Chapter - 1

DNA binding and its degradation by flavones (luteolin, apigenin & chrysin) in the presence of copper ions
RESULTS-I

Formation of polyphenol-Cu(II) complex:

The possibility for the formation of polyphenol-Cu(II) complex was examined. This was carried out by recording the absorption spectra of luteolin, apigenin and chrysin with increasing concentrations of Cu(II). The results given in figures 6, 7 and 8 show that the addition of Cu(II) to luteolin, apigenin and chrysin results in an enhancement in the absorption peak appearing at their respective $\lambda_{\text{max}}$. The absorption spectra of these polyphenols in the presence of copper suggests a simple mode of interaction between them and Cu(II).

Formation of complexes involving calf thymus DNA and polyphenolic compounds:

Figures 9, 10 and 11 show the effect of addition of increasing molar base pair ratios of calf thymus DNA on the fluorescence emission spectra of luteolin, apigenin and chrysin at 560 nm, 545 nm and 540 nm, respectively. Such an addition resulted in a progressive enhancement of the fluorescence. There was however, no significant shift in the $\lambda_{\text{max}}$-emission suggesting a simple mode of binding between DNA and these flavones. When DNA alone was excited at the respective excitation wavelengths (270 nm for luteolin, 272 nm for apigenin and 273 nm for chrysin), no interference with the fluorescence emission spectra of flavone alone/flavone + DNA was found, thus confirming the binding results.

Binding of copper ions to polyphenols:

Binding of copper ions to luteolin, apigenin and chrysin was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of these flavones. The results shown in figures 12, 13 and 14 clearly indicate the binding as addition of Cu(II) causes progressive quenching of flavone
fluorescence. These results support the results of absorption studies shown in figures 6, 7 and 8, where formation of polyphenol-copper complex was demonstrated.

**Detection of polyphenol induced Cu(I) production by bathocuproine:**

The production of Cu(I), formed as a result of reduction of Cu(II) by luteolin/apigenin/chrysin, was analyzed using bathocuproine which is a selective Cu(I) sequestering agent that binds specifically to the reduced form of copper, i.e. Cu(I), but not to the oxidized form (Simpson et al., 1992). The Cu(I)-chelates exhibit an absorption maximum at 480 nm. As shown in figure 15, Cu(II) does not interfere with the maxima, whereas luteolin + Cu(II), apigenin + Cu(II) and chrysin + Cu(II) react to generate Cu(I) which complexes with bathocuproine to give a peak appearing at 480 nm. The results show that these flavones are able to reduce Cu(II) to Cu(I) and contribute to the redox cycling of the metal.

**Generation of oxygen radicals by polyphenols:**

*Superoxide production:* The production of superoxide anion was determined by the method of Nakayama et al. (1983), which involves reduction of NBT by luteolin/apigenin/chrysin to a formazan. The time dependent generation of superoxide anion by these flavones, as evidenced by the increase in absorbance at 560 nm, is shown in figure 16. 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 undergoes automatic dismutation at neutral pH to form H₂O₂ which in the presence of transition metals such as copper favours Fenton type reaction to generate hydroxyl radicals which could act as a proximal DNA cleaving agent leading to oxidative DNA breakage.
**Hydroxyl radical generation:** 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 luteolin, apigenin and chrysin to generate hydroxyl radicals 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 colored adduct with TBA whose absorbance is read at 532 nm (Quinlan and Gutteridge, 1987). The results given in figure 17 clearly show that increasing concentrations of the flavones lead to a progressive increase in the formation of hydroxyl radicals.

**Cleavage of plasmid pBR322 DNA by luteolin/apigenin/chrysin:**

In order to examine the efficacy of flavone-Cu(II) system in DNA cleavage, as shown in figure 18, I have tested the ability of luteolin, apigenin and chrysin 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, apigenin/luteolin/chrysin alone show generation of only some open circular form of plasmid DNA. However, addition of copper to these flavones resulted in the generation of greater open circular as well as some linearized (in case of luteolin) forms of plasmid DNA, demonstrating that flavones are capable of plasmid DNA cleavage in the presence of copper ions.

**Breakage of calf thymus DNA by polyphenols in the presence of Cu(II):**

Apigenin, luteolin and chrysin 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. Table IV gives the dose response data of such a reaction. However, apigenin, luteolin and chrysin in the absence of Cu(II) did
not show appreciable 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 apigenin, luteolin and chrysin resulted in an increase in nuclease sensitive sites in DNA leading to increased DNA hydrolysis.

**DNA breakage by polyphenol-Cu(II) system in lymphocytes as measured by Comet Assay:**

Increasing concentrations of luteolin, apigenin and chrysin [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 (Figure 19). The corresponding tail length is plotted as a function of polyphenol concentration. It can be seen in figure 19 that whereas all the three flavones alone were able to cause some breakage of cellular DNA, the degree of such breakage was significantly 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 flavone-Cu(II) system is capable of DNA breakage in a biological system such as isolated lymphocytes.

**A comparison of DNA breakage induced by polyphenols in intact lymphocytes and permeabilized lymphocytes as measured by Comet Assay:**

As shown in the previous experiment (Figure 19) flavones luteolin, apigenin and chrysin are able to cause single strand breaks in a cellular system and that the degree of such DNA breakage is greater in the presence of copper. In the present experiment (Figure 20), increasing concentrations [0-50 µM] of luteolin, apigenin and chrysin are tested for DNA breakage in permeabilized lymphocytes and are compared with that observed in intact lymphocytes. Use of permeabilized lymphocytes allows the direct interaction of flavones with
cell nuclei at physiological pH. It also eliminates the need to first lyse the cells at alkaline pH and then resuspend them at neutral pH. Thus, considerably greater DNA breakage should be observed in permeabilized lymphocytes as compared to the intact cells. Figure 20 shows that the rate of comet tail formation induced by all the three polyphenols is indeed greater in the case of permeabilized lymphocytes suggesting that these compounds are able to interact with the cell nuclei when a permeabilized system is used. However, in both intact and permeabilized cells, the rate of DNA breakage is greater in the case of luteolin, followed by apigenin and chrysin.

**Effect of neocuproine and bathocuproine on polyphenol induced DNA breakage in intact lymphocytes and permeabilized lymphocytes:**

In the experiment shown in figure 21, the DNA breakage induced by luteolin in intact lymphocytes and permeabilized lymphocytes has been assessed in the presence of Cu(I) specific chelators neocuproine and bathocuproine. Incubation of lymphocytes with neocuproine (a cell membrane permeable copper chelator) inhibited luteolin induced DNA degradation in intact lymphocytes. Bathocuproine disulphonate (the water soluble membrane impermeable analog of neocuproine) which is unable to permeate through the cell membrane did not cause such inhibition (Figure 21 A). This study has further shown (Figure 21 B) that luteolin is able to degrade DNA in permeabilized cells and that such DNA degradation is inhibited by neocuproine as well as bathocuproine disulphonate (both of which are able to permeate the nuclear pore complex), suggesting that nuclear copper is mobilized in this reaction. These results indicate that oxidative DNA breakage by flavones involve mobilization of chromatin bound copper and that Cu(I) is an intermediate in the pathway that leads to DNA breakage.
Figure 6: Absorption spectra of luteolin in the presence of Cu (II)

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

(1) Luteolin alone

(2) Luteolin + 50 µM Cu(II)

(3) Luteolin + 100 µM Cu(II)
Figure 7: Absorption spectra of apigenin in the presence of Cu (II)

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

(1) Apigenin alone

(2) Apigenin + 50 µM Cu(II)

(3) Apigenin + 100 µM Cu(II)
Figure 8: Absorption spectra of chrysin in the presence of Cu (II)

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

(1) Chrysin alone

(2) Chrysin + 50 µM Cu(II)

(3) Chrysin + 100 µM Cu(II)
Figure 9: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of luteolin

Luteolin (in 10 mM Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 270 nm and the emission spectra were recorded between 520-600 nm.

- [ ___ ] Luteolin alone (30 $\mu$M)
- [.......] Luteolin: DNA base pair molar ratio (1:1)
- [__ __] Luteolin: DNA base pair molar ratio (1:2)
- [— · —] Luteolin: DNA base pair molar ratio (1:4)
- [ ___ ___ ] Luteolin: DNA base pair molar ratio (1:6)
- [ _ .. _ ] Luteolin: DNA base pair molar ratio (1:8)
Figure 10: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of apigenin

Apigenin (in 10 mM Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 272 nm and the emission spectra were recorded between 520-600 nm.

[   ] Apigenin alone (30 $\mu$M)

[........] Apigenin: DNA base pair molar ratio (1:1)

[   _ _ _ ] Apigenin: DNA base pair molar ratio (1:2)

[    ⋅ ⋅ ⋅] Apigenin: DNA base pair molar ratio (1:4)

[   __ __ ] Apigenin: DNA base pair molar ratio (1:6)

[ _ .. _ _ ] Apigenin: DNA base pair molar ratio (1:8)
Figure 11: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of chrysin

Chrysin (in 10 mM Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 273 nm and the emission spectra were recorded between 520-580 nm.

[ _ _ ]  Chrysin alone (30 μM)

[........]  Chrysin: DNA base pair molar ratio (1:1)

[ _ _ _ ]  Chrysin: DNA base pair molar ratio (1:2)

[—·—]  Chrysin: DNA base pair molar ratio (1:4)

[ _ _ _ ]  Chrysin: DNA base pair molar ratio (1:6)

[ _ .. _ ]  Chrysin: DNA base pair molar ratio (1:8)
Figure 12: Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of luteolin

Luteolin (in 10 mM Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 270 nm and the emission spectra were recorded between 520-600 nm.

- [ _ _ ] Luteolin alone (30 $\mu$M)
- [ _ _ _ _ _ _ _ _ ] Luteolin: Cu(II) molar ratio (1:1)
- [ _ _ _ _ _ _ _ _ ] Luteolin: Cu(II) molar ratio (1:2)
- [ _ _ _ _ _ _ _ _ ] Luteolin: Cu(II) molar ratio (1:4)
- [ _ _ _ _ _ _ _ _ ] Luteolin: Cu(II) molar ratio (1:6)
- [ _ _ _ _ _ _ _ _ ] Luteolin: Cu(II) molar ratio (1:8)
Figure 13: Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of apigenin

Apigenin (in 10 mM Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 272 nm and the emission spectra were recorded between 520-600 nm.

[ ... ] Apigenin alone (30 μM)

[.......] Apigenin: Cu(II) molar ratio (1:1)

[ _ _ _ ] Apigenin: Cu(II) molar ratio (1:2)

[ – · – ] Apigenin: Cu(II) molar ratio (1:4)

[ __ __ ] Apigenin: Cu(II) molar ratio (1:6)

[ _ . _ ] Apigenin: Cu(II) molar ratio (1:8)
Figure 14: Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of chrysin

Chrysin (in 10 mM Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 273 nm and the emission spectra were recorded between 520-580 nm.

[ ___ ] Chrysin alone (30 $\mu$M)

[.......] Chrysin: Cu(II) molar ratio (1:1)

[    ] Chrysin: Cu(II) molar ratio (1:2)

[— · —] Chrysin: Cu(II) molar ratio (1:4)

[ ___ ___ ] Chrysin: Cu(II) molar ratio (1:6)

[ _ .. _ ] Chrysin: Cu(II) molar ratio (1:8)
Figure 15: Detection of flavone induced Cu(I) production by Bathocuproine

Reaction mixture (3.0 ml) contained 3 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 luteolin + 100 µM Cu(II)
[4] Bathocuproine + 50 µM apigenin + 100 µM Cu(II)
[5] Bathocuproine + 50 µM chrysin + 100 µM Cu(II)

The Bathocuproine alone or bathocuproine in the presence of respective compounds did not interfere with the Bathocuproine-Cu(I) complex peak at 480 nm (not shown).
Figure 16: Photogeneration of superoxide anion by luteolin/ apigenin/ chrysin on illumination under fluorescent light as a function of time

Reaction mixture contained 50 mM phosphate buffer (pH 7.5) and 100 µM of luteolin (♦), apigenin (□) and chrysin (▲). 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 17: Hydroxyl radical generation by luteolin/ apigenin/ chrysin

Reaction mixture (0.5 ml) contained 100 µg calf thymus DNA as substrate, 50 µM Cu(II) and indicated concentrations of luteolin (♦), apigenin (□) and chrysin (▲). The reaction mixture was incubated at 37°C for 30 min. Hydroxyl radical formation was measured by determining the TBA reactive material. All values reported are means of three independent experiments. Error bars represent standard error of mean.
Figure 18: Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 plasmid DNA after treatment with apigenin, luteolin and chrysin 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 three flavones and Cu(II). Incubation was carried out at 37°C for 2 hour.

Lane a: DNA alone; Lane b: DNA + Cu(II) 100 µM; Lane c: DNA + Apigenin (150µM); Lane d: DNA + Luteolin (150µM); Lane e: DNA + Chrysin (150µM)
Lane f: DNA + Apigenin (150µM) + Cu(II) 100 µM; Lane g: DNA + Luteolin (150µM) + Cu(II) 100 µM; Lane h: DNA + Chrysin (150µM) + Cu(II) 100µM.

SC=Supercoiled DNA; OC= Open circular DNA; LIN=Linear DNA
Table IV: Degradation of calf thymus DNA by the flavones in the presence of Cu(II) as measured by the degree of single strand specific S1-nuclease digestion

<table>
<thead>
<tr>
<th>Flavone</th>
<th>Concentration (µM)</th>
<th>% DNA Hydrolysed</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without Cu(II)</td>
<td>With Cu(II)</td>
<td></td>
</tr>
<tr>
<td>APIGENIN</td>
<td>50</td>
<td>3.72 ± 0.24</td>
<td>15.44 ± 1.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5.45 ± 0.51</td>
<td>22.24 ± 2.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>8.12 ± 0.67</td>
<td>32.62 ± 2.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>10.57 ± 0.86</td>
<td>41.58 ± 3.44</td>
<td></td>
</tr>
<tr>
<td>LUTEOLIN</td>
<td>50</td>
<td>3.67 ± 0.23</td>
<td>17.54 ± 1.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.3 ± 0.51</td>
<td>23.47 ± 1.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>11.24 ± 1.03</td>
<td>37.71 ± 3.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>13.59 ± 1.32</td>
<td>49.3 ± 3.46</td>
<td></td>
</tr>
<tr>
<td>CHRYSIN</td>
<td>50</td>
<td>2.48 ± 0.15</td>
<td>12.75 ± 0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5.64 ± 0.52</td>
<td>17.89 ± 1.56</td>
<td></td>
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<tr>
<td></td>
<td>200</td>
<td>7.61 ± 0.67</td>
<td>26.21 ± 2.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>9.38 ± 0.77</td>
<td>34.1 ± 3.11</td>
<td></td>
</tr>
</tbody>
</table>

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 respective polyphenol alone or polyphenol with Cu(II) (50 µM). All values represent mean ± SEM of three independent experiments.
Figure 19: DNA breakage by flavones in human peripheral lymphocytes in the absence and presence of Cu(II)

Reaction mixture (1.0 ml) contained 1 x10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} free, increasing concentrations of respective polyphenol (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. Comet tail length (µ metres) plotted as a function of increasing concentrations of polyphenol (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 20: Comparison of DNA breakage by polyphenols in intact lymphocytes and permeabilized lymphocytes as measured by Comet assay

Intact lymphocytes / Permeabilized lymphocytes were incubated with the reaction mixture (2.0 ml) containing PBS Ca\(^{2+}\) and Mg\(^{2+}\) free (intact cells) / 0.4 M phosphate buffer (pH 7.5) (permeabilized cells) and indicated concentrations of respective polyphenol (0-50 µM) at 37°C for 1 hr / 30 min respectively and processed further for Comet Assay as described in ‘Methods’. Comet tail length (µ metres) plotted as a function of increasing concentrations of polyphenol (0-50 µM) in intact lymphocytes (○) and permeabilized lymphocytes (■). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control (in the absence of polyphenol).
Lymphocytes were incubated with the reaction mixture (2.0 ml) containing luteolin (50 µM) and indicated concentrations of neocuproine / bathocuproine at 37°C for 1 hr (intact cells)/ 30 min (permeabilized cells) and processed further for Comet Assay. Comet tail length (µ metres) plotted as a function of increasing concentrations of neocuproine (○) and bathocuproine (■) in intact lymphocytes (A) and permeabilized lymphocytes (B). Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated 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 (Ahmad et al., 1992; Inoue et al., 1994). The results presented in this chapter lead to the conclusions that all the three flavones tested, namely luteolin, apigenin and chrysin, (i) are able to interact with DNA as well as Cu(II) and possibly form a ternary complex of DNA-Cu(II)-flavone; (ii) are able to reduce Cu(II) to generate Cu(I); (iii) cause redox cycling of copper leading to the generation of various reactive oxygen species, particularly the hydroxyl radical, (iv) are able to induce strand scission in plasmid DNA and calf thymus DNA in the presence of copper ions; (v) show a similar copper dependent activation leading to enhanced DNA degradation in a cellular system of human peripheral lymphocytes; and (vi) mobilize nuclear, possibly chromatin bound copper in the DNA breakage reaction. These observations suggest that such a prooxidant mechanism of DNA breakage involving flavone-Cu(II) system is physiologically feasible and could be of biological significance.

These results place the flavones luteolin, apigenin and chrysin among the other classes of plant derived polyphenolic antioxidants such as isoflavones (Ullah et al., 2009), anthocyanidins (Hanif et al., 2008), stilbene (Azmi et al., 2006), catechins (Azam et al., 2004), curcumin (Ahsan and Hadi, 1998) and tannins (Bhat and Hadi, 1994), which also exhibit prooxidant DNA damaging properties in the presence of copper ions. Previous studies on polyphenols
from this laboratory have shown that a ternary complex of DNA, Cu(II) and polyphenol is formed which generates oxygen radicals in situ via Cu(I) (Rahman et al., 1989). The results presented here demonstrate that flavones are also 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 flavone-Cu(II) mediated DNA cleavage.

Interestingly, certain properties of plant derived polyphenolic compounds such as binding and cleavage of DNA and the generation of ROS in the presence of transition metal ions (Rahman et al., 1990) are similar to those of some 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 et al., 1984; Haidle and McKinney, 1985) as well as flavonoids (Rahman et al., 1989; Ahmad et al., 1992) are based on mechanisms involving reactive oxygen 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). Moreover, 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 various metal ions in living cells. However, it is copper that is known to be present in the nucleus as a normal component of chromatin, being associated with guanine bases (Kagawa et al., 1991). Such endogenous copper can be mobilized by chemical agents such as 1,10-phenanthroline to cause internucleosomal DNA
fragmentation (Burkitt et al., 1996). Wolfe et al. (1994) have proposed that a copper mediated Fenton reaction, generating site-specific hydroxyl radicals, is capable of inducing apoptosis in thymocytes. Conceivably, direct interaction of flavone compounds apigenin, luteolin and chrys in with the DNA bound copper ions in a ternary complex and localized generation of non-diffusible hydroxyl radical is a likely mechanism involved in the flavone-Cu(II) induced DNA breakage.

Also, it is evident from the results presented in this chapter that the efficiency of DNA cleavage by the flavones tested is in the order - luteolin > apigenin > chrysin, and correlates with their relative abilities to cause copper reduction and ROS generation. A possible explanation for this is provided by the structural difference between these molecules (Figure 22).

![Figure 22. Structures of the three flavones used in our studies.](image)

In a previous publication (Jain et al., 1999), this laboratory had studied the reactivities of flavonoids with different hydroxyl substituents for the cleavage of DNA in the presence of Cu(II). One of the structural feature that was identified was that Cu(II) complexes formed by 3-4, 4-5 and ortho substituents can generate species that are capable of attacking DNA. The above results are consistent with this finding where luteolin, in addition to 4-5 hydroxyl substituents, also possesses two ortho hydroxyl groups in ring B. Apigenin possesses only one hydroxyl group in the B ring and possibly this accounts for
its intermediate DNA cleavage efficiency among the three flavones tested. The present findings are further supported by the results of some other studies (Cao et al., 1997; Kozics et al. 2011) which have shown that the antioxidant and copper mediated prooxidant activities of flavonoids have a certain correlation with the number and arrangement of hydroxyl groups in their molecular structures.

Furthermore, the observation that bathocuproine, which is a membrane impermeable copper chelator, inhibits flavone-induced DNA breakage only in permeabilized cells and not in intact cells whereas its membrane permeable analogue neocuproine causes such an inhibition in both intact as well as permeabilized cells, strongly indicates that flavone-induced cellular DNA breakage involves mobilization of nuclear copper. Thus, similar to earlier findings in this laboratory, flavones also exhibit a copper dependent prooxidant action leading to oxidative breakage of cellular DNA. Therefore, it is likely that flavones belong to the class of polyphenolic compounds whose prooxidant action is possibly responsible for their apoptosis induction and anticancer activities.