). Although some DNA hydrolysis is also seen with Zn(II), Mg(II), Co(II), Ca(II), Mn(II) and Ni(II). These results are similar to those seen with other polyphenols such as flavonoids (Rahman *et al.*, 1989).

**Production of hydroxyl radicals by tea extracts**

I demonstrated that tea extracts and Cu(II) generate hydroxyl radicals. The assay involves incorporation of salicylate as a reporter
Figure 8: Effect of different Cu(II) concentrations at a fixed concentration of green tea extract on calf thymus DNA degradation.

DNA was treated with (▼) 50 μM, (○) 100 μM, (●) 200 μM of Cu(II) and 400 μg/ml of green tea extract at 37°C for different time intervals and subjected to single strand specific nuclease digestion. All points represent triplicate samples and mean values have been plotted.
Table I: $S_1$ nuclease hydrolysis of DNA treated with green and black tea extracts in the presence of different metal ions.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>DNA hydrolyzed (%)</th>
<th>Green tea extract</th>
<th>Black tea extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(II)</td>
<td>22.7 ± 1.38</td>
<td>16.28 ± 1.19</td>
<td></td>
</tr>
<tr>
<td>Fe(II)</td>
<td>11.73 ± 1.02</td>
<td>7.65 ± 1.22</td>
<td></td>
</tr>
<tr>
<td>Zn(II)</td>
<td>3.82 ± 0.72</td>
<td>3.04 ± 0.64</td>
<td></td>
</tr>
<tr>
<td>Mg(II)</td>
<td>3.36 ± 0.44</td>
<td>3.34 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>Co(II)</td>
<td>2.63 ± 0.19</td>
<td>2.57 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>Ca(II)</td>
<td>2.61 ± 0.23</td>
<td>2.44 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Mn(II)</td>
<td>2.41 ± 0.21</td>
<td>2.40 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Ni(II)</td>
<td>1.29 ± 0.29</td>
<td>1.26 ± 0.23</td>
<td></td>
</tr>
</tbody>
</table>

Treatment of calf thymus DNA (1000µg/ml) in Tris-HCl (10 mM, pH 7.5), with the tea extracts (400µg/ml) was carried out with respective metal ions (0.1 mM) in sterile tubes at room temperature for 1 hour. At the end of the incubation period the reaction mixtures were subjected to $S_1$ nuclease hydrolysis in the standard assay.

All values are expressed as Mean ± SE for three different experiments.
molecule (Richmond et al., 1981). Table II compares the rate of formation of hydroxyl radicals by green and black tea extracts in the presence of Cu(II). As seen green tea extract is a more efficient generator of the hydroxyl radicals than the black tea. Figure 9 shows that tea extracts — Cu(II) generated hydroxyl radicals that react with calf thymus DNA. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA reactive material, which forms a coloured adduct readable at 532 nm (Quinlan and Gutteridge, 1987). The results confirm the relatively higher rate of formation of hydroxyl radicals by green tea extract and correlate with the rate of DNA degradation by the two extracts.

The most probable mechanism for the formation of hydroxyl radicals would be a Fenton type reaction involving H₂O₂ and Cu(I). However prior presence of H₂O₂ may not be necessary because it is known that generation of superoxide anion may lead to the formation of H₂O₂ (Haliwell and Gutteridge, 1984). The addition of a second electron to the O₂⁻ anion gives the peroxide ion (2O₂⁻), which has no unpaired electron and is not a radical. However, at neutral pH the peroxide ion immediately protonates to give hydrogen peroxide (H₂O₂). Alternatively, in aqueous solutions the superoxide anion undergoes dismutation to form H₂O₂ and O₂. I have therefore compared the rate of formation of superoxide anion by green and black tea extracts by the NBT reduction method (see ‘Methods’). The results in Figure 10 show that the anion is generated by green tea at a higher rate than black tea.
Table II: Formation of hydroxy radical as a function of green and black tea extracts concentrations.

<table>
<thead>
<tr>
<th>Green and Black tea extract (µg/ml)</th>
<th>Hydroxylated product formed (n mol)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Green tea extract</td>
<td>Black tea extract</td>
</tr>
<tr>
<td>5</td>
<td>4.40 ± 0.27</td>
<td>1.89 ± 0.36</td>
</tr>
<tr>
<td>25</td>
<td>9.94 ± 1.04</td>
<td>4.33 ± 1.09</td>
</tr>
<tr>
<td>50</td>
<td>23.01 ± 3.27</td>
<td>10.16 ± 1.53</td>
</tr>
<tr>
<td>100</td>
<td>38.93 ± 2.34</td>
<td>13.61 ± 1.09</td>
</tr>
<tr>
<td>200</td>
<td>59.46 ± 0.59</td>
<td>19.42 ± 1.56</td>
</tr>
</tbody>
</table>

Reaction conditions are described in ‘Methods’ and values of tea extracts are final reaction concentrations.

All values are expressed as Mean ± SE for three different experiments.
Figure 9: Production of hydroxyl radicals as a function of increasing green tea extract and black tea extract concentrations.

The reaction mixtures containing increasing concentrations of tea extracts (50-800μg/ml) in the presence of 0.1 mM Cu (II) were incubated for half an hour at 37°C. Reaction conditions are described in ‘Methods’.

(●) green tea extract
(○) black tea extract
All points represent triplicate samples and mean values have been plotted.
Figure 10: Photogeneration of superoxide anion by tea extracts as a function of time.

(●): 66μg/ml green tea extract under fluorescent light
(○): 66μg/ml black tea extract under fluorescent light
The detailed procedure is given in ‘Methods’.
All points represent triplicate samples and mean values have been plotted.
This observation is in conformity with the higher rate of hydroxyl radical formation and DNA degradation by green tea extract.

**Effect of oxygen radical scavengers on calf thymus DNA breakage by green and black tea extracts**

Table III shows the effect of various oxygen radical scavengers on DNA breakage by green and black tea extracts and copper ions. In agreement with the previous results green tea extract was considerably more efficient in DNA degradation in the absence of any scavenger. The scavengers tested showed varying degree of inhibition suggesting the essential role of oxygen radicals. Of the scavengers of the hydroxyl radical, thiourea was the most effective, whereas mannitol showed twice as much inhibition in the case of green tea. Catalase and sodium azide as removers of hydrogen peroxide and singlet oxygen respectively showed significant inhibition. Whereas superoxide dismutase (SOD), which scavenges superoxide anion was more effective in green tea mediated degradation. Neocuproine which sequesters Cu(I) also had a differential effect being considerably less effective in the case of black tea.

**Effect of green tea and Cu(II) on the viability of phage T4**

In order to explore the biological consequence of the DNA breakage reaction, inactivation of bacteriophage T4 by the green tea –
Table III: Percent inhibition of S1 nuclease hydrolysis of DNA after treatment with 800 μg/ml of tea extracts and 100 μM Cu(II) in the presence of scavengers.

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Green Tea</th>
<th>Black Tea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% inhibition</td>
<td>% inhibition</td>
</tr>
<tr>
<td>Thiourea (20 mM)</td>
<td>85.4 ± 0.71</td>
<td>84.1 ± 0.55</td>
</tr>
<tr>
<td>Catalase (0.1 mg/ml)</td>
<td>46.1 ± 0.55</td>
<td>42.3 ± 0.76</td>
</tr>
<tr>
<td>Sodium Azide (20 mM)</td>
<td>45.4 ± 0.41</td>
<td>34.1 ± 0.84</td>
</tr>
<tr>
<td>Mannitol (20 mM)</td>
<td>41.2 ± 0.69</td>
<td>19.7 ± 0.53</td>
</tr>
<tr>
<td>SOD (20 mM)</td>
<td>36.1 ± 0.76</td>
<td>17.3 ± 0.66</td>
</tr>
<tr>
<td>Neocuproine (1 mM)</td>
<td>29.9 ± 0.56</td>
<td>11.7 ± 0.44</td>
</tr>
<tr>
<td>Boiled catalase (0.1 mg/ml)</td>
<td>19.95 ± 0.46</td>
<td>3.3 ± 0.24</td>
</tr>
</tbody>
</table>

Concentration of scavengers shown are final reaction concentrations. Details of the reaction mixture are given in 'Methods'.

All values are expressed as Mean ± SE for three different experiments.
Cu(II) system was tested. The inactivating activity was determined by incubating the phage with green tea extract and Cu(II) and then measuring the loss of biological activity. Figure 11 gives the loss of survival of phage as a function of green tea concentration. Increasing concentrations of green tea at a fixed concentration of Cu(II) (100μM) after an hour of incubation at 37°C, resulted in a progressive loss of survival of the phage. The percentage of phage surviving at 200 μg/ml of green tea extract is only about 15%. Increasing concentrations of Cu(II) with a fixed green tea concentration of 200 μg/ml also resulted in a similar loss of survival of the phage (results not shown).

As shown above Cu(I) is an essential intermediate in the DNA breakage reaction. In order to examine its role in phage inactivation I have studied the effect of increasing concentrations of neocuproine on phage survival. The concentration of Cu(II) used was 50 μM. The result given in Figure 12 show that the phage inactivation is inhibited to about 90% indicating the essential involvement of Cu(I) in the reaction. This result indicate that the formation of Cu(I) by green tea is an essential step in the phage inactivation mechanism.

The inactivating activity of green tea – Cu(II) system was substantially but differentially reduced by prior addition of quenchers of oxygen free radicals (Table IV). Maximum inhibition was afforded by sodium benzoate and catalase followed by sodium azide and thiourea indicating the involvement of hydroxyl radical, hydrogen
Effect of increasing concentrations of green tea extract in the presence of 100 μM Cu(II) on the viability of bacteriophage T₄. Phage suspension was treated with indicated concentrations of green tea extract at 37°C for 1 hour in a reaction mixture containing 10mM Tris-HCl/Mg²⁺ (pH 8.0). The detailed procedure is given in ‘Methods’. All points represent triplicate samples and mean values have been plotted.
Figure 12: Effect of increasing concentrations of neocuproine on the viability of green tea extract – Cu(II) treated T₄ phage.

The concentrations of green tea extract and Cu(II) were 400μg/ml and 50 μM respectively. Other details of the procedure are given in ‘Methods’. All points represent triplicate samples and mean values have been plotted.
Table IV: Percent inhibition of T₄ phage inactivation after treatment with green tea (100μg/ml) and Cu(II) (25μM) in the presence of scavengers of reactive oxygen species.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% inhibition of T₄ inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium benzoate (50mM)</td>
<td>56.11 ± 1.51</td>
</tr>
<tr>
<td>Catalase (100μg/ml)</td>
<td>49.38 ± 1.83</td>
</tr>
<tr>
<td>Boiled catalase (100 μg/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Sodium Azide (50 mM)</td>
<td>34.49 ± 1.86</td>
</tr>
<tr>
<td>Thiourea (50 mM)</td>
<td>18.2 ± 0.10</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± SE for three different experiments.
peroxide and singlet oxygen in T4 phage inactivation reaction by green tea. These results and those of the previous experiments strongly indicate that the DNA degradation and phage inactivation by green tea and copper ions essentially follow a similar mechanism of action.

Table V shows the sensitivity of bacteriophage T4 to green tea – Cu(II) and the effect of UV irradiation on the host cells. Control incubations of phage with 400 μg/ml green tea alone or 100 μM Cu(II) alone resulted in <5% inactivation. The fractions of surviving phage, after green tea – Cu(II) treatment, were 0.028 and 0.38 in the case of untreated and UV treated host cells, respectively. It may be noted that at 400 μg/ml green tea concentration used in this experiment, the fraction of phage survival (0.028) matches the degree of inactivation in Figure 11 (0.05). This result indicate that UV treatment of the host bacteria enhance the recovery of the phage. One possibility could be that the UV inducible pathway is activated on such treatment leading to enhanced recovery of the phage.
Table V: Sensitivity of $\lambda$ phage to green tea-Cu(II) and the effect of UV-irradiation on host cells.

<table>
<thead>
<tr>
<th>Host Strain</th>
<th>Host pretreatment</th>
<th>Phage pretreatment</th>
<th>PFU/ml</th>
<th>Survival$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli BB</td>
<td>None</td>
<td>Control</td>
<td>$(9.51\pm0.049)\times10^9$</td>
<td>1</td>
</tr>
<tr>
<td>E. coli BB</td>
<td>None</td>
<td>GTE-Cu(II)$^b$</td>
<td>$(2.73\pm0.079)\times10^8$</td>
<td>0.028</td>
</tr>
<tr>
<td>E. coli BB</td>
<td>UV</td>
<td>Control</td>
<td>$(7.81\pm0.063) \times 10^9$</td>
<td>1</td>
</tr>
<tr>
<td>E. coli BB</td>
<td>UV</td>
<td>GTE-Cu(II)$^b$</td>
<td>$(3.01\pm0.051) \times 10^9$</td>
<td>0.384</td>
</tr>
</tbody>
</table>

Incubation was done for 1 hour at $37^\circ$C followed by dilution and plating.

All values are expressed as Mean ± SE for three different experiments.

$^a$The value of survival was calculated by dividing values in the presence of green tea-Cu(II) by control values.

$^b$Concentrations of green tea and Cu(II) used were 400$\mu$g/ml and 100$\mu$M respectively.
DISCUSSION (Part I)

The major conclusions of the above experiments may be stated as follows: (i) In agreement with earlier reports (Wei et al., 1999) green tea extract contains catechins which include epicatechin, epigallocatechin, epigallocatechin-3-gallate and these are absent in black tea extract; (ii) the rate of DNA degradation in the presence of Cu(II) by water extract of green tea is considerably greater than by the extract of black tea; (iii) this correlates with the higher rate of Cu(II) reduction and hydroxyl radical generation by green tea extract; (iv) it is further suggested that ‘oxidized’ polyphenolic constituents of tea extract are also capable of catalyzing the reduction of redoxcycled copper ions; (v) Phage inactivation studies indicate that the DNA breakage reaction is biologically significant.

The structural units of catechins in green tea, shown in Figure 1 are epicatechin and gallic acid. Previously it has been shown that both these polyphenols catalyze strand breakage in DNA in the presence of copper ions (Khan and Hadi, 1998; Ahmad et al., 1992). It was also shown that the rate of DNA degradation correlates with the number of hydroxyl groups present particularly with ortho-dihydroxy configuration. Indeed, when two of the three hydroxyl groups of gallic acid are methylated (syringic acid) the DNA degrading capacity declines sharply (Khan and Hadi, 1998). This result correlates with the
observations (Inoue et al., 1994) which showed that whereas gallic acid exhibits apoptotic activity, syringic acid is inactive. In the manufacture of black tea, the monomeric flavan-3-ol (epicatechin) undergoes polyphenol oxidase dependent oxidative polymerization leading to the formation of bis-flavanols, theaflavins, thearubigins and other oligomers. The formation of these compounds involves the hydroxyl groups, leading to a reduction in the number of copper ion chelating positions relative to the size of the molecule. This possibly accounts for the reduced DNA degradation and copper reducing capability of black tea extract.

The results of bacteriophage T4 inactivation studies indicate that green tea–Cu(II) mediated action also causes bacteriophage inactivation through a mechanism similar to DNA breakage. This is particularly suggested by (i) the effect of scavengers on phage inactivation and (ii) that the phage inactivation is inhibited by the neocuproine which sequesters Cu(I). These results further indicate that UV treatment of host bacteria enhances the recovery of the phage, indicating the involvement of UV inducible pathway in the repair of green tea – Cu(II) mediated damage of phage DNA.

Most clinically used anticancer drugs can activate late events of apoptosis (DNA degradation and morphological changes) and the essential signaling pathways differ between pharmacological cell death and physiological induction of programmed cell death (Smets, 1994).
Fe$^{3+}$ and Cu$^{2+}$ are the most redox-active of the metal ions in living cells. Although iron is considerably more abundant in cells the major ions in the nucleus are copper and zinc (Bryan, 1979). It has been proposed (Wolfe et al., 1994) that a copper mediated Fenton reaction, generating site-specific hydroxyl radicals, is capably of inducing apoptosis in thymocytes. Further, it has been shown (Burkitt et al., 1996) that the internucleosomal DNA fragmentation might be caused not only by an endonuclease but also by metal chelating agents such as 1,10-phenanthroline (OP), which promotes the redox activity of endogenous copper ions and the resulting production of hydroxyl radicals. Thus, the internucleosomal DNA “laddering” often used as an indicator of apoptosis may also reflect DNA fragmentation by non-enzymatic processes. Several reports indicate that serum (Ebadi and Swanson, 1988) and tissue (Yoshida et al., 1993) concentrations of copper are greatly increased in various malignancies. Indeed, such concentrations have been described as a sensitive index of disease activity of several hematologic and non-hematologic malignancies (Pizzolo et al., 1978). Irrespective of the exact mechanism involved these facts and other observations in literature suggest that the various anticancer effects and apoptotic DNA fragmentation activities of several plant derived polyphenols, including those in green tea, may be explained by their ability to mobilize endogenous or otherwise increased concentrations of copper in cancer cells. However, it is recognized that the results presented above provide only partial support
to our hypothesis (Hadi et al., 2000), which implies a prooxidant effect, involving mobilization of copper ions by green tea polyphenols, on cancer cells. Therefore, further experiments on the effect of green tea extract and the constituent polyphenols, on the induction of oxidative damage to certain critical cellular targets have to be carried out. Further, the fact that these polyphenolic compounds are ingested by human populations as a part of the normal diet in relatively higher concentrations without adverse reactions points to their great potential as putative chemopreventive or therapeutic agents.
Part - II
RESULTS (Part-II)

As already mentioned, gallic acid is structural constituent of tannins and polyphenols of green tea. It shows a number of biological properties including induction of apoptosis. Similar to green tea it also exhibits antioxidant and pro-oxidant properties. Thus, with the aim of identifying the structural features of green tea polyphenols, I have compared the prooxidant DNA cleavage properties and antioxidant properties of gallic acid and green tea.

Breakage of calf thymus DNA by gallic acid and green tea extract

Figure 13 gives the acid soluble material produced from calf thymus DNA with increasing concentrations of gallic acid and green tea extract at a fixed concentration of Cu(II). The rate of DNA hydrolysis and the maximum hydrolysis achieved was greater with gallic acid than with green tea.

Production of hydroxyl radical by gallic acid and green tea extract

Figure 14 shows that gallic acid and green tea – Cu(II) generate hydroxyl radicals that react with calf thymus DNA. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA reactive material, which forms a coloured adduct readable at 532 nm (Quinlan and Gutteridge, 1987). The results confirm the relatively higher rate of formation of hydroxyl radicals by
Figure 13: Degradation of calf thymus DNA as a function of increasing concentrations of gallic acid and green tea extract in the presence of Cu(II) as measured by the degree of S$_1$ nuclease digestion.

DNA was incubated with increasing concentrations (50-400 μg/ml) of gallic acid (●) and green tea extract (▼) in the presence of 0.1 mM Cu(II) at 37°C for 1 hour.
All points represent triplicate samples and mean values have been plotted.
Gallic acid/Green tea extract (µg/ml)

% DNA hydrolyzed

Gallic acid/Green tea extract (µg/ml)
Figure 14: Production of hydroxyl radicals as a function of increasing gallic acid and green tea extract concentrations.

The reaction mixtures containing increasing concentrations of gallic acid and green tea extract (50-400 μg/ml) in the presence of 0.1 mM Cu(II) were incubated for half an hour at 37°C. Reaction conditions are described in ‘Methods’.

gallic acid (●)
green tea extract (▼)

All points represent triplicate samples and mean values have been plotted.
gallic acid and correlate with the rate of DNA degradation by the two compounds.

**Photogeneration of superoxide anion by gallic acid and green tea extract**

I have compared the rate of formation of superoxide anion by gallic acid and green tea extract by NBT reduction method (See Methods). The result in Figure 15 show that the anion is generated by gallic acid at a higher rate than green tea. This observation is in conformity with the higher rate of hydroxyl radical formation and DNA degradation by gallic acid. It may be mentioned that superoxide anion spontaneously leads to the formation of hydrogen peroxide (Halliwell and Gutteridge, 1984), which in the presence of reduced copper (Fenton reaction) can form hydroxyl radicals.

**Effect of alternative metal ions**

Of the several metal ions tested only Cu(II) and Fe(III) complemented green tea extract and gallic acid in the DNA breakage reaction to any significant extent (Table VI). Although some hydrolysis is also seen with Fe(II), Zn(II), Ca(II), Mg(II), Co(II), Mn(II) and Ni(II). These results are similar to those seen with other polyphenols such as flavonoids (Rahman et al., 1989). However in agreement with the relative DNA hydrolysis by the two systems, gallic acid is considerably more active in the presence of Cu(II) and Fe(III).
Figure 15: Photogeneration of superoxide anion by gallic acid and green tea extract as a function of time.

(●): 66 µg/ml gallic acid under fluorescent light
(▼): 66 µg/ml green tea extract under fluorescent light
The detailed procedure is given in ‘Methods’.
All points represent triplicate samples and mean values have been plotted.
Table VI: \( \text{S}_1 \) nuclease hydrolysis of DNA treated with gallic acid and green tea extract in the presence of different metal ions.

| Metal ion | DNA hydrolyzed (%) |  |  |
|-----------|---------------------|  |  |
|           | Gallic acid         | Green tea extract |
| Cu(II)    | 20.23 ± 0.48        | 14.35 ± 0.41 |
| Fe(III)   | 11.5 ± 0.71         | 7.67 ± 0.66 |
| Fe(II)    | 7.47 ± 0.11         | 4.64 ± 0.15 |
| Zn(II)    | 4.38 ± 0.17         | 3.56 ± 0.13 |
| Ca(II)    | 3.66 ± 0.14         | 3.48 ± 0.15 |
| Mg(II)    | 3.33 ± 0.21         | 2.86 ± 0.19 |
| Co(II)    | 2.58 ± 0.13         | 2.26 ± 0.14 |
| Mn(II)    | 2.09 ± 0.16         | 1.64 ± 0.11 |
| Ni(II)    | 1.26 ± 0.10         | 0.63 ± 0.04 |

Treatment of calf thymus DNA (1000\( \mu \)g/ml) in Tris-HCl (10 mM, pH 7.5), with compounds (400\( \mu \)g/ml) was carried out with respective metal ions (0.1 mM) in sterile tubes at room temperature for 1 hour. At the end of the incubation period the reaction mixtures were subjected to \( \text{S}_1 \) nuclease hydrolysis in the standard assay.

All values are expressed as Mean ± SE for three different experiments.
Effect of gallic acid and green tea extract on Fe(II)-EDTA mediated degradation of calf thymus DNA as measured by the degree of S1 nuclease digestion

As mentioned above the polyphenolic constituents of green tea and gallic acid are considered to be physiological antioxidants in plants. I have therefore tested these activities on hydroxyl radical mediated degradation of DNA. The generation of hydroxyl radical was carried out by the Fe(II) – EDTA hydroxyl radical generating system (Prigodich and Martin, 1990). Figure 16 shows the effect of increasing concentrations of gallic acid and green tea extract on Fe(II) – EDTA mediated degradation of calf thymus DNA in the presence of H2O2 and ascorbate. The result shows that green tea exhibited a greater protective effect than gallic acid.

Formation of hydroxyl radicals by Fe(II) – EDTA and their quenching by gallic acid and green tea extract

Using Fe(II) – EDTA system (Prigodich and Martin, 1990), I have directly tested the effect of increasing concentrations of gallic acid and green tea extract on the generation of hydroxyl radicals. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA reactive material, which forms a coloured adduct with TBA, readable at 532 nm (Quinlan and Gutteridge, 1987). Figure 17 shows that gallic acid has a quenching effect on the production of hydroxyl radicals. Green tea is also shown
Figure 16: Inhibition of hydroxyl radical [generated by Fe(II)-EDTA-H$_2$O$_2$] mediated degradation of calf thymus DNA by increasing concentrations of gallic acid and green tea extract as measured by the degree of $S_1$ nuclease digestion.

Calf thymus DNA was treated with 1mM sodium ascorbate (pH 7.0), 40 µM Fe(II), 80 µM EDTA and 0.03% H$_2$O$_2$ in the presence of increasing concentrations of gallic acid (●) and green tea extract (▼) (100-600 µg/ml) for 45 minutes at 37°C. At the end of the incubation period the reaction mixture was subjected to $S_1$ nuclease hydrolysis. All points represent triplicate samples and mean values have been plotted.
Figure 17: Formation of hydroxyl radicals by Fe(II) – EDTA – H₂O₂ system and their quenching by increasing concentrations of gallic acid and green tea extract.

Calf thymus DNA (400μg/ml) was treated with 0.4mM sodium ascorbate (pH 7.0), 40μM Fe(II), 80μM EDTA and 0.03% H₂O₂ in the presence of increasing concentrations of gallic acid (●) and green tea extract (▼) (100-600 μg/ml) for half an hour at 37°C. Reaction conditions are described in ‘Methods’. All points represent triplicate samples and mean values have been plotted.
to quench the production of radicals but its effect is relatively less than that of gallic acid.

**Effect of gallic acid and green tea extract on riboflavin and light mediated DNA cleavage**

Riboflavin being a photo oxidant is known to generate singlet oxygen and cause subsequent DNA degradation in the presence of visible light (Naseem *et al.*, 1993). In order to confirm the relative antioxidant property of gallic acid and green tea extract, the effect of the two compounds on such DNA degrading system was examined. In agreement with the above finding (Figure 16), Figure 18 shows that green tea is a considerably better inhibitor of DNA breakage by riboflavin as compared to gallic acid.
Figure 18: Inhibition of photosensitized riboflavin mediated degradation of calf thymus DNA by increasing concentrations of gallic acid and green tea extract as measured by the degree of S1 nuclease digestion.

Calf thymus DNA in 10mM Tris-HCl (pH 7.5) was incubated with 20 μM riboflavin in the presence of increasing concentrations of gallic acid (●) and green tea extract (▼) (100-600 μg/ml) for 2 hours at room temperature under fluorescent light. At the end of the incubation period the reaction mixture was subjected to S1 nuclease hydrolysis.
All points represent triplicate samples and mean values have been plotted.
Discussion (Part-II)

The major polyphenolic constituent of green tea, which has shown chemopreventive properties in a number of studies, is epigallocatechin-3-gallate (EGCG). The major structural constituent of EGCG is gallic acid (Figure 1). Similar to EGCG, gallic acid itself is considered to be an antioxidant and induces apoptosis in cancer cells (Inoue et al., 1994). As already mentioned in the ‘Introduction’ most plant polyphenols also exhibit prooxidant properties particularly in the presence of transition metal ions such as copper, therefore in the above experiments I have compared the prooxidant and antioxidant properties of green tea extract with gallic acid. The major conclusions of these experiments may be stated as follows: (i) the rate of DNA degradation in the presence of Cu(II) by gallic acid is considerably greater than by green tea extract; (ii) this correlates with the higher rate of hydroxyl radical and super oxide anion generation by gallic acid; (iii) gallic acid is also a more efficient quencher of hydroxyl radical generation (Figure 17).

The other polyphenols in green tea that have been implicated in chemoprevention against cancer and cardiovascular disease are epigallocatechin (EGC) and epicatechin (EC). The former also contains gallic acid as a structural component. It is thus possible that gallic acid contributes significantly to the pharmacological action of green tea polyphenols. Structure activity studies carried out in our laboratory
with tannic acid and gallic acid indicate that if two of the three hydroxyl groups of gallic acid are methylated (syringic acid), the DNA degrading capacity decreases sharply (Khan and Hadi, 1998). These results correlate with those of Inoue et al. (1994) who showed that modification of phenolic hydroxyl groups, such as that resulting in the formation of syringic acid, abolishes the apoptotic activity of gallic acid. Several lines of evidence in the literature strongly suggest that the antioxidant properties of plant polyphenolics may not entirely account for their chemopreventive effects (Hadi et al., 2000). For example, Burkitt et al. (1996) suggested that the internucleosomal DNA fragmentation might be caused not only by an endonuclease but also by metal chelating agents such as 1,10-phenanthroline (OP), which promote the redox activity of endogenous copper ions and the resulting production of hydroxyl radicals. In addition, several reports indicate that serum (Ebadi and Swanson, 1988) and tissue (Yoshida et al., 1993) concentrations of copper are greatly increased in various malignancies. Indeed, such concentrations have been described as a sensitive index of disease activity of several hematologic and nonhematologic malignancies (Pizzolo et al., 1978).
Part - III
RESULTS (Part-III)

Breakage of calf thymus DNA and cleavage of plasmid DNA by gallic acid, ellagic acid and tannic acid

Figure 19 gives the rate of S1 nuclease hydrolysis of calf thymus DNA following damage induced by gallic acid, ellagic acid and tannic acid in presence of Cu(II). The reaction was assayed by recording the proportion of double stranded DNA converted to acid soluble nucleotides by S1 nuclease. Control experiments (data not shown) established that heat denatured DNA underwent 100% hydrolysis following the treatment with S1 nuclease, whereas only 3% of native DNA was hydrolyzed. As seen in the Figure 19 the efficacy of the compounds tested was as follows: gallic acid > ellagic acid > tannic acid. There is a gradual increase of DNA degradation upon treatment with increasing concentrations of the compounds tested at a fixed concentration of Cu(II). It may be seen that gallic acid causes the largest degree of hydrolysis and lowest degree is achieved by tannic acid.

In order to further substantiate the above results, conversion of supercoiled plasmid DNA to relaxed open circles and linear forms was also examined. This is a sensitive test for just one nick per molecule. Figure 20 shows the ethidium bromide stained banding pattern of pBR 322 DNA with two different concentrations (50 μM
Figure 19: Degradation of calf thymus DNA as a function of increasing concentration of various compounds in the presence of Cu(II) as measured by the degree of S1 nuclease digestion.

DNA was incubated with increasing concentrations (5 µM – 100 µM) of gallic acid (O), ellagic acid (▼) and tannic acid (●) in the presence of 0.1 mM Cu(II) at 37°C for 2 hours. All points represent triplicate samples and mean values have been plotted.
Gallic acid/ Ellagic acid/ Tannic acid (µM)

% DNA hydrolyzed

0 10 20 30 40

0 20 40 60 80 100
Figure 20: Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 DNA after treatment with increasing concentration of various compounds in presence of Cu(II).

Reaction mixtures in a volume of 30 μl containing 0.4 μg plasmid pBR322 DNA, 0.1 mM Cu(II) and increasing concentrations of tannic acid, gallic acid and ellagic acid were incubated for 1 hour at 37°C.
Lane a: DNA alone
Lane b: DNA + Cu(II) + tannic acid (50μM)
Lane c: DNA + Cu(II) + tannic acid (100μM)
Lane d: DNA + Cu(II) + gallic acid (50μM)
Lane e: DNA + Cu(II) + gallic acid (100μM)
Lane f: DNA + Cu(II) + ellagic acid (50μM)
Lane g: DNA + Cu(II) + ellagic acid (100μM)
and 100 μM) of gallic acid, ellagic acid and tannic acid. At both the concentrations tannic acid gives partial conversion to relaxed open circle (lanes b and c), gallic acid degrades DNA to small heterogeneous sized molecules giving rise to a smear in lanes d and e and ellagic acid shows conversion to relaxed open circles as well as some linear molecules (lanes f and g). Thus these results are in conformity with the relative DNA degrading capacity of the three compounds seen in Figure 19.

**Stoichiometry of Cu(II) reduction by gallic acid, ellagic acid and tannic acid**

To determine the stoichiometry of Cu(II) reduction by gallic acid, ellagic acid and tannic acid, the Cu(I) sequestering agent namely neocuproine was used. A Job plot (Wong et al., 1984) of absorbance versus [Cu(II)/tannic acid] reveals maximum absorbance at the Cu(II)/tannic acid molar ratio of 6.0 at which maximum conversion of Cu(II) to Cu(I) is achieved (Figure 21) (Bhat and Hadi, 1994). Such ratio was determined to be 6 and 4 for the gallic acid and ellagic acid respectively. The ratio obtained gives the stoichiometry for the reduction of Cu(II) by the free compounds. Except for tannic acid these values correlate with the relative rates of DNA degradation by gallic acid and ellagic acid.
Figure 21: Determination of stoichiometry of Cu(II) reduction by various compounds.

Concentration of compounds used was (●) 0.01 mM in the presence of 0.4 mM neocuproine. The absorbance at 450 nm of samples with added Cu(II) is plotted versus molar equivalents of Cu(II) per molar equivalent of compound. The value of independent variable at the intersection of the two lines is a measure of the moles of Cu(II) converted to Cu(I) per mole of compound. The values obtained for various compounds are:
(a) Tannic acid, 6:1, (b) Gallic acid, 6:1 and (c) Ellagic acid, 4:1.
**Generation of hydroxyl radical (OH') by gallic acid, ellagic acid and tannic acid.**

The capacity of gallic acid, ellagic acid and tannic acid to generate hydroxyl radical in the presence of Cu(II) was also compared (Figure 22). This assay uses salicylate as the reporter molecule (Richmond *et al.*, 1981). The result shows that ellagic acid is the least efficient in generating hydroxyl radical whereas tannic acid is the most efficient, followed by gallic acid.

**Effect of gallic acid, ellagic acid and tannic acid on Fe(II)-EDTA mediated degradation of calf thymus DNA.**

The relative antioxidant effect of gallic acid, ellagic acid and tannic acid was examined by their ability to protect DNA breakage by hydroxyl radicals. The generation of hydroxyl radicals was carried out by the Fe(II)-EDTA hydroxyl radical generating system (Prigodich and Martin, 1990). Figure 23 shows the effect of increasing concentrations of gallic acid, ellagic acid and tannic acid on Fe(II)-EDTA mediated degradation of calf thymus DNA in the presence of H$_2$O$_2$ and ascorbate. The result shows that tannic acid is a considerable better inhibitor of such DNA breakage compared to gallic acid, which shows least inhibition followed by ellagic acid.
Figure 22: Production of hydroxyl radicals as a function of increasing gallic acid, ellagic acid and tannic acid concentrations.

The reaction mixtures containing increasing concentrations of compounds (2-25 μM) were incubated for 2 hours at 37°C. Reaction conditions are described in ‘Methods’.

(○) gallic acid
(▼) ellagic acid
(●) tannic acid

All points represent triplicate samples and mean values have been plotted.
Figure 23: Inhibition of hydroxyl radical [generated by Fe(II)-EDTA-H$_2$O$_2$] mediated degradation of calf thymus DNA by increasing concentration of the compounds as measured by the degree of S$_1$ nuclease digestion.

Calf thymus DNA was treated with 1mM sodium ascorbate (pH 7.0), 40 $\mu$M Fe(II), 80 $\mu$M EDTA and 0.03% H$_2$O$_2$ in the presence of increasing concentrations of gallic acid (○), ellagic acid (▼) and tannic acid (●) (25 – 100 $\mu$M) for 1 hour 30 minutes at 37°C. At the end of the incubation period the reaction mixture was subjected to S$_1$ nuclease hydrolysis. All points represent triplicate samples and mean values have been plotted.
Gallic acid/ Ellagic acid/ Tannic acid (μM)
Interaction of gallic acid, ellagic acid and tannic acid with Cu(II)

Absorption spectrum of gallic acid alone gave $\lambda_{max}$ at 211nm and 260nm (Figure 24(a)). When Cu(II) was added a shift occurred in $\lambda_{max}$ of gallic acid from 211nm to 226nm and 260nm to 290nm (Figure 24(b)). As with tannic acid when Cu(II) was added there was a decrease in the absorbance at $\lambda_{max}$ of 215nm and a shift occurred in the $\lambda_{max}$ of tannic acid from 280 nm to 323nm (Figure 24(c) and 24(d)). Ellagic acid alone gave peaks at 225nm, 255nm, 275nm and 366 nm (Figure 24(e)) and addition of Cu(II) lead to the formation of a single peak absorbing at 280 nm (Figure 24(f)). These results are indicative of the binding of Cu(II) to gallic acid, tannic acid and ellagic acid.
Figure 24: Effect of Cu(II) on the absorption spectrum of gallic acid, tannic acid and ellagic acid.

The absorption solution contained 50µM gallic acid, 10 µM tannic acid, 10 µM ellagic acid and 100 µM Cu(II) in 10 mM Tris-HCl (pH 7.5).

(a) gallic acid alone
(b) gallic acid and Cu(II)
(c) tannic acid alone
(d) tannic acid and Cu(II)
(e) ellagic acid alone
(f) ellagic acid and Cu(II)
Wavelength (nm)  

Absorbance

Wavelength (nm)

(a)  

(b)
Studies in previous section (Part-II) showed that gallic acid possess a potent prooxidant activity as evidenced by its capacity to degrade DNA in the presence of Cu(II) and to generate hydroxyl radicals. It is a structural constituent of tannic acid present as a digalloyl moiety. Ellagic acid is another plant-derived polyphenol which is considered to possess anticarcinogenic properties (Gali et al., 1992; Khanduja et al., 1999). However, various polyphenols exhibit anticancer and apoptosis inducing properties to variables degrees. Thus it is not clear as to what structural features of polyphenols are important in imparting the various pharmacological properties. For example although ellagic acid is 10 times more potent an antioxidant than is tannic acid, the latter was more effective than the former in inhibiting the promotion of skin tumour by 12-O-tetradecanoyl phorbol-13-acetate (Gali et al., 1992). The authors suggested that the antioxidant effects of those polyphenols may be essential but not sufficient for their activity against tumour promotion. As already mentioned it has been proposed in this laboratory that the cytotoxic action of plant polyphenols against cancer cells possibly involves mobilization of endogenous copper ions and the consequent prooxidant action. I have therefore compared the prooxidant properties of gallic acid (GA), tannic acid (TA) and ellagic acid (EA) with respect to their ability to oxidatively degrade DNA and to reduce copper ions. The relative rates of DNA degradation by GA,
TA and EA was established as GA > EA > TA (Figure 19). Although the stoichiometry of Cu(II) reduction by gallic acid and tannic acid is the same (1:6), gallic acid degrades DNA at a considerable greater rate. On the other hand stoichiometry of Cu(II) reduction by ellagic acid could not be determined as it does not gives a clear maximum of Cu(I) formation where absorption becomes constant. This suggests a possible recycling of copper ions in the reaction. Also the number of free hydroxyl groups in these molecules does not seem to be important as tannic acid would contain a much larger number of such groups compared to both ellagic acid and gallic acid. The rate of hydroxyl radical generation by the three compounds also does not correlate with rate of DNA degradation. The lesser DNA degrading capacity of tannic acid may be possibly explained by the reduced electron density of gallic acid when it is part of the tannic acid molecule. The decreased electron density at phenolic hydroxyls would lead to a reduced Cu(II) chelating efficiency. Steric hindrances in the case of tannic acid may further effect DNA cleavage rate. Similarly a reduced electron density of hydroxyls may explain the decreased DNA degrading capacity of ellagic acid compared to gallic acid.