CHAPTER-I

DNA binding and its degradation by caffeic acid and its analogs para and ortho coumaric acids in the presence of copper ions.
**RESULTS**

Formation of hydroxycinnamate phenol-Cu(II) complex

As Cu(II) is reduced by polyphenols, the possibility for the formation of a hydroxycinnamate phenolic-Cu(II) complex was examined. This was carried out by recording the absorption spectra of caffeic acid and \( p- \) and \( o- \)coumaric acids with increasing concentrations of Cu(II). The results of figures 3 and 4 shows that the addition of 50 \( \mu \)M Cu(II) to caffeic acid/\( p- \)coumaric acid results in an enhancement of the absorption band at 220 nm indicative of the binding of the phenols to copper ions. \( o- \)coumaric acid (Figure 5) on the other hand does not show any enhancement at 50 \( \mu \)M concentration of copper suggesting the absence of binding to the phenol. At the higher ratio of Cu(II) (500 \( \mu \)M) an enhancement in the absorption of \( o- \)coumaric acid is seen with the emergence of a broad absorption band. The results of figures 3, 4 and 5 taken together suggest that the binding of \( o- \)coumaric acid to Cu(II) is qualitatively different from that observed in the case of caffeic acid and \( p- \)coumaric acid.

Formation of complexes involving calf thymus DNA and hydroxycinnamate phenols

Figures 6 and 7 shows the effect of addition of increasing base pair molar ratios of calf thymus DNA on the fluorescence emission of caffeic acid and \( p- \)coumaric acid. Such an addition resulted in a dose-dependant quenching of the fluorescence. There was however, no shift in the \( \lambda_{\text{max}} \) emission suggesting a simple mode of binding of DNA and the phenols. The control native DNA alone when excited at the same wavelength (324/310 nm) did not interfere with the emission spectrum of phenol alone/phenol + DNA.

Binding of copper ions to hydroxycinnamate phenols

Binding of copper ions to caffeic acid and \( p- \)coumaric acid was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of the two compounds. The results shown in figures 8 and 9 clearly indicate the
Results - I

binding action as addition of Cu(II) causes quenching of the fluorescence of the phenols. These results support the results of absorption studies shown in figures 3 and 4 where formation of phenol-copper complex was demonstrated.

**Reduction of Cu(II) by hydroxycinnamate phenols**

The production of Cu(I), formed as a result of reduction of Cu(II) by the three hydroxycinnamates, was analyzed using neocuproine which is a selective Cu(I) sequestering agent that binds specifically to the reduced form of copper (Cu(I)) but not to the oxidized form (Cu(II)) (Simpson et al., 1992). The Cu(I)-neocuproine complex formed exhibits an absorption maximum at 453 nm. Under the experimental conditions employed, neither Cu(II) nor any of the three hydroxycinnamates interfere with this maximum, whereas all the three hydroxycinnamates and Cu(II) react to generate Cu(I). As can be inferred from figure 10 caffeic acid reduces Cu(II) most efficiently followed by p-coumaric acid whereas o-coumaric acid is least efficient in reducing Cu(II). The implication of this finding is that Cu(II) is reduced by hydroxycinnamates in the complex to generate Cu(I).

**Reducing power of hydroxycinnamate phenols**

In a Fenton type reaction, Fe(II) or Cu(I) reacts with H$_2$O$_2$ resulting in the production of hydroxyl radicals, which is considered to be the most reactive radical towards biological macromolecules in cells. Fe(II) is oxidized to Fe(III) in the Fenton reaction. The resultant oxidized form of iron can be reduced to form Fe(II) by phenols, which can enhance the generation of hydroxyl radicals. To elucidate the role of reducing power in prooxidant effects, activities of caffeic acid, p-coumaric acid and o-coumaric acid towards the reduction of Fe(III) to Fe(II) was studied. As shown in figure 11 caffeic acid exhibited a greater reducing power than p-coumaric acid and o-coumaric acid at all the concentrations tested.
Figure 3. Detection of caffeic acid-Cu(II) complex:

The 3 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 
μM caffeic acid and increasing amounts of Cu(II). The spectra 
were recorded after addition of the components indicated.

[—] caffeic acid alone

[— —] caffeic acid + 50 μM Cu(II)

[ - -] caffeic acid + 500 μM Cu(II)
Figure 4. Detection of \( p \)-coumaric acid-Cu(II) complex:

The 3 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 µM \( p \)-coumaric acid and increasing amounts of Cu(II). The spectra were recorded after addition of the components indicated.

[—] \( p \)-coumaric acid alone

[— —] \( p \)-coumaric acid + 50 µM Cu(II)

[- -] \( p \)-coumaric acid + 500 µM Cu(II)
Figure 5. Detection of o-coumaric acid-Cu(II) complex:

The 3 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 μM o-coumaric acid and increasing amounts of Cu(II). The spectra were recorded after addition of the components indicated.

[—] o-coumaric acid alone

[——] o-coumaric acid + 50 μM Cu(II)

[- -] o-coumaric acid + 500 μM Cu(II)
Figure 6. Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectrum of caffeic acid:

Caffeic acid (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 324 nm and the emission spectra were recorded between 425-455 nm.

[−] caffeic acid alone (25 μM)

[− −] caffeic acid: DNA base pair molar ratio (1:1)

[− -] caffeic acid: DNA base pair molar ratio (1:2)

[−'-] caffeic acid: DNA base pair molar ratio (1:4)

[−'−] caffeic acid: DNA base pair molar ratio (1:6)
Figure 7. Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectrum of p-coumaric acid:

*p*-coumaric acid (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 310 nm and the emission spectra were recorded between 415-465 nm.

[-] p-coumaric acid alone (25 µM)

[- -] p-coumaric acid: DNA base pair molar ratio (1:1)

[- -] p-coumaric acid: DNA base pair molar ratio (1:2)

[- -] p-coumaric acid: DNA base pair molar ratio (1:4)

[- -] p-coumaric acid: DNA base pair molar ratio (1:6)
Figure 8. **Effect of increasing concentration of Cu(II) on the fluorescence emission spectrum of caffeic acid:**

Caffeic acid (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 324 nm and the emission spectra were recorded between 420-460 nm.

[—] caffeic acid alone (25 μM)

[— —] caffeic acid: Cu(II) molar ratio (1:1)

[ - - ] caffeic acid: Cu(II) molar ratio (1:2)

[ -.-] caffeic acid: Cu(II) molar ratio (1:4)

[-"-] caffeic acid: Cu(II) molar ratio (1:6)
Figure 9. **Effect of increasing concentration of Cu(II) on the fluorescence emission spectrum of p-coumaric acid:**

*p*-coumaric acid (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 310 nm and the emission spectra were recorded between 400-490 nm.

[-]  $p$-coumaric acid alone (25 $\mu$M)

[- -]  $p$-coumaric acid: Cu(II) molar ratio (1:1)

[- -]  $p$-coumaric acid: Cu(II) molar ratio (1:2)

[- -]  $p$-coumaric acid: Cu(II) molar ratio (1:4)

[- -]  $p$-coumaric acid: Cu(II) molar ratio (1:6)
Figure 10. Reduction of Cu(II) by caffeic acid, p-coumaric acid and o-coumaric acid:

The 3 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 µM caffeic acid or p-coumaric acid or o-coumaric acid, 200 µM neocuproine and 100 µM Cu(II).

[—] neocuproine alone

[— —] neocuproine + caffeic acid + Cu(II)

[- -] neocuproine + p-coumaric acid + Cu(II)

[.- -] neocuproine + o-coumaric acid + Cu(II)
Figure 11. Detection and comparison of reducing power of caffeic acid, p-coumaric acid and o-coumaric acid:

Reaction mixture (5 ml) contained 0.2 M potassium phosphate buffer (pH 6.6), increasing concentrations of caffeic acid, p-coumaric acid and o-coumaric acid (0-300 μM), 1% potassium ferricyanide and distilled water. The reaction mixture was incubated at 37 °C for 30 minutes and processed further as described in “Methods”. (●) caffeic acid; (○) p-coumaric acid and (□) o-coumaric acid.
Breakage of calf thymus DNA by hydroxycinnamate phenols in the presence of Cu(II)

Caffeic acid, p-coumaric acid and o-coumaric acid in the presence of Cu(II) were found to generate single strand nuclease sensitive sites in calf thymus DNA indicating the formation of single strand breaks. The reaction was assessed by recording the proportion of DNA converted to acid soluble-nucleotides by the nuclease. Figure 12 gives the dose response curve of such a reaction. Control experiments (data not shown) established that heat denatured DNA underwent 100% hydrolysis following the treatment with nuclease. In the presence of Cu(II) (50 μM), increasing concentrations of all the three plant phenols resulted in progressive increase in nuclease sensitive sites in DNA.

Thermal Melting profile of DNA in the presence of caffeic acid

When S1 nuclease was used to determine the thermal melting profile of DNA in the presence of caffeic acid (Figure 13), a slight increase in melting temperature (80 °C versus 78 °C) was seen. The experiment suggests the possibility of caffeic acid binding to DNA through non-covalent interactions.

Generation of oxygen radicals by hydroxycinnamate phenols

**Superoxide production:** The production of superoxide anion was determined by the method of Nakayama et al. (1983), which involves reduction of NBT by hydroxycinnamate phenols to a formazan. The time dependant generation of superoxide anion by 100 μM caffeic acid, p-coumaric acid and o-coumaric acid, as evidenced by the increase in absorbance at 560 nm is shown in figure 14. The fact that NBT was genuinely assaying superoxide was confirmed by SOD (100 μg/ml) inhibiting the reaction (results not shown).

**Hydroxyl radical generation:** It has been previously shown that during the reduction of Cu(II) to Cu(I) by plant polyphenols, reactive oxygen species such as hydroxyl radicals are formed which serve as the proximal cleaving agent
(Rahman et al., 1989). Therefore, the capacity of caffeic acid, p-coumaric acid and o-coumaric acid to generate hydroxyl radicals in the presence of Cu(II) was compared. The assay is based on the principle that degradation of DNA by hydroxyl radicals results in the release of TBA (thiobarbituric acid) reactive material which forms a colored adduct with TBA absorbing at 532 nm (Quinlan & Gutterige, 1987). Results of figure 15 clearly show that increasing concentrations of phenols leads to a progressive increase in the formation of hydroxyl radicals. As can be seen at all the concentrations tested the formation of TBA reactive material was greater in the case of caffeic acid followed by p- and o-coumaric acids.

**Cleavage of plasmid pBR322 DNA by caffeic acid, p-coumaric acid and o-coumaric acid**

In order to understand the chemical basis of DNA breakage by caffeic acid-Cu (II) system, I have compared the relative DNA cleavage efficacy of caffeic acid, p-coumaric acid and o-coumaric acid in plasmid pBR322 supercoiled DNA. In the results given in figure 16 only caffeic acid causes conversion of supercoiled plasmid molecules into linear molecules. This is in conformity with the observation that the relative efficiency of Cu(II) reduction is highest in the case of caffeic acid (Figure 10). It is also shown subsequently that the rate of generation of superoxide anion (Figure 14) and hydroxyl radical (Figure 15) is also highest in the case of caffeic acid as compared with o- and p-coumaric acids. Presumably the presence of two catecholic hydroxyls in caffeic acid accounts for these observations as this leads to a ready and efficient binding of Cu(II) and its reduction to Cu(I).
Figure 12. Degradation of calf thymus DNA by caffeic acid, p-coumaric acid and o-coumaric acid in the presence of Cu(II) as measured by the degree of single strand specific nuclease digestion:

500 µg calf thymus DNA was incubated at 37 °C with indicated concentrations of caffeic acid, p-coumaric acid and o-coumaric acid and Cu(II) (50 µM) in a total reaction volume of 0.5 ml containing 10 mM Tris-HCl (pH 7.5). (●) caffeic acid; (○) p-coumaric acid and (□) o-coumaric acid. Single strand specific digestion was performed as described in “Methods”. All points represent triplicates and mean values have been plotted. Error bars denote ±SEM of three independent experiments.
Figure 13. Thermal melting profile of caffeic acid treated calf thymus DNA:
Calf thymus DNA in TNE (0.01 M Tris-HCl pH 7.4, 0.01 M NaCl and 20 × 10⁻⁴ mM EDTA) was treated with indicated concentrations of caffeic acid. The treated solution was dialyzed against 0.01 M TNE to remove unbound caffeic acid. 500 µg aliquots of native as well as treated DNA were incubated for 8 minutes at the desired temperature and immediately quenched in ice for 2 minutes. S1 nuclease digestion was performed as described in “Methods”.

[●] untreated calf thymus DNA

[○] DNA + 250 µM caffeic acid

[×] DNA + 500 µM caffeic acid

[□] DNA + 1mM caffeic acid
Figure 14. Photogeneration of superoxide anion by caffeic acid, p-coumaric acid and o-coumaric acid on illumination under fluorescent light as a function of time:

The concentration of phenolic acids were 100 μM. Details of the reaction are given in “Methods”. The samples were placed 10 cm from the light source. (●) caffeic acid; (○) p-coumaric acid and (□) o-coumaric acid. Error bars represent ±SEM of three independent experiments.
Figure 15. Hydroxyl radical generation by caffeic acid, *p*-coumaric acid and *o*-coumaric acid:

Reaction mixture (0.5 ml) contained 100 μg calf thymus DNA as substrate, 50 μM Cu(II) and indicated concentrations of the three hydroxycinnamate phenols. The reaction mixture was incubated at 37 °C for 30 minutes. (●) caffeic acid; (○) *p*-coumaric acid and (□) *o*-coumaric acid. Hydroxyl radical formation was measured by determining the TBA reactive material as described in “Methods”. Error bars represent ±SEM of three independent experiments.
Figure 16. Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 DNA after treatment with caffeic acid, p-coumaric acid and o-coumaric acid in the absence and presence of Cu(II):

The reaction mixtures (30 μl) contained 0.50 μg pBR322 DNA, 10 mM Tris-HCl (pH 7.5), indicated concentrations of the three hydroxycinnamate phenols and Cu(II). Incubation was carried out at 37 °C for 1 hour.

Lane 1, DNA alone; lanes 2-4, DNA + caffeic acid, p-coumaric acid and o-coumaric acid (50 μM); lanes 5-7, DNA + caffeic acid, p-coumaric acid and o-coumaric acid (50 μM) + Cu(II) (20 μM); SC, supercoiled DNA; OC, open circular; LIN, linear molecules.
DISCUSSION -I
The major conclusions of the experiments described in this chapter are: (i) caffeic acid as well as \( p \)- and \( o \)-coumaric acids are able to induce strand scission in calf thymus DNA. However only caffeic acid could cleave pBR322 supercoiled DNA at the concentrations tested, (ii) they form a complex with both DNA as well as Cu(II) and possibly in the presence of all three a ternary complex of hydroxycinnamate-Cu(II)-DNA is formed, (iii) redox cycling of copper leads to the generation of various reactive oxygen species, particularly the hydroxyl radical, which serve as the proximal DNA cleaving agents, and (iv) the position and number of hydroxyl groups play an important role in the DNA cleavage efficacy of hydroxycinnamates.

These results place hydroxycinnamates in a class of plant derived polyphenolic antioxidants such as flavonoids (Ahmad et al., 1992), tannins (Bhat & Hadi, 1994) and catechins (Azam et al., 2004), which also exhibit prooxidant DNA damaging properties. The generation of oxygen radicals in the proximity of DNA is well established as a cause of strand scission (Ahmad et al., 1992; Bhat & Hadi, 1994; Rahman et al., 1989). It is generally recognized that such reactions with DNA are preceded by association of ligand with DNA, followed by the production of oxygen radicals at that site (Pryor, 1988). Metal-ion dependant degradation of DNA by 4-(9-acridinylamino) methanesulphonem-anisidine (Wong et al., 1984), 1,10-phenanthroline (Gutterige & Halliwell, 1982), beleomycin (Ehrenfeld et al., 1987), adriamycin (Eliot et al., 1984; Haidle & McKinney, 1985) as well as flavonoids (Ahmad et al., 1992; Rahman et al., 1989) are based on mechanisms involving oxygen-derived radicals. In these studies it was shown that a ternary complex of the drug, DNA and Cu(II) is formed which generates oxygen radical \textit{in situ} via Cu(I). The results presented here show that the catechol caffeic acid as well as \( p \)- and \( o \)-coumaric acids are capable of binding to DNA as well as copper and thus it would be reasonable to assume that a similar mechanism operates in the case of...
hydroxycinnamate-Cu(II) mediated DNA cleavage. Further, the results with caffeic acid and \( p \)- and \( o \)-coumaric acids (Figures 12 and 16) indicate that the DNA cleavage property of the hydroxycinnamates is dependent on the number of hydroxyl groups and their respective position on the aromatic ring. Caffeic acid, which has two catecholic hydroxyl groups, is efficient and most effective in the DNA cleavage reaction. This presumably is because the catecholic hydroxyls are able to reduce Cu\(^{2+}\) more readily which in turn leads to a higher rate of production of ROS. The DNA cleavage efficacy decreases with the decrease in the number of hydroxyl groups on the single aromatic ring of hydroxycinnamates with \( o \)-coumaric acid being least efficient among the three. The reason why coumaric acids are also able to bind Cu(II) and cleave DNA could be that these phenols are further hydroxylated in the reaction mixture leading to the formation of di-hydroxy moieties capable of binding Cu(II). The generation of superoxide by these phenols would lead to the formation of H\(_2\)O\(_2\) which may generate hydroxyl radicals either with the superoxide anion (Haber-Wiess reaction) or in the presence of reduced copper (Fenton reaction).

In addition to chemoprevention of cancer, caffeic acid has also been shown to possess apoptotic activity in human tumor cells (Satoh et al., 1997; Xu et al., 2005). Caffeic acid and \( p \)-coumaric acid can be absorbed from dietary material as shown by its presence in plasma (Nardini et al., 1997). Also, it is known that red wines are a good source of copper (Carno, 1988; Darret et al., 1986). Copper is an essential constituent of many enzymes such as tyrosinases, superoxide dismutase and topoisomerase II. Normal serum contains upto 8 \( \mu \)M loosely bound copper and other biological fluids may also contain comparable amounts (Gutterige, 1984). Loosely bound copper has been defined by Gutterige (1984) as that copper which is available for binding to the chelating agent 1,10-phenanthroline. It is possible that such loosely bound copper can also be mobilized by hydroxycinnamates. Copper has also been reported to be a normal component of chromatin and such endogenous copper can be mobilized by chelating agents such as 1,10-phenanthroline to cause internucleosomal
DNA fragmentation (Burkitt et al., 1996). Since caffeic acid and \( p \)-coumaric acid have been detected in human plasma, it is reasonable to assume that these hydroxycinnamates are transported across cell membranes because of their lipophilic nature. The polyphenolic antioxidant curcumin has also been shown to induce apoptosis in human leukemic cells and such apoptosis is prevented by superoxide dismutase and catalase (Kuo et al., 1996). It would thus appear that prooxidant action of polyphenolics rather than the antioxidant activity is responsible for the induction of apoptosis in these studies. One of the mechanisms could be that in tumor cells endogenous copper in serum or chromatin is relatively easily mobilized by phenolic compounds and similar apoptosis inducing agents. This further appears possible, as it has been shown that serum, tissue and cellular copper levels are significantly elevated in a number of malignancies (Yoshida et al., 1993; Nazulewis et al., 2004; Ebadi & Swanson, 1998). Irrespective of the physiological significance of the results it is clear that hydroxycinnamates have implications for development as novel antitumor and cancer chemopreventive agents.