CHAPTER-I

DNA binding and its degradation by aloin and aloe-emodin in the presence of copper ions.
RESULTS-I
RESULTS-I

Formation of aloin/aloe-emodin-Cu(II) complex:
The possibility for the formation of aloin/aloe-emodin-Cu(II) complex was examined. This was carried out by recording the absorption spectra of aloin and aloe-emodin with increasing concentrations of Cu(II). The results given in figure 8 and 9 show that the addition of Cu(II) to aloin/aloe-emodin results in an enhancement in the peak appearing at the λmax of aloin and aloe-emodin. The absorption spectra of aloin and aloe-emodin suggests a simple mode of interaction between these polyphenols and Cu(II).

Detection of aloin/aloe-emodin-induced Cu(I) production by neocuproine/bathocuproine:
The production of Cu(I), formed as a result of reduction of Cu(II) by aloin/aloe-emodin, was analyzed using neocuproine/bathocuproine which are selective Cu(I) sequestering agents that bind 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 450 nm and 480 nm respectively. In the case of aloin, neither Cu(II) nor aloin interfere with the maxima, whereas aloin + Cu(II) react to generate Cu(I) which complexes with neocuproine (figure 10) and bathocuproine (figure 12) to give peaks appearing at 450 nm and 480 nm respectively. However, the polyphenol aloe-emodin itself forms a complex with neocuproine (figure 11) and bathocuproine (figure 13) that absorbs at 425 nm. This possibly interferes with the complex formation of the two chelators with Cu(I) and therefore no absorbance band is seen at 450 nm and 480 nm respectively.

Stoichiometry of Cu(II) reduction by aloin/aloe-emodin:
Plant polyphenols are known to reduce Cu(II) to Cu(I) as well as reduce molecular oxygen to superoxide anion (Rahman et al., 1990). The superoxide
thus formed spontaneously, gives rise to $\text{H}_2\text{O}_2$ which in the presence of Cu(I) generates hydroxyl radical (Fenton type reaction). We have therefore deduced the stoichiometry of Cu(II) reduction by aloin and aloe-emodin. In the experiment shown in figure 14, increasing concentrations of Cu(II) were added to fixed concentrations of aloin and aloe-emodin (50 $\mu$M) in the presence of 300 $\mu$M bathocuproine (a Cu(I) specific sequestering agent). The results are plotted as absorbance at 480 nm Vs equivalents of Cu(II)/polyphenol. As seen in figure 14, no clear maxima, where absorption plateaued, was obtained thereby suggesting a possible recycling of copper ions in the reaction. However, it does appear that aloin is a more effective copper reducing agent than aloe-emodin.

**Formation of complexes involving calf thymus DNA and aloin/aloe-emodin:**

Figure 15 and figure 16 shows the effect of addition of increasing molar base pair ratios of calf thymus DNA on the fluorescence emission at 580 nm and 540 nm of aloin and aloe-emodin respectively. Such an addition resulted in a dose-dependent enhancement of aloin fluorescence (figure 15) whereas quenching of the fluorescence was observed in the case of aloe-emodin (figure 16). There was however, no shift in the $\lambda_{\text{max}}$ emission suggesting a simple mode of binding of DNA and aloin/aloe-emodin. The control (native DNA alone) when excited at the same wavelength (360 nm for aloin and 410 nm for aloe-emodin) did not interfere with the emission spectrum of polyphenol alone/polyphenol + DNA, thus confirming the binding results.

**Binding of copper ions to aloin/aloe-emodin:**

Binding of copper ions to aloin/aloe-emodin was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of aloin/aloe-emodin. The result shown in figure 17 clearly indicate the binding as addition of Cu(II) causes quenching of aloin fluorescence whereas the
addition of Cu(II) to aloe-emodin causes enhancement of the fluorescence (figure 18). These results support the result of absorption studies shown in figure 8 and figure 9 where formation of aloin-copper and aloe-emodin-copper complex were demonstrated.

**Generation of oxygen radicals by aloin/aloe-emodin:**

**Superoxide production:** The production of superoxide anion was determined by the method of Nakayama et al. (1983), which involves reduction of NBT by aloin/aloe-emodin to a formazan. The time dependent generation of superoxide anion by 100 μM aloin/aloe-emodin, as evidenced by the increase in absorbance at 560 nm is shown in figure 19. The fact that NBT was genuinely assaying superoxide was confirmed by SOD (100 μg/ml) inhibiting the reaction (results not shown).

**Hydroxyl radical generation by aloin/aloe-emodin:** 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 aloin and aloe-emodin 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 (Quinlan and Gutteridge, 1987). The result of figure 20 clearly show that increasing concentrations of aloin lead to a progressive increase in the formation of hydroxyl radicals whereas aloe-emodin is considerably less effective in generating hydroxyl radicals at the concentrations used.
Breakage of calf thymus DNA by aloin/aloe-emodin in the presence of Cu(II):

Aloin and aloe-emodin 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. Figure 21 and figure 22 gives the dose response curve of such a reaction. However, aloin and aloe-emodin in the absence of Cu(II) did not generate 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. In the presence of Cu(II) (50 μM), increasing concentrations of aloin (figure 21) and aloe-emodin (figure 22) resulted in increase in nuclease sensitive sites in DNA leading to increased DNA hydrolysis. Although, as can be seen from the figures, % DNA hydrolysis is greater in the case of aloin than aloe-emodin.

Standardization of alkaline single cell gel electrophoresis/Comet Assay:

Alkaline single cell gel electrophoresis is a sensitive technique for detecting DNA single strand breaks at the level of a single cell. 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 23. 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
Results-I

comet tails is observed. In figure 24, the results of the same experiment are plotted as comet tail length (µm) as a function of increasing H₂O₂ concentration.

**DNA breakage by aloin-Cu(II) and aloe-emodin-Cu(II) system in lymphocytes as measured by Comet Assay:**

Increasing concentrations of aloin (0-200 µM) either alone or in the presence of 50 µM CuCl₂ was tested for DNA breakage in isolated human peripheral lymphocytes using the Comet Assay. The corresponding tail length is plotted as a function of polyphenol concentration. It is seen in figure 25 that whereas aloin alone causes some breakage of cellular DNA, the degree of such breakage is considerably greater in the presence of Cu(II). Untreated lymphocyte controls were similar to aloin alone or Cu(II) alone without any significant DNA breakage. A similar experiment with increasing concentrations of aloe-emodin (0-200 µM) alone and in the presence of 50 µM Cu(II) was also performed. As can be seen in figure 26, aloe-emodin was unable to cause significant and progressive DNA breakage either alone or in the presence of Cu(II) as compared to aloin. The results clearly establish that aloin-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 aloin and increasing concentrations of Cu(II). The spectra were recorded after addition of components indicated.

(—) Aloin alone

(---) Aloin + 100μM Cu(II)

(- - -) Aloin + 200 μM Cu(II)
The 3.0 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 µM aloe-emodin and increasing concentrations of Cu(II). The spectra were recorded after addition of components indicated.

(—) Aloe-emodin alone

(---) Aloe-emodin + 100µM Cu(II)

(----) Aloe-emodin + 200 µM Cu(II)
Results-I

Figure 10. Detection of aloin-induced Cu(I) production by neocuproine:

Testscan GBC DBUV Instrument

Absorbance

Wavelength

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

(a) Neocuproine + 100 μM Cu(II)
(b) Neocuproine + 100 μM Cu(I)
(c) Neocuproine + 50 μM Aloin
(d) Neocuproine + 50 μM Aloin + 100 μM Cu(II)
Figure 11. Detection of aloe-emodin-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:

(a) Neocuproine + 100 μM Cu(II)
(b) Neocuproine + 100 μM Cu(I)
(c) Neocuproine + 50 μM Aloe-emodin
(d) Neocuproine + 50 μM Aloe-emodin + 100 μM Cu(II)
Figure 12. Detection of aloin-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:

(a) Bathocuproine + 100 μM Cu(II)
(b) Bathocuproine + 100 μM Cu(I)
(c) Bathocuproine + 50 μM Aloin
(d) Bathocuproine + 50 μM Aloin + 100 μM Cu(II)
Figure 13. Detection of aloe-emodin-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:
(a) Bathocuproine + 100 μM Cu(II)
(b) Bathocuproine + 100 μM Cu(I)
(c) Bathocuproine + 50 μM Aloe-emodin
(d) Bathocuproine + 50 μM Aloe-emodin + 100 μM Cu(II)
Figure 14. Detection of stoichiometry of aloin/aloe-emodin and Cu(II) interaction:

Reaction mixture contained 10 mM Tris-HCl (pH 7.5), 300 μM bathocuproine and fixed concentration of aloin (•) / aloe-emodin (△) (50 μM), with increasing concentrations of Cu(II) (shown as molar ratios of Cu(II)/polyphenol). Absorbance was recorded at 480 nm after incubating the reaction mixture at room temperature for 10 min. All points represent triplicates and mean values have been plotted.
Figure 15. Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of aloin:

Aloin (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 360 nm and the emission spectra were recorded between 530-610 nm.

- [ - ] Aloin alone (25 µM)
- [ - - - ] Aloin : DNA base pair molar ratio (1:1)
- [ ----- ] Aloin : DNA base pair molar ratio (1:2)
- [ - - - - ] Aloin : DNA base pair molar ratio (1:4)
- [ - - - - - ] Aloin : DNA base pair molar ratio (1:6)
Aloe-emodin (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 410 nm and the emission spectra were recorded between 510-580 nm.

[ — ] Aloe-emodin alone (25 μM)

[— — ] Aloe-emodin : DNA base pair molar ratio (1:1)

[----] Aloe-emodin : DNA base pair molar ratio (1:2)

[ — — ] Aloe-emodin : DNA base pair molar ratio (1:4)
Figure 17. Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of aloin:

Aloin (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 360 nm and the emission spectra were recorded between 530-610 nm.

[ - ] Aloin alone (25 µM)

[ -- ] Aloin : Cu(II) molar ratio (1:1)

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

[ - - - ] Aloin : Cu(II) molar ratio (1:4)
Figure 18. Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of aloe-emodin:

Aloe-emodin (in Tris-HCl, pH 7.5) was excited at its $\lambda_{\text{max}}$ (absorption) of 410 nm and the emission spectra were recorded between 510-580 nm.

[—] Aloe-emodin alone (25 μM)

[— —] Aloe-emodin : Cu(II) molar ratio (1:1)

[———] Aloe-emodin : Cu(II) molar ratio (1:2)

[— — —] Aloe-emodin : Cu(II) molar ratio (1:4)
Figure 19. Photogeneration of superoxide anion by aloin/aloe-emodin on illumination under fluorescent light as a function of time:

Reaction mixture contained 100 mM phosphate buffer (pH 7.5) and 100 μM of aloin (●)/aloe-emodin (▲). 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 20. Hydroxyl radical generation by aloin/aloë-emodin:

Reaction mixture (0.5 ml) contained 100 μg calf thymus DNA as substrate, 50 μM Cu(II) and indicated concentrations of aloin (•) and aloë-emodin (▲). 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 21. Degradation of calf thymus DNA by aloin 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 aloin alone (○) and aloin with Cu(II) (50 μM) (■). Single strand specific nuclease 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.
Results-I

Figure 22. Degradation of calf thymus DNA by aloe-emodin 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 aloe-emodin alone (○) and aloe-emodin with Cu(II) (50 µM) (■). Single strand specific nuclease 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 23. Standardization of Comet Assay with H$_2$O$_2$:

Reaction mixture (1.0 ml) contained $1 \times 10^5$ cells, RPMI 400 µl, PBS (Ca$^{2+}$ and Mg$^{2+}$ free) and indicated concentrations of H$_2$O$_2$ (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 H$_2$O$_2$.

(A) Untreated lymphocytes
(B) Lymphocytes + 10 µM H$_2$O$_2$
(C) Lymphocytes + 20 µM H$_2$O$_2$
(D) Lymphocytes + 50 µM H$_2$O$_2$
Figure 24. Standardization of Comet Assay with H$_2$O$_2$:

Reaction mixture (1.0 ml) contained 1 x $10^5$ cells, RPMI 400 µl, PBS (Ca$^{2+}$ and Mg$^{2+}$ free) and indicated concentrations of H$_2$O$_2$ (0-50 µM). The reaction mixture was incubated at 37°C for 30 min and processed further for Comet Assay as described in “Methods”. Comet tail length (µ metres) is plotted as a function of increasing concentrations of H$_2$O$_2$ (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 25. DNA breakage by aloin-Cu(II) in human peripheral lymphocytes with increasing aloin concentrations:

Reaction mixture (1.0 ml) contained 1 × 10^5 cells, RPMI 400 μl, PBS (Ca^{2+} and Mg^{2+} free), increasing concentrations of aloin (0-200 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) is plotted as a function of increasing concentrations of aloin (0-200 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 26. DNA breakage by aloe-emodin-Cu(II) in human peripheral lymphocytes with increasing aloe-emodin concentrations:

Reaction mixture (1.0 ml) contained 1 x 10^5 cells, RPMI 400 µl, PBS (Ca^{2+} and Mg^{2+} free), increasing concentrations of aloe-emodin (0-200 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) is plotted as a function of increasing concentrations of aloe-emodin (0-200 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
DISCUSSION-I

The major conclusions of the experiments described in this chapter are: (i) aloin and aloe-emodin form a complex with both DNA as well as Cu(II) and possibly in the presence of all three, a ternary complex of the polyphenol-Cu(II)-DNA is formed, (ii) Cu(II) is reduced by aloin in the complex to generate Cu(I), (iii) aloin is able to cause DNA breakage in the presence of copper ions in lymphocytes whereas aloe-emodin is relatively ineffective, (iv) redox cycling of copper leads to the generation of various reactive oxygen species, particularly the hydroxyl radical which serves as the proximal DNA cleaving agent.

These results place aloin in a class 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. The generation of oxygen radicals in the proximity of DNA is well established as a cause of strand scission (Ahmad et al., 1992; Bhat and Hadi, 1994; Rahman et al., 1989). It is generally recognized that such reactions with DNA are preceded by association of the ligand with DNA, followed by the production of oxygen radicals at that site (Pryor, 1988). Metal ion dependent degradation of DNA by 4-(9-acridinylamino) methanesulphone-m-anisidine (Wong et al., 1984), 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 (Ahmad et al., 1992; Rahman et al., 1989) are based on mechanisms involving oxygen-derived radicals. Studies on flavonoids from this laboratory have shown that a ternary complex of the polyphenol, DNA and Cu(II) is formed which generates oxygen radicals insitu via Cu(I). The results presented here show that aloin is 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 aloin-Cu(II) mediated DNA cleavage.
It has been reported that several chemopreventive agents that are antioxidants at some concentrations become prooxidants at other concentrations (Lee and Park, 2003). One of the studies has shown that aloin exerts prooxidant effect leading to DNA breakage at lower concentrations i.e. 8-300 μM (Tian and Hua, 2005). Aloin leads to the generation of hydroxyl radical which serves as DNA cleaving agent through the Fenton type reaction (Tian and Hua, 2005). As we can see from the conclusions drawn from the results of this chapter, aloe-emodin is relatively ineffective in causing DNA breakage in lymphocytes when compared to aloin. This also correlates with the copper reducing efficiency of the two compounds. Studies have shown that aloe-emodin exerts a prooxidant effect on DNA only at higher concentrations i.e. 1.25-2.5 mM (Tian and Hua, 2005). At concentrations used in the present study, it is possible that free radical scavenging activity of aloe-emodin predominates over its prooxidant action. Fe^{3+} and Cu^{2+} are the most redox active of the metal ions in living cells. Copper is an essential constituent of many enzymes such as tyrosinase and superoxide dismutase. Normal serum contains upto 8 μM loosely bound copper and other biological fluids may also contain comparable amounts (Gutteridge, 1984). Loosely bound copper has been defined by Gutteridge (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 aloin. Copper has also been reported to be a normal component of chromatin and such endogenous copper can be mobilized by chemical agents such as 1,10-phenanthroline to cause internucleosomal DNA fragmentation (Burkitt et al., 1996).

Most of the plant polyphenols are known to be potential antioxidants, a property which is implicated in their chemopreventive effects. However, evidence in literature suggests that antioxidant properties of these compounds may not fully account for their anticancer properties (Gali et al., 1992; Ahmad et al., 2000). Earlier studies in our laboratory have shown that
polyphenols such as curcumin (Ahsan and Hadi, 1998), resveratrol (Azmi et al., 2006) and delphinidin (Hanif et al., 2008) exhibit prooxidant property in the presence of copper ions. These compounds are also known to induce apoptosis in cancer cells (Kuo et al., 1996; Clement et al., 1998; Hou et al., 2003). Similar to our earlier findings, aloin also exhibits copper dependant prooxidant action leading to oxidative breakage of cellular DNA. Thus, aloin also belongs to the class of polyphenolic antioxidants whose prooxidant action is possibly responsible for apoptosis induction and anticancer activity.