CHAPTER V

THE EFFECT OF THE DRUG ON APOPTOSIS
5.1 INTRODUCTION

We have been able to find by preliminary experiments that the drug may act as an anti cancer drug on the principles of apoptosis. Recent studies have been indicated a role of apoptosis in a variety of human diseases including cancer. Several anti cancer drugs isolated from plants have been found to be helpful in the prevention of cancer development. The term apoptosis was coined in a new classic paper by Kerr JFR et al in 1972 as a means of distinguishing a morphologically distinctive form of cell death, which was associated with normal physiology. Nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum and membrane blebbing characterize Apoptosis. Mitochondria remain unchanged morphologically (Wyllie AH et al, 1980). This visible transformation is accompanied by biochemical changes. Those at the cell surface include the externalization of phosphatidylserine and other alterations that promote recognition by phagocytes. Intracellular changes include the degradation of the chromosomes of the chromosomal DNA into high molecular weight and oligonucleosomal fragments, and cleavage of a specific subset of cellular polypeptides (Kerr JFR et al 1972 and Wyllie A H et al 1980). This cleavage is accomplished by a family of intracellular proteases called caspases.

5.2 Biochemistry of apoptosis

It is to be found that how specific normal or cancer cells are selected for death and how cells that should die are protected from activation of the death programme. Our present knowledge indicate that apoptosis is the result of an active, gene-directed process, we should eventually be able to manipulate this process by developing drug that interact with cell death proteins. Works have been carried out regarding the role of mitochondria in apoptosis. Connection between cell cycle and apoptosis including p53 and Rb (Scheer C. J et al 2000 and Harbour J W et al 2000) oncogenes (Green
The major apoptosis regulator gene family is the human Bcl-2 homologues. The BC22 family of proteins can be divided into two groups; suppressors of apoptosis (e.g.: BCL2, BCLXI, BCLW, BAG I, MCL I, AI) and activations of apoptosis [e.g.: BAX, BOK, HRK, BNIP3, BIM, BIK, BCLXS, BIK, BLK, BID, BAK and BAD] (Adams J.M et al 1998). Pathological increases in the amounts of one or more of the apoptosis suppressing proteins have been observed in several types of cancer. The accumulating data suggest that the protein functions at several stages of the signaling cascade, leading to apoptosis. Mitochondria play an important part in apoptosis. Several apoptosis stimuli induce translocation of BAX from cytosol to mitochondria, where it induces these organelles to release the caspases activating protein cytochrome C. BAX seems to create pores in the outer membranes of mitochondria of sufficient size to allow cytochrome C to escape.

BCL2 genes are regulated by p53. The p53 protein induces cell cycle arrest and apoptosis when anticancer drugs or radiation damages DNA. The promoter region of BAX was found to contain typical p53 binding sites and the BAX gene was shown to be a direct transcriptional target of p53. Over expression of BAX or other proapoptotic family members renders tumour cells more sensitive to many anticancer drugs, whereas ablation of its expression reduces drug-induced apoptosis, demonstrating the importance of this family of proteins, not only as modulators of chemo responses, but also as possible direct mediators of their ultimate cytotoxic actions.

Most apoptotic proteolytic cleavage results from the action of caspases. Survivin is over expressed in a large proportion of human cancers, providing evidence that altered expression of these proteins can occur during tumorigenesis. (Ambrosini et al 1997 and Tarnn et al 1998) At the beginning of mitosis, survivin associates with the mitotic spindle, and disruption of this interaction results in the loss of its anti-apoptosis function. The over expression of survivin in cancer may thus overcome this apoptosis-related cell-cycle checkpoints and favour aberrant progression of
transformed cells through mitosis. Apoptosis induced by various chemotherapeutic drugs may involve the FAS/FAS ligand systems (Friesen C et al 1996). However, in cells deficient in caspase 8 or over expressing its inhibitor cFLIP, apoptosis mediated by FAS but not by anti cancer drugs was inhibited. Moreover, in the absence of caspase 8, anticancer drugs still induce the processing of caspase 9 and 3, indicating that chemotherapy induced cell death does not require caspase 8. Cells resistant to FAS were equally susceptible to anticancer drugs and activated caspase 8, with the same dose response as FAS sensitive cells. These data suggest that drug induced apoptosis does not require FAS.

5.3 Apoptosis induced by Medicinal plants

Programmed cell death (apoptosis), a form of cell death, described by Kerr and Wyllie some 20 years ago, has generated much attention in recent years. The mechanisms by which this mode of cell death (seen both in animal and plant cells), takes place have been examined in detail. The attempt to influence the natural phenomenon of programmed cell death stems from the fact that it is reduced (like in cancer) or increased (like in neuro degenerative diseases) in several clinical situations. Thus, chemicals that can modify programmed cell death are likely to be potentially useful drugs. From foxglove, which gave digitalis to the Pacific Yew from which came taxol, plants have been a source of research material for useful drugs. Recently, a variety of plant extracts has been investigated for their ability to influence the apoptotic process. Various cell-lines like HI-60, human hepatocellular carcinoma cell line (KIM-I), a cholangio carcinoma cell-line (KMC-1), B-cell hybridomas U937 a monocytic cell-line, HeLa cells, human Lymphoid leukemia (MOLT-48) cells and K562 cells have been studied. The agents found to induce apoptosis (measured either morphologically or flow cytometrically) included extracts of plants like mistletoe and Semicarpus anacardium. Isolated compounds like bryonolic acid (from Trichosanthes kirilowii var japonica), crocin (from saffron) and allicin (from Allium sativum) have also been found to induce programmed cell death and therefore arrest
proliferation. Even Chinese herbal medicine “Sho-saiko-to” induces programmed cell death in selected cancerous cell-lines. It is very interesting to note that Panax ginseng prevents irradiation induced programmed cell death in hair follicles, suggesting important therapeutic implications. Nutraceuticals (dietary plants) like soybean, garlic, ginger, green tea, etc., which has been suggested in epidemiological studies, to reduce the incidence of cancer might do so by inducing, programmed cell death. Soybean extracts have been shown to prevent development of diseases like polycystic kidneys. Another peculiar feature is that a number of food items as well as herbal medicines have been reported to produce toxic effect by inducing programmed cell death. For example, programmed cell death in isolated rat hepatocytes has been implicated in the hepatitis induced by an herbal medicine containing diterpenoids from germander. Several studies suggest that rapid progression of the betel and tobacco related oral squamous cell carcinomas might be associated with a simultaneous involvement of p53 and c-myc leading to inhibition of programmed cell death. Several mechanisms have been identified to underlie the modulation of programmed cell death by plants including endonuclease activation, induction of p53, activation of caspase 3 protease via a BCL-2 insensitive pathway, potentiate free-radical formation and accumulation of sphinguanine. Programmed cell death is a highly conserved mechanism of self-defense, also found to occur in plants. Hence, it is natural to assume that chemicals must exist in them to regulate programmed cell death in them. Thus, plants are likely to prove to be important sources of agents that will modulate programmed cell death (Ingo Tamm et al 2001).

5.4 Materials and Methods

5.4.1 Mice: Inbred strains of male Swiss albino mice, 20-25 gm were used for the study.
5.4.2 DLA Cell-line: The cells were obtained from tumour bearing mice in which the tumour cells were maintained by weekly serial passages.

5.4.3 Purification of *B. racemosa* seed extract: Detailed procedure was mentioned in section 2.2.2.2. The purified fraction $F_8$ (2:8) was used for this experiment.

5.4.4 *In vitro* study: It was carried out by incubating $1 \times 10^6$ DLA cells in RPMI 1640 with 5% FCS containing various concentrations (2, 4, 6, 8 & 10 μg/ml) of the purified fraction $F_8$ (2:8) in a CO$_2$ incubator at 37°C for 6 and 12 hours of treatment (Kirsch Volders et al. 1997).

5.4.5 *In vivo* study: Three groups of mice were used for this study. The first group was used as control. The second (2$^{nd}$) and third (3$^{rd}$) group of mice was given 3 & 6 mg/kg, respectively for 16 days. On the 14$^{th}$ day, the cells were aspirated from the peritoneal cavity of mice 4 hours after drug administration using a syringe. Similarly on the 16$^{th}$ day, the same procedure was repeated and evaluated apoptotic cells.

5.4.6 Morphologic determination of apoptosis

(a) Giemsa staining: The microscopic observation of apoptotic cells was performed as reported by Kirsch Volders et al., 1997. Briefly, after 6 and 12 hours of treatment, the cells were methanol fixed and seeded on glass slides, then stained with Giemsa 10% solution and examined by optical microscope. At least 1000 cells were blind scored for each experimental point.

(b) Acridine orange - Ethidium bromide dual staining: For assessment of apoptosis after treatment of DLA cells for 6 and 12 hrs, acridine orange-ethidium bromide dual staining of unfixed DLA cells was used. The mixture of fluorescent dyes consisted of acridine orange at 5μg/ml and ethidium bromide at 5μg/ml in PBS. These dyes stain the DNA and allow visualization of the condensed chromatin of apoptosis cells. Slides were observed under a fluorescent microscope. Acridine orange was observed using standard narrow band FITC excitation (excitation wavelength 450-
490nm and barrier filter 520-560nm). Ethidium bromide only stains cells in late stages of apoptosis and secondary necrosis when membrane integrity has been lost. Early apoptotic cells are impermeable to the dye. The early stages of apoptosis are readily detectable using acridine orange (Haque et al 1997).

5.5 Result

The in vitro study shows that at a concentration of 4µg/ml DLA cells showed apoptotic characters such as membrane blebbing, chromatin condensation, cytoplasmic vacuolation and nuclear fragmentation. When the cells were stained with acridine orange and ethidium bromide, it showed chromatin condensation with membrane blebbing (Plate no. 4 a). The normal (without drug) and treated cells were also stained with 10% giemsa. All these cells showed the same characters that mentioned above (Plate no.5). Early apoptotic cells stained with AO + El have dense chromatin which lines the nuclear membrane in crescent shape (Plate no. 4 f & c). It was found that the percentage of apoptotic cells were 4.62% at the above concentration during 12 hrs of drug treatment as compared to 9% in the case of control (Table-18). The same concentration showed only less apoptotic cells i.e., 0.62% during 6 hrs of treatment. The concentration at 6µg/ml exhibited only few apoptotic cells of 1.95% during 12 hrs of drug treatment. The higher concentration such as 8 and 10 µg/ml showed necrosis instead of apoptosis. (Plate no.4 h)The concentration at 6 µg/ml exhibited only very few apoptotic cells. At a concentration of 2 µg/ml, most of the cells are comparable to control (without drug). The result indicates that maximum apoptotic cells were obtained at a concentration of 4µg/ml. Hence, this dose can be considered as optimum dose for inducing apoptosis in DLA cells. This proves that the purified fraction F₈ (2:8) of B. racemosa seed extract can definitely induce apoptotic changes. In the in vivo study at a lower concentration of 3 mg/kg showed more apoptotic characters as compared to 6mg/kg. No significant increase in apoptosis was observed on 14h and 16th day and results are shown in (Table-19).
Figure A - DLA cells were cultured for 12 hrs and stained with acridine orange and ethidium bromide and examined using a fluorescence microscope to determine the extent of apoptosis. This is the control (normal) cells treated with vehicle only (400x).

Figure B - DLA cells were cultured for 12hrs and stained with acridine orange and ethidium bromide and examined using a fluorescence microscope to determine the extent of apoptosis. It illustrates the change in morphology when DLA cells are treated with 4μg/ml of B. racemosa purified fraction (F8:2:8). The majority of cells shrink, with a significant number of nuclei becoming bright with condensed chromatin (400x).

Figure C - DLA cells were cultured for 12 hrs and stained with acridine orange and ethidium bromide and examined using a fluorescence microscope to determine the extent of apoptosis. It illustrates the change in morphology when DLA cells are treated with 4μg/ml of B. racemosa purified fraction F8 (2:8). The majority of cells shrink, with a significant number of nuclei becoming bright with condensed chromatin which lines the nuclear membrane in crescent shape (400x).

Figure D - DLA cells were cultured for 6 hours and stained with acridine orange and ethidium bromide and examined using a fluorescence microscope to determine the extent of apoptosis. The cells were treated with 4μg/ml of B. racemosa seed purified fraction F (2:8). Treated cells showing chromatin condensation with membrane blebbs (400x).

Figure E - DLA cells were cultured and 6 hours stained with acridine orange and ethidium bromide and examined using a fluorescence microscope to determine the extent of apoptosis. The cells were treated with 4μg/ml of B. racemosa seed purified fraction F (2:8). Treated cells are significantly smaller than control, with acridine orange and ethidium bromide fluorescence and evidence of chromatin condensation (400x).

Figure F - DLA cells were aspirated from tumour bearing mice treated with 3mg/kg of B. racemosa purified fraction F (2:8). On 14th day 4hr after drug administration. The cells were stained with acridine orange and ethidium bromide and examined using a fluorescence microscope to determine the extent of apoptosis. Figure showing early apoptotic cells have dense chromatin which lines the nuclear membrane in crescent shape (400x).

Figure G - DLA cells were aspirated from tumour bearing mice treated with 3mg/kg of B. racemosa purified fraction F (2:8). On 16th day 4hr after drug administration. The cells were stained with acridine orange and ethidium bromide and examined using a fluorescence microscope to determine the extent of apoptosis. Figure showing chromatin condensation with membrane blebbs (400x).

Figure H - DLA cells were cultured for 12 hours and stained with acridine orange and ethidium bromide and examined using a fluorescence microscope to determine the extent of apoptosis. It illustrates the change in morphology when DLA cells are treated with 8μg/ml of B. racemosa purified fraction F8 (2:8). The majority of cells showing necrosis instead of apoptosis (400x).
Plate No.5

Figure A - DLA cells were treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) for 12 hours and stained with 10% giemsa. The nuclei of the cell has broken into two densely stained discrete fragments which is typical feature of apoptosis. And it also showing chromatin condensation (400x).

Figure B - DLA cells were treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) for 12 hours and stained with 10% giemsa. The nuclei of the cell has broken into two densely stained discrete fragments which is typical feature of apoptosis. And it also showing chromatin condensation (400x).

Figure C - DLA cells were treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) for 12 hours and stained with 10% giemsa. The cells showing chromatin condensation with membrane blebbing (400x).

Figure D - DLA cells were treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) for 12 hours and stained with 10% giemsa. The nuclei of the cell has broken into a number of densely stained discrete fragments which is typical feature of apoptosis. And it also showing chromatin condensation (400x).

Figure E - DLA cells were treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) for 12 hours and stained with 10% giemsa. The cells showing chromatin condensation with membrane blebbing (400x).

Figure F - DLA cells were treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) for 6 hours and stained with 10% giemsa. The cells showing membrane blebbing and chromatin condensation (400x).

Figure G - DLA cells were aspirated from the peritoneal cavity of mice treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) 4 hours after drug administration for 16 days. The nuclei of the cell has broken into two densely stained discrete fragments which is typical feature of apoptosis. And it also showing chromatin condensation (400x).

Figure H - DLA cells were aspirated from the peritoneal cavity of mice treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) 4 hours after drug administration for 16 days. The nuclei of the cell has broken into a number of densely stained discrete fragments which is typical feature of apoptosis. And it also showing chromatin condensation (400x).

Figure I - DLA cells were aspirated from the peritoneal cavity of mice treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) 4 hours after drug administration for 16 days. The figure showing the characteristic chromatin rearrangement and it is strongly different from its normal organisation and it also exhibits membrane blebbing (400x).

Figure J - DLA cells were aspirated from the peritoneal cavity of mice treated with purified fraction of *B. racemosa* seed extract F<sub>8</sub> (2:8) 4 hours after drug administration for 16 days. The figure showing the characteristic chromatin rearrangement and it is strongly different from its normal organisation and it also exhibits membrane blebbing (400x).
Table - 18

Apoptosis Induction in DLA cells treated with *B. racemosa* as determined by Microscopic morphological observation (giemsa staining)

<table>
<thead>
<tr>
<th>Concentration of <em>B. racemosa</em> seed extract</th>
<th>Scored cells</th>
<th>Body weight (gm)</th>
<th>No. of cells</th>
<th>Apoptotic cells(%)</th>
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<tr>
<td></td>
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<td></td>
<td>Treatment time</td>
<td>Treatment time</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>6hrs</td>
<td>12hrs</td>
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<tr>
<td>0 (Control)</td>
<td>4000</td>
<td></td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>4000</td>
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<tr>
<td>10</td>
<td>4000</td>
<td></td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

Values are the average of 3 separate assay.
Table - 19

Apoptosis induction in inoculated DLA cells in the peritoneal cavity of mice treated with *B.racemosa* seed extract as determined by microscopic morphological observation (giemsa staining)

<table>
<thead>
<tr>
<th>Concentration of <em>B.racemosa</em> seed extract</th>
<th>Body weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scored cells</td>
</tr>
<tr>
<td></td>
<td>Treatment time</td>
</tr>
<tr>
<td></td>
<td>14th day</td>
</tr>
<tr>
<td>Control</td>
<td>4000</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>4000</td>
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<tr>
<td>6mg/kg</td>
<td>4000</td>
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</table>

Values are the average of 3 separate assays

5.6 Discussion

The present work shows for the first time the induction of apoptosis by the *B.racemosa* seed extract. Apoptosis is a genetically controlled form of cell suicide, which is also involved, in the specific elimination of damaged cells. At a lower concentration of 6 μg/ml it showed maximum apoptotic characters as compared to higher concentration of 8 and 10 μg/ml. The very lower dose 2 μg/ml showed only less apoptotic behavior and it was almost similar to control cells. There are a number of reports that several crude and purified fractions induce apoptosis in various cell lines. It has already reported that the extract of the stem bark of *Acanthopanax senticosus*
HARMS induce apoptosis a quit KATO III cells and morphological change showing apoptotic bodies was observed (Hibasami et al 2000). Shikonin, isolated from Lithospermum erythrorhizori, a traditional oriental medicinal herb, was observed to induce apoptosis in HL-60 human promyleocytic leukemia cell line (Yoon Y et al 1999). Most chemotherapeutic drugs exert this anti tumor effects by inducing apoptosis. Therefore, an effective compound inducing apoptosis appears to be a relevant strategy to suppress various human tumours. In search for tumour inhibitors from various kinds of plants, Ran et al found that extracts from Solanum muricatum can inhibit tumour growth in vivo and in vitro by inducing apoptosis. It was found that the suppressive effect of novel water extracts of Uncaria tomentosa on tumour cell lines (K56 and HL60) appears to be mediated through induction of characteristic morphological changes, inter nucleosomal DNA fragmentation after agarose gel electrophoresis and DNA fragmentation quantification (Sheng-Y-et al 1998). During our cytotoxic experiment the drug was administered directly to the peritoneal cavity and from there it might have been absorbed so that the concentration is less and at this concentration it may be possible to have apoptotic effect on the tumour cells at the incited level of production. Hence, the multiplication of the cell can be prevented by this mechanism. All the results indicate that B. racemosa seed extract induces apoptosis in DLA cells and it is possible to confirm apoptosis as one of the methods of anticancer activity.