Chapter I

DNA binding and its degradation by gossypol and its semi-synthetic derivative, apogossypolone, in the presence of copper ions.
RESULTS I

Formation of gossypol/ApoG2-Cu(II) complex:

The absorption spectra of gossypol and ApoG2 with increasing concentrations of Cu(II) was recorded to examine the formation of gossypol/ApoG2-Cu(II) complex. The results given in figure 7 and 8 show that the addition of Cu(II) to gossypol and ApoG2 results in an enhancement in the peak of the spectra of both the compounds. In the case of ApoG2, a shift in the absorption towards a higher wavelength is also seen. The absorption spectra of gossypol and ApoG2 suggests a simple mode of interaction between these polyphenols and Cu(II).

Detection of gossypol/ApoG2-induced Cu(I) production by neocuproine:

The generation of Cu(I) from Cu(II), as a result of reduction of Cu(II) by gossypol and ApoG2, was analyzed using neocuproine. Neocuproine 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 chelates exhibit an absorption maximum at 450 nm. The compounds alone, as well as Cu(II) alone, do not lead to any change in spectra with neocuproine. However, gossypol+Cu(II) and ApoG2+Cu(II) react to generate Cu(I), as indicated by the peaks appearing at 450 nm (figure 9).

Formation of complexes involving calf thymus DNA with gossypol and ApoG2:

Figure 10 and figure 11 show the effect of the addition of increasing molar base pair ratios of calf thymus DNA on the fluorescence emission spectra of gossypol and ApoG2 excited at 360 nm and 340 nm, respectively. Such an addition resulted in a dose-dependent enhancement of fluorescence of gossypol and ApoG2. However, no significant shift in the λ\text{max} emission suggested a simple mode of binding of DNA with gossypol/ApoG2. The control (native DNA alone) when excited at the same wavelength (360 nm and 340 nm, respectively, result not
shown) did not interfere with the emission spectrum of the compounds alone thus confirming the binding results.

**Binding of copper ions to gossypol and ApoG2:**

Binding of copper ions to gossypol and ApoG2 was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of the compounds. The results shown in figure 12 and figure 13 clearly indicate the binding of Cu(II) to compounds, as addition of Cu(II) causes quenching of gossypol and ApoG2 fluorescence. These results support the result of absorption studies shown in figure 9 where formation of gossypol-copper and ApoG2-copper complex was demonstrated.

**Generation of oxygen radicals by gossypol and ApoG2:**

**Superoxide production:** The production of superoxide anion was determined by the method of Nakayama et al. (1983), which involves reduction of NBT by gossypol/ApoG2 to a formazan. The time dependent generation of superoxide anion by the compounds, 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 H₂O₂ which in the presence of transition metals, such as copper, favors Fenton type reaction to generate hydroxyl radicals which can 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), ROS such as hydroxyl radicals are formed which serve as the proximal DNA cleaving agent (Rahman et al., 1989). Therefore, the capacity of gossypol and ApoG2 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 colored adduct with TBA whose absorbance is read at 532 nm (Quinlan and Gutteridge, 1987). The result of figure 15 clearly shows that
increasing concentrations of the compounds lead to a progressive increase in the formation of hydroxyl radicals. Moreover, ApoG2 was found to be a more effective producer of hydroxyl radical at tested concentrations.

**Agarose gel electrophoresis of pBR322 plasmid DNA treated with gossypol/ApoG2 with copper:**

To examine the efficacy of gossypol/ApoG2-Cu(II) system in DNA cleavage pBR322 plasmid DNA was treated with gossypol/ApoG2 in presence of copper ions. As shown in figure 16, whereas copper alone or compounds alone do not cause DNA cleavage, the addition of copper to the compounds resulted in the generation of open circular topological structures of plasmid DNA, demonstrating that both gossypol and ApoG2 are capable of DNA degradation in presence of copper ions.

**Breakage of calf thymus DNA by gossypol/ApoG2 in the presence of Cu(II):**

Gossypol and ApoG2 in the presence of Cu(II) were found to generate single strand specific nuclease (Aspergillus oryzae) sensitive sites in calf thymus DNA. The reaction was assessed by recording the proportion of DNA converted to acid soluble-nucleotides by the nuclease. Figure 17 and figure 18 gives the dose response curve of such a reaction. In absence of Cu(II) gossypol and ApoG2 generated significantly lower levels of single strand nuclease sensitive sites in calf thymus DNA. Control experiments (data not shown) established that heat denatured DNA underwent 100% hydrolysis following the treatment with nuclease. However, in the presence of Cu(II) (50 µM), increasing concentrations of gossypol (figure 17) and ApoG2 (figure 18) resulted in an increase of nuclease sensitive sites in DNA leading to increased DNA hydrolysis.

**DNA breakage by gossypol-Cu(II) and ApoG2-Cu(II) system in lymphocytes as measured by Comet Assay:**

Increasing concentrations of gossypol (figure 19) and ApoG2 (figure 20) (0-50 µM) alone or in the presence of 20 µM CuCl₂ were tested for DNA breakage in
intact isolated human peripheral lymphocytes using alkaline single cell gel electrophoresis (comet assay). The corresponding tail length is plotted as a function of compound concentration. It is seen in both the figures that whereas the compounds alone cause breakage of cellular DNA, the degree of such DNA breakage is significantly enhanced in the presence of Cu(II). Cu(II) (20 µM) controls were similar to untreated lymphocyte without any significant DNA breakage. Moreover, it is seen that ApoG2 causes a significantly greater degree of DNA breakage in whole lymphocytes. The results clearly establish that gossypol/ApoG2-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.

Comparison of DNA breakage by gossypol and ApoG2 in whole lymphocytes and lymphocyte nuclei:

Since in the lysed version of Comet assay, membrane and cytoplasmic barrier are eliminated, it would be reasonable to assume that the compounds are able to directly interact with the cell nuclei. Thus, considerably greater DNA breakage should be observed in the lysed version as compared with the standard version (where intact lymphocytes are used). Increasing concentrations of (0 — 50 µM) of gossypol (figure 21) and ApoG2 (figure 22) were tested for DNA breakage in intact lymphocytes and compared with DNA breakage induced by gossypol and ApoG2 (0 - 50 µM) in lymphocyte nuclei (lysed version). It is seen that the rate of tail formation is greater in the case of lysed version suggesting that the compounds are able to directly interact with the nuclei.

Effect of neocuproine and bathocuproine disulphonic acid on gossypol/ApoG2 induced DNA breakage:

Copper chelators (neocuproine and bathocuproine disulphonic acid) were used to study their effect on DNA breakage by gossypol/ApoG2 in whole lymphocytes (figure 23A and figure 24A) as well as in lymphocyte nuclei (figure 23B and figure 24B). In whole lymphocytes, a clear inhibition was seen in presence of neocuproine (a cell membrane permeable Cu(I) specific chelator) on gossypol-
induced as well as ApoG2-induced DNA breakage. However, no such DNA breakage inhibition was observed in whole lymphocytes with bathocuproine disulphonic acid (a water soluble cell membrane impermeable analogue of neocuproine). Further, in case of lymphocyte nuclei treated with gossypol and ApoG2, both neocuproine and bathocuproine disulphonic acid were found to inhibit DNA breakage (figure 24B and figure 24B). Here both neocuproine and bathocuproine disulphonic acid are directly able to interact with nuclei.

**Effect of desferrioxamine and histidine on gossypol/ApoG2 induced DNA breakage:**

Fe$^{3+}$ and Cu$^{2+}$ are the most redox active of the metal ions present in living cells. Also, copper and zinc are the major metal ions present in the nucleus (Bryan, 1979). To examine the possible involvement of iron and zinc in gossypol/ApoG2-induced DNA breakage, the effect of desferrioxamine mesylate (a Fe(II) specific chelator) and histidine (a zinc specific chelator) has been examined on DNA breakage in lymphocytes and lymphocyte nuclei. The results of figure 25 and 26 (A&B) indicate that desferrioxamine mesylate as well as histidine were unable to inhibit gossypol/ApoG2-induced DNA breakage in intact lymphocytes or lymphocyte nuclei. This indicates that gossypol and ApoG2-induced DNA breakage occurs by the same mechanism through the mobilization of endogenous copper, possibly chromatin bound copper.

**Effect of scavengers of ROS on gossypol and ApoG2 induced DNA breakage in whole lymphocytes and lymphocyte nuclei:**

Table II gives the results of an experiment where various scavengers of reactive oxygen species such as superoxide dismutase (SOD), catalase, thiourea and mannitol were tested for their effect on gossypol/ApoG2-induced DNA breakage in lymphocytes. SOD and catalase remove superoxide and H$_2$O$_2$ respectively and thiourea and mannitol remove hydroxyl radicals. All scavengers caused a significant inhibition of DNA breakage as evidenced by decreased tail lengths. It may be mentioned that due to the site specific nature of the reaction of hydroxyl radicals with DNA it is difficult for any trapping molecules to intercept them
completely (Czene et al., 1997). This possibly accounts for the incomplete inhibition of DNA breakage by the scavengers. The results indicate that superoxide anion and H₂O₂ are essential components in the pathway that leads to the formation of hydroxyl radical and other species which would be the proximal DNA cleaving agents. In a similar experiment given in Table III, effect of the scavengers was studied in DNA breakage induced by the compounds in lymphocyte nuclei. The results clearly demonstrate inhibition of such DNA breakage by scavengers in nuclei. This further confirms that a similar mechanism involving ROS is responsible for cellular DNA breakage in intact lymphocytes as well as in the lymphocyte nuclei.

Effect of preincubation of lymphocytes with thiourea and neocuproine on TBARS generated in presence of gossypol/ApoG2:

As mentioned above, it is suggested that lymphocyte DNA breakage induced by gossypol and ApoG2 is the result of the generation of reactive oxygen species in situ. Oxygen radical damage to deoxyribose or DNA is considered to give rise to TBA reactive material (Quinlan & Gutterige, 1987). In the experiment shown in figure 27A and 27B, the formation of TBA reactive substance (TBARS) as a measure of oxidative stress in lymphocytes with increasing concentrations of gossypol and ApoG2 has been determined. The effect of preincubating the cells with neocuproine and thiourea was also studied. Results given in figure show that there is a dose dependent increase in the formation of TBA reactive substance in lymphocytes. However, when cells were preincubated with neocuproine and thiourea there was a considerable decrease in the rate of formation of TBA reactive substance by these compounds. The results given in figure 23/24 along with these results indicate that both DNA breakage and oxidative stress in cells is inhibited by Cu(I) chelation and scavenging of reactive oxygen. Thus it can be concluded that the formation of reactive oxygen species by the compounds in lymphocytes involves their interaction with intracellular copper as well as its reduction to Cu(I).
Detection of \( \text{H}_2\text{O}_2 \) generation in incubation medium by gossypol and ApoG2 using FOX assay:

Polyphenols have been shown to auto-oxidize in cell culture media and lead to the release of \( \text{H}_2\text{O}_2 \) and quinone that can enter the cells/nuclei causing damage to various biomacromolecules (Long et al., 2000). This extraneous production of ROS could also account for DNA breakage observed above. Therefore, we determined the production of \( \text{H}_2\text{O}_2 \) by gossypol and ApoG2 in the incubation medium RPMI 1640 and compared it to a known producer, tannic acid. As can be seen in Figure 28, where the generation of \( \text{H}_2\text{O}_2 \) is notably significant by tannic acid, gossypol and ApoG2 do not produce \( \text{H}_2\text{O}_2 \) in any significant amount. This indicates that the DNA breakage by the compounds observed above is not a result of extraneous generation of \( \text{H}_2\text{O}_2 \) in the reaction medium. Moreover, we have earlier shown that there exists no correlation between the extraneous generation of \( \text{H}_2\text{O}_2 \) and the ability of polyphenols to cause oxidative DNA breakage (Ullah et al., 2009).

Stoichiometry of Cu(II) reduction by gossypol/ApoG2:

It has been previously suggested that the redox cycling of Cu(II)/Cu(I) is an essential element in the induction of copper assisted oxidative DNA breakage (Hadi et al 2000). To assess the relative efficiency of reduction of Cu(II) by gossypol and ApoG2, stoichiometric studies were carried out. The result in figure 29 shows that a clear stoichiometry of Cu(II) reduction by both compounds is not obtained. The absorbance of neocuproine-Cu(I) complex continues to increase with increasing concentration of the compounds possibly reflecting the various oxidized states, which by themselves are able to reduce copper.
Figure 7: Absorption spectra of gossypol in the presence of Cu(II).

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

1. Gossypol alone
2. Gossypol + 50 µM Cu(II)
3. Gossypol + 100 µM Cu(II)
Figure 8: Absorption spectra of ApoG2 in the presence of Cu(II).

The 3.0 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 µM ApoG2 and increasing concentrations of Cu(II). The spectra were recorded after the addition of components indicated:

1. ApoG2 alone (——)
2. ApoG2 + 50 µM Cu(II) (-----)
3. ApoG2 + 100 µM Cu(II) (- - - - -)
Figure 9: Detection of gossypol and ApoG2 induced Cu (I) production by neocuproine.

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

1. Neocuproine + 100 µM Cu(II)
2. Neocuproine + 50 µM gossypol
3. Neocuproine + 50 µM ApoG2
4. Neocuproine + 50 µM gossypol + 100 µM Cu(II)
5. Neocuproine + 50 µM ApoG2 + 100 µM Cu(II)
Figure 10: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of gossypol.

Gossypol (in 10 mM Tris-HCl, pH 7.5) was excited at 360 nm and the emission spectra were recorded between 375-535 nm.

[---] Gossypol alone (25 μM)
[---] Gossypol: DNA base pair molar ratio (1:1)
[-----] Gossypol: DNA base pair molar ratio (1:2)
[------] Gossypol: DNA base pair molar ratio (1:4)
Figure 11: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of ApoG2.

ApoG2 (in 10 mM Tris-HCl, pH 7.5) was excited at 340 nm and the emission spectra were recorded between 365-545 nm.

[——] ApoG2 alone (25 µM)
[———] ApoG2: DNA base pair molar ratio (1:1)
[————] ApoG2: DNA base pair molar ratio (1:2)
[—————] ApoG2: DNA base pair molar ratio (1:4)
Figure 12: Effect of increasing concentrations of Cu(II) on the fluorescence emission spectra of gossypol.

Gossypol (in 10 mM Tris-HCl, pH 7.5) was excited at λ 360 nm and the emission spectra were recorded between 370-550 nm.

[———] Gossypol alone (25 µM)
[——] Gossypol: Cu(II) molar ratio (1:1)
[———] Gossypol: Cu(II) molar ratio (1:2)
[———] Gossypol: Cu(II) molar ratio (1:4)
[———] Gossypol: Cu(II) molar ratio (1:6)
Figure 13: Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of ApoG2.

2 (in 10 mM Tris-HCl, pH 7.5) was excited at \( \lambda = 340 \) nm and the emission spectra recorded between 365-535 nm.

- — — ApoG2 alone (25 µM)
- — — ApoG2: Cu(II) molar ratio (1:1)
- — — ApoG2: Cu(II) molar ratio (1:2)
- — — ApoG2: Cu(II) molar ratio (1:4)
- — — ApoG2: Cu(II) molar ratio (1:6)
Chapter I: Results

Figure 14: Photogeneration of superoxide anion by gossypol and ApoG2 on illumination under fluorescent light as a function of time.

Reaction mixture contained 100 mM phosphate buffer (pH 7.5) and 100 μM of gossypol (□)/ApoG2 (●). The samples were placed at a distance of 10 cm from the light source. All values reported are mean of three independent experiments.
Figure 15: Hydroxyl radical generation by gossypol/ApoG2.

Reaction mixture (0.5 ml) contained 100 µg calf thymus DNA as substrate, 50 µM Cu(II) and indicated concentrations of gossypol (Δ) and ApoG2 (□). 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 mean ± SEM of three independent experiments.

**p value<0.05 when compared to untreated control.

*p value<0.01 when compared to untreated control.
Figure 16: Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 plasmid DNA after treatment with gossypol and ApoG2 in the absence and presence of copper.

Lane D: pBR322 DNA alone
Lane C: pBR322 DNA + Cu (II) (30 µM)
Lane 1,2: pBR322 DNA + gossypol (100, 200 µM)
Lane 3: pBR322 DNA + gossypol (100 µM) + Cu(II) (30 µM)
Lane 4,5: pBR322 DNA + ApoG2 (100, 200 µM)
Lane 6: pBR322 DNA + ApoG2 (100 µM) + Cu(II) (30 µM)
Figure 17: Degradation of calf thymus DNA by gossypol in the presence of Cu(II) as measured by the degree of single strand specific nuclease (*Aspergillus oryzae*) 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 concentration of gossypol, alone (♦) and with Cu(II) (50 µM) (■). Single strand specific digestion was performed using S1 Nuclease as described in “Methods”. All points represent triplicates and mean values have been plotted.
Figure 18: Degradation of calf thymus DNA by ApoG2 in the presence of Cu(II) as measured by the degree of single strand specific nuclease (*Aspergillus oryzae*) 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 concentration of ApoG2, alone (○) and with Cu(II) (50 µM) (■). Single strand specific digestion was performed using SI Nuclease as described in “Methods”. All points represent triplicates and mean values have been plotted.
Figure 19: DNA breakage by gossypol in human peripheral lymphocytes in the absence and presence of Cu(II).

1 x 10^5 lymphocyte cells were incubated in microfuge tubes with reaction mixture at 4°C for 1 hr. Reaction mixture contained RPMI (400 µL), Ca²⁺ and Mg²⁺ free PBS, increasing concentrations of gossypol (0-50 µM), alone (•) and with fixed concentration of Cu(II) (20 µM) (■) and processed further for comet assay as given in “Methods”. All points represent mean ± SEM of three independent experiments. **p value<0.05 when compared to *(untreated control cells).
Figure 20: DNA breakage by ApoG2 in human peripheral lymphocytes in the absence and presence of Cu(II).

1 x 10^5 lymphocyte cells were incubated in microfuge tubes with reaction mixture at 4°C for 1 hr. Reaction mixture contained RPMI (400 µL), Ca^{2+} and Mg^{2+} free PBS, increasing concentrations of ApoG2 (0-50 µM), alone (•) and with fixed concentration of Cu(II) (20 µM) (■) and processed further for comet assay as given in "Methods". All points represent mean ± SEM of three independent experiments. **p value<0.05 when compared to *(untreated control cells).
Figure 21: Comparison of DNA breakage by gossypol in intact lymphocytes and lymphocyte nuclei as measured by Comet assay.

Whole lymphocytes (♦)/lymphocyte nuclei (■) embedded in agarose were layered with the reaction mixture (2.0 ml) containing RPMI (400 µL) and Ca²⁺ and Mg²⁺ free PBS [for whole lymphocytes] or 0.4 M phosphate buffer (pH 7.5) [for lymphocyte nuclei] and indicated concentrations of gossypol (0-50 µM) at 4°C for 1 hr and processed further for comet assay as given in “Methods”. All points represent mean of three independent experiments.

Error bars denote ± SEM.

**p value<0.05 and significant when compared to *control (untreated cells).
Figure 22: Comparison of DNA breakage by ApoG2 in intact lymphocytes and lymphocyte nuclei as measured by comet assay.

Whole lymphocytes (●)/lymphocyte nuclei (■) embedded in agarose were layered with the reaction mixture (2.0 ml) containing RPMI (400 µL) and Ca²⁺ and Mg²⁺ free PBS [for whole lymphocytes] or 0.4 M phosphate buffer (pH 7.5) [for lymphocyte nuclei] and indicated concentrations of ApoG2 (0-50 µM) at 4°C for 1 hr and processed further for comet assay as given in “Methods”. All points represent mean of three independent experiments.

Error bars denote ± SEM.

**p value<0.05 and significant when compared to *control (untreated cells).
Figure 23: Effect of neocuproine/bathocuproine on gossypol induced DNA breakage in whole lymphocytes (A) and lymphocyte nuclei (B).

(A)

(B)

Whole lymphocyte (A)/lymphocyte nuclei (B) embedded in agarose were layered with the reaction mixture (2.0 ml) containing gossypol (50 µM) and indicated concentrations of neocuproine (♦)/bathocuproine (■) at 4°C for 1hr and processed further for comet assay as given in “Methods”.

Values reported are mean ± SEM of three independent experiments.

**p value<0.05 and significant when compared to control.
Figure 24: Effect of neocuproine/bathocuproine on ApoG2 induced DNA breakage in whole lymphocytes (A) and lymphocyte nuclei (B).

Whole lymphocyte (A)/lymphocyte nuclei (B) embedded in agarose were layered with the reaction mixture (2.0 ml) containing ApoG2 (50 µM) and indicated concentrations of neocuproine (●)/bathocuproine (■) at 4°C for 1hr and processed further for comet assay as given in “Methods”. Values reported are mean ± SEM of three independent experiments. **p value<0.05 and significant when compared to control.
Figure 25: Effect of desferrioxamine mesylate/histidine on gossypol induced DNA breakage in lymphocytes (A) and lymphocyte nuclei (B).

Lymphocyte/lymphocyte nuclei were incubated with the reaction mixture (2.0 ml) containing gossypol (50 µM) and indicated concentrations of desferrioxamine mesylate/histidine at 4°C for 1hr and processed further for comet assay as given in “Methods”. Comet tail length (µ-meters) plotted as a function of increasing concentrations of desferrioxamine mesylate (■) and histidine (▲) in lymphocytes (A) and lymphocyte nuclei (B). Values reported are mean±SEM of three independent experiments.
Figure 26: Effect of desferrioxamine mesylate/histidine on ApoG2 induced DNA breakage in lymphocytes (A) and lymphocyte nuclei (B).

Lymphocyte/lymphocyte nuclei were incubated with the reaction mixture (2.0 ml) containing ApoG2 (50 µM) and indicated concentrations of desferrioxamine mesylate/histidine at 4°C for 1 hr and processed further for comet assay as described in "Methods". Comet tail length (µ-meters) plotted as a function of increasing concentrations of desferrioxamine mesylate (■) and histidine (▲) in lymphocytes (A) and lymphocyte nuclei (B). Values reported are mean±SEM of three independent experiments.
Table II: Effect of scavengers of reactive oxygen species on gossypol/ApoG2 induced DNA breakage in intact lymphocyte.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tail Length</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.03 ± 0.07&quot;</td>
<td>-</td>
</tr>
<tr>
<td><strong>Gossypol (50 µM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ SOD (100 µg/ml)</td>
<td>11.93 ± 0.98&quot;</td>
<td>55</td>
</tr>
<tr>
<td>+ Catalase (100 µg/ml)</td>
<td>12.42 ± 0.74&quot;</td>
<td>53</td>
</tr>
<tr>
<td>+ Thiourea (1 mM)</td>
<td>15.46 ± 1.35&quot;</td>
<td>41</td>
</tr>
<tr>
<td>+ Mannitol (1 mM)</td>
<td>14.23 ± 1.23&quot;</td>
<td>46</td>
</tr>
<tr>
<td><strong>Apogossipolone (50 µM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ SOD (100 µg/ml)</td>
<td>15.21 ± 1.22&quot;</td>
<td>50</td>
</tr>
<tr>
<td>+ Catalase (100 µg/ml)</td>
<td>14.13 ± 1.04&quot;</td>
<td>54</td>
</tr>
<tr>
<td>+ Thiourea (1 mM)</td>
<td>12.64 ± 0.62&quot;</td>
<td>59</td>
</tr>
<tr>
<td>+ Mannitol (1 mM)</td>
<td>13.84 ± 0.97&quot;</td>
<td>55</td>
</tr>
</tbody>
</table>

Whole lymphocytes embedded in agarose were layered with reaction mixture containing gossypol or ApoG2 in presence of scavengers of ROS at concentrations indicated at 4 °C for 1 hr and further processed for comet assay as described in “Methods.”

Values reported are mean±SEM of three independent experiments.

* and # p<0.05 when compared to **
+ p value<0.05 when compared to *
† p value<0.05 when compared to #
Table III: Effect of scavengers of reactive oxygen species on gossypol/ApoG2 induced DNA breakage in lymphocyte nuclei.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tail Length</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.11 ± 0.09”</td>
<td>-</td>
</tr>
<tr>
<td>Gossypol (50 µM)</td>
<td>33.80 ± 1.41”</td>
<td>-</td>
</tr>
<tr>
<td>+ SOD (100 µg/ml)</td>
<td>13.40 ± 0.82”</td>
<td>60</td>
</tr>
<tr>
<td>+ Catalase (100 µg/ml)</td>
<td>14.82 ± 0.88”</td>
<td>56</td>
</tr>
<tr>
<td>+ Thiourea (1 mM)</td>
<td>16.55 ± 0.95”</td>
<td>51</td>
</tr>
<tr>
<td>+ Mannitol (1 mM)</td>
<td>17.56 ± 1.02”</td>
<td>52</td>
</tr>
<tr>
<td>Apogossypolone (50 µM)</td>
<td>37.04 ± 1.82”</td>
<td>-</td>
</tr>
<tr>
<td>+ SOD (100 µg/ml)</td>
<td>15.54 ± 1.08”</td>
<td>58</td>
</tr>
<tr>
<td>+ Catalase (100 µg/ml)</td>
<td>16.24 ± 1.21”</td>
<td>56</td>
</tr>
<tr>
<td>+ Thiourea (1 mM)</td>
<td>15.16 ± 0.92”</td>
<td>59</td>
</tr>
<tr>
<td>+ Mannitol (1 mM)</td>
<td>15.44 ± 0.97”</td>
<td>58</td>
</tr>
</tbody>
</table>

Lymphocyte nuclei embedded in agarose were layered with reaction mixture containing gossypol or ApoG2 in presence of scavengers of ROS at concentrations indicated at 4 °C for 1 hr and further processed for comet assay. Values reported are mean±SEM of three independent experiments.

* and # p<0.05 when compared to **
+ p value<0.05 when compared to *
‖ p value<0.05 when compared to #
Figure 27: Effect of preincubation of lymphocyte with neocuproine and thiourea on TBARS generated by increasing concentrations of (A) gossypol and (B) ApoG2.

Lymphocyte cells were preincubated with fixed concentration of neocuproine and thiourea for 30 min at 37°C after which they were further incubated for 1hr in the presence of increasing concentrations of gossypol (A) and ApoG2 (B). Compound alone (○), Compound+ neocuproine (1 mM) (□), Compound + thiourea (1 mM) (△). Values reported are mean of three independent experiments.

*p value < 0.05 when compared to untreated control

p value < 0.05 when * value compared to ** value
Figure 28: \( \text{H}_2\text{O}_2 \) generation by tannic acid, gossypol and ApoG2 in the incubation medium.

A comparison of the rate of \( \text{H}_2\text{O}_2 \) formation by tannic acid (■) with gossypol, ApoG2 (not so distinct) in the incubation medium of the lymphocytes as determined by Fox assay.
Chapter 1: Results

Figure 29: Stoichiometry of copper reduction by gossypol and ApoG2.

Detection of stoichiometry of compounds-Cu(II) interaction. The concentration of gossypol (△) and ApoG2 (□) were 25 μM in the presence of 0.4 mM neocuproine. The absorbance of samples at 450 nm is plotted vs equivalents of Cu(II) per molar equivalent of compounds. All points represent triplicate samples and the mean values are plotted.
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, radiations etc. However, some data in the literature suggests that the antioxidant properties of the polyphenolic compounds may not fully account for their chemopreventive effects (Gall 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 following conclusions: (i) gossypol and ApoG2 are directly able to interact with DNA and copper and possibly form a ternary complex of DNA-Cu-compound; (ii) both the compounds are able to reduce Cu(II) to Cu(I); (iii) redox cycling of copper in the presence of gossypol/ApoG2 leads to the generation of ROS, such as superoxide, hydroxyl radicals, moreover, ApoG2 generates a significantly higher degree of hydroxyl radicals than gossypol; (iv) gossypol and ApoG2 induce strand scission in calf thymus DNA and plasmid DNA in presence of copper ions; (v) cellular DNA breakage induced by gossypol and ApoG2 in normal lymphocytes involves redox-cycling of endogenous copper, possibly chromatin bound copper; (vi) the relatively higher DNA breakage efficiency of ApoG2 is possibly accounted for by the presence of quinonoid moieties which impart a greater degree of membrane permeability. Copper mediated further oxidation of ortho-hydroxyl groups in ApoG2 may lead to a greater degree of DNA breakage capacity. Possibly, this further accounts for the enhanced DNA breakage capacity of ApoG2. As already mentioned both gossypol and ApoG2 have been shown to possess anticancer and apoptosis inducing properties in various cancer cell lines (Huang et al., 2006; Zhang et al., 2009; Mi et al., 2008; Arnold et al., 2008). Thus it may be suggested that gossypol and ApoG2 fall under the category of plant polyphenols such as flavonoids, catechins etc (considered to possess anticancer properties) that are able to mobilize endogenous copper leading to the formation of reactive oxygen.
species and consequent cellular DNA breakage (Hadi et al., 2000; Hadi et al. 2007, Hanif et al., 2008, Shamim et al., 2008, Ullah et al., 2009, Khan et al., 2011). This could be an important mechanism for the anticancer properties of gossypol and ApoG2.

The results presented above are in further confirmation of our hypothesis which envisages the mobilization of endogenous copper and the consequent generation of ROS. Since tumor cells are already under considerable oxidative stress any further increase in ROS levels is likely to be cytotoxic (Schumacker, 2006). Thus, the mechanism proposed by us is an alternative, non-enzymatic and copper dependent pathway for the cytotoxic action of certain anticancer agents that are capable of mobilizing and reducing endogenous copper. Such a common mechanism better explains the anticancer effects of antioxidants with diverse chemical structures as also the preferential cytotoxicity towards cancer cells. Further, this also leads to the prospect of synthesizing novel anticancer compounds with greater permeability and half-life in cells and more efficient copper-chelation and -reducing capabilities.
Chapter II

DNA binding and its degradation by thymoquinone in the presence of copper ions.