Chapter - I

DNA binding and its degradation by isoflavones genistein and biochanin A in the presence of copper ions
RESULTS-I

Formation of genistein/biochanin A-Cu(II) complex:

The possibility for the formation of genistein / biochanin A- Cu(II) complex was examined. This was carried out by recording the absorption spectra of genistein and biochanin A with increasing concentrations of Cu(II). The results given in figure 6 and 7 show that the addition of Cu(II) to genistein and biochanin A results in an enhancement in the peak appearing at their respective $\lambda_{\text{max}}$. The absorption spectra of genistein and biochanin A in the presence of copper suggests a simple mode of interaction between these isoflavones and Cu(II).

Formation of complexes involving calf thymus DNA and genistein/biochanin A:

Figure 8 and figure 9 show the effect of addition of increasing molar base pair ratios of calf thymus DNA on the fluorescence emission spectra of genistein and biochanin A at 580 nm and 560 nm respectively. Such an addition resulted in a dose-dependent enhancement of the fluorescence. There was however, no significant shift in the $\lambda_{\text{max}}$ emission suggesting a simple mode of binding of DNA and genistein/biochanin A. The control (native DNA alone) when excited at the same wavelength (268 nm for genistein and 260 nm for biochanin A) did not interfere with the emission spectrum of isoflavone alone/isoflavone - DNA, thus confirming the binding results.

Binding of copper ions to genistein/biochanin A:

Binding of copper ions to genistein and biochanin A was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of the isoflavones. The result shown in figure 10 and figure 11 clearly indicate the binding as addition of Cu(II) causes quenching of isoflavone fluorescence.
These results support the result of absorption studies shown in figure 6 and 7 where formation of genistein /biochanin A-copper complex was demonstrated.

**Detection of genistein/biochanin A induced Cu(I) production by bathocuproine:**

The production of Cu(I), formed as a result of reduction of Cu(II) by genistein/biochanin A, was analyzed using bathocuproine 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)-chelates exhibits an absorption maximum at 480 nm. In both the cases as shown in figure 12 and figure 13, neither Cu(II) nor isoflavones interfere with the maxima, whereas genistein + Cu(II) (fig 12) and biochanin A + Cu(II) (fig 13) react to generate Cu(I) which complexes with bathocuproine to give a peak appearing at 480 nm. The results show that the isoflavones are able to reduce Cu(II) to Cu(I) and may contribute to the redox cycling of the metal.

**Generation of oxygen radicals by genistein/biochanin A:**

**Superoxide production:** The production of superoxide anion was determined by the method of Nakayama et al (1983), which involves reduction of NBT by genistein/biochanin A to a formazan. The time dependent generation of superoxide anion by genistein/biochanin A, 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). It is known that superoxide may undergo automatic dismutation to form H$_2$O$_2$ which in the presence of transition metals such as copper favors Fenton type reaction to generate hydroxyl radicals which could act as a proximal DNA cleaving agent leading to oxidative DNA breakage.

**Hydroxyl radical generation by genistein/biochanin A:** It has been previously shown that during the reduction of Cu(II) to Cu(I), reactive oxygen species such as hydroxyl radicals are formed which serve as the proximal DNA
cleaving agent (Rahman et al., 1989). Therefore, the capacity of genistein and biochanin A to generate hydroxyl radical in the presence of Cu(II) was examined. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA (2-thiobarbituric acid) reactive material, which forms a coloured adduct with TBA whose absorbance is read at 532 nm (Quinanlan and Gutteridge, 1987). The results given in figure 15 clearly show that increasing concentrations of isoflavones lead to a progressive increase in the formation of hydroxyl radicals.

Cleavage of plasmid pBR322 DNA by genistein/biochanin A:

In order to examine the efficacy of isoflavone-Cu(II) system in DNA cleavage, in figure 16, I have tested the ability of genistein and biochanin A to cause cleavage of supercoiled plasmid pBR322 DNA in the presence of copper ions. As can be seen from the ethidium bromide stained agarose gel pattern, whereas copper alone or isoflavone alone did not show DNA cleavage, addition of copper to isoflavone resulted in the generation of open circular and heterogeneous linear fragments of Plasmid DNA, demonstrating that isoflavones are capable of DNA degradation in the presence of copper ions.

Breakage of calf thymus DNA by genistein/biochanin A in the presence of Cu(II):

Genistein and biochanin A in the presence of Cu(II) were found to generate single strand specific nuclease sensitive sites in calf thymus DNA. The reaction was assessed by recording the proportion of DNA converted to acid soluble-nucleotides by the nuclease. Figures 17 and 18 give the dose response curve of such a reaction. However, genistein and biochanin A in the absence of Cu(II) did not show significant degradation of calf thymus DNA. 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 genistein (figure 17) and biochanin A (figure
18) resulted in an increase in nuclease sensitive sites in DNA leading to increased DNA hydrolysis.

**Standardization of alkaline single cell gel electrophoresis/Comet Assay:**

Single cell gel electrophoresis (Comet assay) is a sensitive technique to quantify DNA damage in cellular system. In the original version of the assay, cells embedded in agarose microscopic slides were lysed and subjected to electrophoresis under neutral conditions enabling the detection of DNA double-strand breaks (Ostling and Johansson 1984). A later modification using alkaline conditions made it possible to detect DNA single strand breaks and alkaline-labile sites in DNA (Singh et al., 1988). Further in case two single strand breaks are closely opposed they show up as a double strand break. In this technique a small number of cells are treated with the test agent, layered on glass slides and sandwiched between layers of agarose. The slides are electrophoresed in alkaline conditions, stained and viewed under a fluorescent microscope for DNA single strand breaks. The technique is called Comet Assay because of a comet like appearance of damaged cellular DNA. H₂O₂ is a known genotoxic agent and is routinely used in genotoxicity testing. In order to standardize Comet Assay an experiment was performed with H₂O₂. Photographs of comets (100x) observed after treatment of lymphocytes with increasing concentrations of H₂O₂ are shown in figure 19. As can be seen, untreated cells are not damaged and do not show a tail. However, with increasing concentrations of H₂O₂ a progressive increase in the length of comet tails is observed. In figure 20, the results of the same experiment are plotted as comet tail length (µm) as a function of increasing H₂O₂ concentration.

**DNA breakage by genistein-Cu(II) and biochanin A-Cu(II) system in lymphocytes as measured by Comet Assay:**

Increasing concentrations of genistein (fig 21) and biochanin A (fig 22) [0-50 µM] either alone or in the presence of 50 µM CuCl₂ were tested for DNA
breakage in isolated human peripheral lymphocytes using the Comet Assay. The corresponding tail length is plotted as a function of isoflavone concentration. It is seen in both the figures that whereas isoflavone alone causes some breakage of cellular DNA, the degree of such breakage is enhanced in the presence of Cu(II). Cu(II) [50 μM] controls were similar to untreated lymphocyte without any significant DNA breakage. The results clearly establish that isoflavone-Cu(II) system is capable of DNA breakage in isolated lymphocytes. Thus, such cellular DNA breakage is physiologically feasible and could be of biological significance.
The 3.0 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 μM genistein and increasing concentrations of Cu(II). The spectra were recorded after addition of components indicated.

(1) Genistein alone

(2) Genistein + 50 μM Cu(II)

(3) Genistein + 100 μM Cu(II)
The 3.0 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 µM biochanin A and increasing concentrations of Cu(II). The spectra were recorded after addition of components indicated.

(1) biochanin A alone

(2) biochanin A + 50 µM Cu(II)

(3) biochanin A + 100 µM Cu(II)
Figure 8: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of genistein.

Genistein (in Tris-HCl, pH 7.5) was excited at its λ<sub>max</sub> (absorption) of 268 nm and the emission spectra were recorded between 480-660 nm.

- [ ] Genistein alone (25 μM)
- [ ] Genistein: DNA base pair molar ratio (1:1)
- [ ] Genistein: DNA base pair molar ratio (1:2)
- [ ] Genistein: DNA base pair molar ratio (1:4)
- [ ] Genistein: DNA base pair molar ratio (1:6)
- [ ] Genistein: DNA base pair molar ratio (1:8)
Figure 9: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of biochanin A

Biochanin A (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 260 nm and the emission spectra were recorded between 480-620 nm.

[ ] Biochanin A alone (25 μM)

[ ] Biochanin A: DNA base pair molar ratio (1:1)

[ ] Biochanin A: DNA base pair molar ratio (1:2)

[ ] Biochanin A: DNA base pair molar ratio (1:4)

[ ] Biochanin A: DNA base pair molar ratio (1:6)

[ ] Biochanin A: DNA base pair molar ratio (1:8)
Figure 10: Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of genistein

Genistein (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 268 nm and the emission spectra were recorded between 480-660 nm.

- **[   ]** Genistein alone (50 μM)
- **[· · ·]** Genistein: Cu(II) molar ratio (1:1)
- **[ _ _ ]** Genistein: Cu(II) molar ratio (1:2)
- **[ _ _ · ]** Genistein: Cu(II) molar ratio (1:4)
- **[ _ _ _ ]** Genistein: Cu(II) molar ratio (1:6)
- **[ _ _ _ _ ]** Genistein: Cu(II) molar ratio (1:8)
Biochanin A (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 260 nm and the emission spectra were recorded between 480-620 nm.

[ ] Biochanin A (50 $\mu$M)

[........] Biochanin A: Cu(II) molar ratio (1:1)

[___] Biochanin A: Cu(II) molar ratio (1:2)

[__._] Biochanin A: Cu(II) molar ratio (1:4)

[._._] Biochanin A: Cu(II) molar ratio (1:6)

[-.--) Biochanin A : Cu(II) molar ratio (1:8)
Figure 12: Detection of genistein induced Cu (I) production by Bathocuproine

Reaction mixture (3.0 ml) contained 10 mM Tris-HCl (pH 7.5) along with 300 μM bathocuproine and indicated concentrations of the following:

(1) Bathocuproine + 100 μM Cu(II)

(2) Bathocuproine + 100 μM Cu(I)

(3) Bathocuproine + 50 μM genistein

(4) Bathocuproine + 50 μM genistein + 100 μM Cu(II)
Figure 13: Detection of biochanin A induced Cu (I) production by Bathocuproine

Reaction mixture (3.0 ml) contained 10 mM Tris-HCl (pH 7.5) along with 300 μM bathocuproine and indicated concentrations of the following:

(1) Bathocuproine + 100 μM Cu(II)

(2) Bathocuproine + 100 μM Cu(I)

(3) Bathocuproine + 50 μM biochanin A

(4) Bathocuproine + 50 μM biochanin A + 100 μM Cu(II)
Figure 14: Photogeneration of superoxide anion by genistein/biochanin A on illumination under fluorescent light as a function of time

Reaction mixture contained 100 mM phosphate buffer (pH 7.5) and 100 μM of genistein (♦)/biochanin A (▲). Details of the reaction are given in “Methods”. The samples were placed at a distance of 10 cm from the light source. All values reported are means of three independent experiments. Error bars represent standard error of mean.
Figure 15: Hydroxyl radical generation by genistein/biochanin A

Reaction mixture (0.5 ml) contained 100 μg calf thymus DNA as substrate, 50 μM Cu(II) and indicated concentrations of genistein (●) and biochanin A (●). The reaction mixture was incubated at 37 °C for 30 min. Hydroxyl radical formation was measured by determining the TBA reactive material as described in “Methods”. All values reported are means of three independent experiments. Error bars represent standard error of mean.
Figure 16: Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 plasmid DNA after treatment with genistein and biochanin A in the absence and presence of copper

The reaction mixture (30μl) contained 0.50 μg pBR322 DNA, 10 mM Tris-HCl (pH 7.5), indicated concentrations of the two isoflavones and Cu (II). Incubation was carried out at 37°C for 2 hour

Lane 1: DNA alone; Lane 2: DNA + Cu(II) 100 μM; Lane 3: DNA + Genistein (150μM); Lane 4: DNA + Biochanin A (150μM); Lane 5: DNA + Genistein (150μM) + Cu(II) 100 μM; Lane 6: DNA + Biochanin A (150μM) + Cu(II) 100 μM.

SC=Supercoiled DNA; OC= Open circular; Lin=Linear
Figure 17: Degradation of calf thymus DNA by genistein in the presence of Cu(II) as measured by the degree of single strand specific nuclease digestion

Reaction mixture (0.5 ml) containing 10 mM Tris-HCl (pH 7.5) and 500 µg calf thymus DNA was incubated at 37 °C with indicated concentrations of genistein alone (●) and genistein with Cu(II) (50 µM) (■). Single strand specific digestion was performed as described in “Methods”. All points represent triplicates and mean values have been plotted. Error bars represent SEM of three independent experiments.
Figure 18: Degradation of calf thymus DNA by biochanin A in the presence of Cu(II) as measured by the degree of single strand specific nuclease digestion

Reaction mixture (0.5 ml) containing 10 mM Tris-HCl (pH 7.5) and 500 μg calf thymus DNA was incubated at 37 °C with indicated concentrations of biochanin A alone (○) and biochanin A with Cu(II) (50 μM) (■). Single strand specific digestion was performed as described in “Methods”. All points represent triplicates and mean values have been plotted. Error bars represent SEM of three independent experiments.
Figure 19: Standardization of Comet Assay with H₂O₂

Reaction mixture (1 ml) contained 1 x10^5 cells, RPMI 400 µl, PBS Ca²⁺ and Mg²⁺ free and indicated concentrations of H₂O₂ (0-50 µM). The reaction mixture was incubated at 37 °C for 30 min and processed further for Comet Assay as described in “Methods”.

Photographs of comets (100x) obtained on incubating lymphocytes with varying concentrations of H₂O₂.

(A) Untreated lymphocytes
(B) Lymphocytes + 10 µM H₂O₂
(C) Lymphocytes + 20 µM H₂O₂
(D) Lymphocytes + 50 µM H₂O₂
Figure 20: Standardization of Comet Assay with H₂O₂

The data corresponding to Figure 19 is plotted as tail lengths of comets with increasing concentrations of H₂O₂ (0-50 μM). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.
Figure 21: DNA breakage by genistein in human peripheral lymphocytes in the absence and presence of Cu(II)

Reaction mixture (1.0 ml) contained $1 \times 10^5$ cells, RPMI 400 µl, PBS Ca$^{2+}$ and Mg$^{2+}$ free, increasing concentrations of genistein (0-50 µM) and 50 µM Cu(II). The reaction mixture was incubated for 1 hr at 37 °C. After incubation the cells were processed further for Comet Assay as described in “Methods”. Comet tail length (µ metres) plotted as a function of increasing concentrations of genistein (0-50 µM) in the absence (■) and presence (□) of 50 µM Cu(II). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.
Figure 22: DNA breakage by biochanin A in human peripheral lymphocytes in the absence and presence of Cu(II)

Reaction mixture (1.0 ml) contained 1 x 10^5 cells, RPMI 400 μl, PBS Ca²⁺ and Mg²⁺ free, increasing concentrations of biochanin A (0-50 μM) and 50 μM Cu(II). The reaction mixture was incubated for 1 hr at 37 °C. After incubation the cells were processed further for Comet Assay as described in "Methods". Comet tail length (μ metres) plotted as a function of increasing concentrations of biochanin A (0-50 μM) in the absence (■) and presence (●) of 50 μM Cu(II). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.
DISCUSSION-I

Most of the pharmacological properties of plant polyphenols are considered to reflect their ability to scavenge endogenously generated oxygen radicals or those free radicals formed by various xenobiotics, radiation etc. However, some data in the literature suggests that the antioxidant properties of the polyphenolic compounds may not fully account for their chemopreventive effects (Gali et al., 1992; Hadi et al., 2000). Although most plant polyphenols are considered to have a physiological role as antioxidants, they may also exhibit prooxidant properties in the presence of transition metals such as copper (Inoue et al., 1994; Ahmad et al., 1992). The results presented in this chapter lead to the conclusions that both the isoflavones, genistein and biochanin A; (i) are able to interact with DNA as well as Cu (II) and possibly form a ternary complex of DNA-Cu (II)-isoflavone, (ii) are able to reduce Cu(II) to generate Cu(I), (iii) redox cycling of copper leads to the generation of various reactive oxygen species, particularly the hydroxyl radical, (iv) induce strand scission in calf thymus DNA and plasmid DNA in the presence of copper ions, (v) and show a similar copper dependant activation leading to enhanced DNA degradation in the cellular system of human peripheral lymphocytes. These observations suggest that such a prooxidant mechanism of DNA breakage involving Isoflavone-Cu(II) system is physiologically feasible and could be of biological significance.

These results place the isoflavones genistein and biochanin A among the other classes of plant-derived polyphenolic antioxidants such as flavonoids (Ahmad et al., 1992), tannins (Bhat and hadi, 1994), and catechins (Azam et al., 2004), which also exhibit prooxidant DNA damaging properties. Interestingly, certain properties of naturally occurring antioxidants such as binding and cleavage of DNA and the generation of ROS in the presence of transition metal ions (Rehman et al., 1990) are similar to those of certain known anticancer drugs (Ehrenfeld et al., 1987). Metal ion dependent degradation of DNA by 1,10-phenanthroline (Gutteridge and Halliwell, 1982), bleomycin (Ehrenfeld et al.,
1987), adriamycin (Eliot., 1984; Haidle and McKinney, 1985) as well as flavonoids (Ahmad et al., 1992; Rahman et al., 1989) are based on mechanisms involving oxygen-derived radicals. The generation of hydroxyl radicals in the proximity of DNA is well established as a cause of strand scission. It is generally recognized that such reaction with DNA is preceded by the association of a ligand with DNA followed by the formation of hydroxyl radicals at that site. Among oxygen radicals the hydroxyl radical is most electrophilic with high reactivity and therefore possesses a small diffusion radius. Thus, in order to cleave DNA it must be produced in the vicinity of DNA (Pryor 1988). The location of the redox-active metal is of utmost importance because the hydroxyl radical, due to its extreme reactivity, interacts exclusively in the vicinity of the bound metal (Chevion 1988). Fe$^{3+}$ and Cu$^{2+}$ are the most redox-active of the metal ions in living cells. Wolfe et al. (1994) have proposed that a copper mediated Fenton reaction, generating site-specific hydroxyl radicals, is capable of inducing apoptosis in thymocytes. Copper ions are known to interact with both DNA phosphates and the bases through coordination binding (Zimmer et al., 1971) and are also present naturally in chromatin, associated with guanine bases (Kagawa et al., 1991). Direct interaction of isoflavone compounds genistein and biochanin A with the DNA bound copper ions in a ternary complex and localized generation of non diffusible hydroxyl radical is a likely mechanism involved in isoflavone- Cu (II) induced DNA cleavage.