CHAPTER V

DISCUSSION
Cancer is the leading cause of mortality worldwide and the new approaches for its treatment are critically needed. Throughout medical history, plant products have been shown to be valuable sources of novel anti-cancer drugs and have also played an important role in contributing to the arsenal of the approximately 60 cancer chemotherapeutic drugs in the market (Kinghorn, 2000). Main four structural classes of plant anticancer agents are available, which are constituted by the *Catharanthus* (Vinca) alkaloids (vinblastine, vincristine, vinorelbine), the epipodophyllotoxins (etoposide, etoposide phosphate, teniposide), the taxanes (paclitaxel and docetaxel), and the camptothecin derivatives (irinotecan and topotecan).

Keeping in view the importance of natural products in anticancer drug discovery the present study was planned to explore *Erythrina suberosa* and *Anagallis arvensis* for their anticancer potential, coupled with the fact that plants belonging to their respective families were reported to have anticancer potential and that, these plants have not yet been explored for their activities against cancer. The stem and stem bark of *E. suberosa* and whole plant of *A. arvensis* were used in the present study. The major approach in searching for potential anticancer agents over the last 50 years has been based on selective cytotoxic effects on mammalian cancer cell lines. Cytotoxicity testing is based on one or more mammalian cell lines being grown under conditions where they are actively growing and undergoing mitotic division. Cells are cultured in a microtitre well plate and the rate of multiplication and growth is measured indirectly by formation of a colour, the intensity of which is directly proportional to the number of cells. A variety of experiments viz. SRB assay, MTT assay can be used and the most basic is to compare the rate of proliferation of a cancer cell line in the presence and absence of the test substance, usually after a specified time. Ideally several different cancer cell lines can be used so that selectivity can be assessed and the addition of normal cell lines to the battery enables selectivity of effect between cancer and normal cell lines (Houghton *et al*., 2007).

Three extracts (95% alcoholic, 50% alcoholic and aqueous) were prepared and evaluated against a panel of human cancer cell lines using sulphorhodamine B (SRB) assay. This assay relies on the uptake of the negatively charged pink aminoxanthine dye, SRB by basic amino acids in the cells. The greater the number of live cells, the greater amount of dye is taken up and, after fixing, when the cells are lysed, the released dye will give a more intense colour and greater absorbance (Houghton *et al*., 2007). It was noticed
that, 95% alcoholic extract from both the plant parts i.e. ESS & ESB, were cytotoxic in case of *E. suberosa*. In case of *A. arvensis*, 95% alcoholic extract and 50% aqueous-alcoholic extract showed a pronounced effect. ESB and ESS were further evaluated at 10, 30, 100 µg/ml and 25, 50, 100 µg/ml concentrations respectively and 95% alcoholic & 50% alcoholic extract of *A. arvensis* at 10, 30, 100 µg/ml concentrations against human cancer cell lines of nine tissues at different time intervals. It was found that ESB showed a differential effect with IC\(_{50}\) values between 32.9 - >100 µg/ml and 22.9 - >100 µg/ml, after 24 and 48h treatment respectively, depending on the cell lines. IC\(_{50}\) values calculated in case of ESS was in the range of 90.6 to >100 µg/ml and 56.9 to >100 µg/ml at 24 and 48h respectively. It was observed that, 95% alcoholic extract of *A. arvensis* showed a significant decrease in the IC\(_{50}\) value as the incubation time was increased. After 24h, IC\(_{50}\) values against cell lines treated with 95% alcoholic extract of *A. arvensis* were between 25.1 - >100 µg/ml, which were found to decrease to 6.0 - 54.3 µg/ml after 48h. At 72h, it further decreased from 1.24 µg/ml to 45.7 µg/ml. In case of 50% alcoholic extract of *A. arvensis* IC\(_{50}\) values were between 31.4 - >100 µg/ml, 8.2 - >100 µg/ml and 2.3 - 94.5 µg/ml at 24, 48 and 72h respectively. It is evident that in case of *A. arvensis* also, there was a gradual decline in IC\(_{50}\) values in all the cell lines as the incubation time was increased. Further studies were proceeded with only 95% alcoholic extract of *A. arvensis* (AAE).

From cytotoxicity data, thus it can be inferred, that from all the three extracts the most promising and active was the alcoholic extract. This observation corroborate with the belief of traditional practitioner’s, that the polar compounds are mostly responsible for the claimed anticancer properties (Tan *et al.*, 2005). Likewise, some recent studies on methanolic extract from *Coriolus versicolor* showed cytotoxicity against B16 cells in a dose-dependent manner (Harhaji *et al.*, 2008). An aqueous-ethanol extract prepared from *C. versicolor* also showed a dose-dependent *in vitro* cytotoxicity against HL60 cells (Lau *et al.*, 2004). Grape seed extract strongly inhibits cell growth and induces cell cycle arrest and apoptosis in human colon carcinoma cells (Kaur *et al.*, 2008). The extracts from *Vitis rotundifolia* and *Vitis vinifera* reduced the growth of MOLT-4 cells after 48 h in a concentration-dependent manner (Talcott *et al.*, 2008). Aqueous extracts of *Oldenlandia diffusa* also inhibited the growth of the HL60 cells significantly (Willimott *et al.*, 2007).
Further to have better insight into the cytotoxicity profile of ESB and AAE, they were subjected to flash chromatography and nine fractions were obtained. It was observed that the ethyl acetate fractions (ESB-EA1 & ESB-EA2) of ESB and ethanol:water fraction (AAE-EW1) of AAE were most active at 100 µg/ml. Further, from cytotoxicity data at lower concentrations of 10 and 30 µg/ml, it was interpreted that activity was not enriched as compared to their respective parent extracts.

One of the important criteria for a therapeutic drug for cancer is to have minimum or no side-effects on normal body cells (Badisa et al., 2009). To ascertain this, cytotoxicity of ESB, ESS and AAE was also tested against normal human lung fibroblast (WI-38 and MRC-5) and monkey kidney (CV-1) cell lines. It was observed that effect of the extracts was negligible against normal cell lines, indicating that cytotoxic potential is specific to cancer cells. Likewise, Jaiaree et al. (2011) have reported the selective cytotoxic activities of ethanolic extract of Dioscorea birmanica against lung cancer cells and no cytotoxic activity against normal cancer MRC-5 cells was observed.

Human promyelocytic leukemia HL-60 cells are considered a valid model system for testing antileukemic and general antitumor compounds, because they are known to be sensitive to a large panel of apoptotic stimuli (Kim et al. 2001; Tartier et al., 2000). In the present investigation apoptosis-inducing effect of ESB, ESS and AAE was investigated using HL60 suspension cell lines using MTT colorimetric assay and it was observed that there was inhibition in cell proliferation in a dose- and time-dependent manner. Likewise, Liu et al. (2006) discovered that extract from Narcissus tazetta decreased the survival rate of HL-60 cells and induced apoptosis in the HL-60 cell line. Roy et al. (2007) also reported the inhibitory effect of methanolic extract of the fruits of Oroxylum indicum, against proliferation of HL-60 cells. Moreover, in order to analyze the DNA damaging effect of extracts the BrdU incorporation assay was carried out. The results obtained showed that HL-60 cells treated with ESB, ESS and AAE inhibited BrdU incorporation in concentration dependent manner. Costa et al. (2008) have also showed similar effect of Maytenus ilicifolia extract against human HL-60 cells.

There are many reports in literature which show that natural products having significant in vitro cytotoxicity also exhibit effective in vivo activity. In the present study, murine ascite model was used to have the insight into the anti-cancer potential in general, which is followed by the murine tumor and leukemia models. ESB, ESS and AAE were
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tested against different murine models. It was observed that ESB showed 30.48% and 32.38% tumor growth inhibition against EAC at 125 mg/kg and 150 mg/kg i.p. respectively. In Lymphoid leukemia (L1210) model the median survival time (%T/C) was 103.44% at 200 mg/kg i.p. Further ESS showed 34.55% and 42.80% tumor growth inhibition against Sarcoma-180 (Ascites) at 200 and 300 mg/kg concentration and the median survival time (%T/C) was 91.95% at 200 mg/kg i.p. in Lymphoid leukemia (L1210) model. Furthermore AAE showed concentration dependent tumor growth inhibition against EAC at 3 mg/kg and 5 mg/kg i.p. doses respectively. Median survival time (%T/C) was 124.02% at 4 mg/kg i.p. against lymphoid leukemia (L1210) model. It showed 1.70% and 38.34% tumor growth inhibition against Sarcoma-180 (Solid) at 3 mg/kg and 5 mg/kg i.p. dose level respectively. Thus, the in vivo data showed that the doses used were in the tolerable limits and were not lethal to animals, signifying its preclinical importance. A similar study conducted by Kumar et al. (2011) has shown that an analog of parthenin, had IC_{50} of 3.5µM at 48h, in HL60 cells. They further observed that in L1210 lymphoid leukemia model it significantly extended the median life span of mice to 198% at 10 mg/kg concentration. Further, in Sarcoma-180 (solid) tumor model the analog produced about 64% tumor growth inhibition at 15 mg/kg and in the Ehrlich ascites tumor (solid) upto 63% at 25 mg/kg b.wt. (i.p.). Similarly Chashoo et al. (2011) have also reported that a boswellic acid analog, showing strong in vitro effect inhibited EAC tumor at 60mg/kg by 67.29%. In another study by Khan et al. (2011) a different analogue of boswellic acid exhibited tumor regression by 47.49% at 30 mg/kg, i.p. in EAC.

Induction of apoptosis is the preferred mode of action of cancer chemotherapeutics (Shimoda et al., 2003). This form of cell death is characterized by distinct morphological and biochemical features including condensation of nuclear chromatin, cell shrinkage, externalization of phosphotidylserine, and activation of caspases (Steller, 1995; Wyllie et al., 1980). Several sensitive methods for detecting apoptosis have been developed. Microscopic analysis of the treated cells is the first hand observation to observe the changes in the cells. In the present studies three different dyes [Giemsa stain for light microscopy, acridine orange – ethidium bromide dual staining (AO-EtBr) and Hoechst 33258 for fluorescence microscopy] were used for observing nuclear and morphological changes in cells. Staining of apoptotic cells with fluorescent dyes such as Hoechst 33258 and AO-EtBr is considered as the correct method for evaluating the changes in the nuclear
morphology (Jo et al., 2005, Baskic et al., 2006). Surface alterations, such as cytoplasmic blebbing, the formation of a number of membrane-bounded apoptotic bodies that have a diverse appearance, particularly in regard to their size, were also observed by scanning electron microscopy in different cell lines. Results of the light and fluorescent microscopy showed that ESB-induced morphological alterations, such as chromatin condensation and nuclear fragmentation at 50 and 100 μg/ml. Further ESS caused changes in nuclear and morphological structures at 100 μg/ml. Furthermore in case of AAE treated HL-60 cells, changes were observed at 20 and 50 μg/ml. It was seen with AO-EtBr dual staining that cells undergoing necrosis increased with the increase in the concentration of AAE. The scanning electron micrographs of ESB treated HL-60, COLO-205 and SW-620 cells, ESS treated HL-60 cells and AAE treated HL-60 and A-549 cells were also observed, which showed formation of blebs and apoptotic bodies, loss of cellular processes and smoothening of cell surface.

Likewise, Jo et al. (2005), Yu et al. (2006) and Zhang et al. (2007) have reported the use of Hoechst 33258 in investigating the morphological changes of nuclei in treated cancer cells. They observed shrinkage in cell volume, condensation of chromatin and nuclear fragmentation. Similarly the use of AO-EtBr has been widely used in literature to differentiate between apoptotic and necrotic cells. Liu et al. (2006) and Kania et al. (2007) in their studies, have identified the early and late apoptotic as well as necrotic population of treated cells using the dual stain method. Baskic et al. (2006) have analyzed the alterations in cells by utilizing Giemsa stain and AO/EtBr dual staining and observed nuclear shrinkage, chromatin condensation, cytoplasmic membrane blebbing, reduction of cell volume and formation of apoptotic bodies, in case of treated cells undergoing apoptosis. Necrotic cells were separated by nuclear and cytoplasmic swelling, chromatin flocculation, cytoplasmic and nuclear membrane dissolution or lysis. Furthermore, in the studies done by Sharma et al. (2010), scanning electron microscopy has been used in analyzing the surface morphology of cells. It has been shown that in treated cells there was uniform loss of cellular processes, smoothening of cell surface and formation of numerous apoptotic bodies.

There are several methods that can be used to quantify apoptosis and flow cytometry is one of them. Apoptotic cells exhibit some morphological modifications that are readily detected according to their light scatter properties (FSC/ SCC) by flow
Many anticancer agents can cause cell death by damaging DNA or arresting cell cycle at the G_{0}/G_{1}, S, G_{2}/M phase and then induce apoptosis. Use of a fluorochrome, such as PI, that is capable of binding and labeling DNA makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis and subsequent identification of hypodiploid cells (Riccardi and Nicoletti, 2006). The proportion of hypodiploid cells in total cell population represents the intensity of apoptosis-inducing activity of the tested sample (Li et al., 2005). The DNA content of cells treated with ESB showed significant increase in sub-G_{0}/G_{1} population. Cell cycle analysis showed a significant concentration dependent increase in sub G_{0} population of cells from 6.0% - 46.0% at 18h. ESS treated HL-60 cells showed 4.08% – 54.42% increase at 24h and AAE treated HL-60 showed an increase of 5.4% - 39.9% at 12h. Similarly, Bhushan et al. (2007) and Kumar et al. (2008) have observed increase in hypodiploid sub-G_{0} DNA fraction of cell cycle phase distribution in treated cells as compared to control group.

One of the earliest events of apoptosis is the loss of plasma membrane polarity, accompanied by translocation of phosphatidylinerse (PS) from the inner to outer membrane leaflets, thereby exposing PS to the external environment. The phospholipid-binding protein annexin V has high affinity for PS and can bind to cells with fluorescently labeled annexin V, which correlates with loss of membrane polarity during apoptosis. It precedes the complete loss of membrane integrity that accompanies later stages of cell death, resulting from either apoptosis or necrosis. In contrast, PI can only enter cells after loss of membrane integrity. Thus, dual staining with annexin V and PI allows clear discrimination between affected cells (annexin V negative, PI negative), early apoptotic cells (annexin V positive, PI negative), and the late apoptotic or necrotic cells (annexin V positive, PI positive) (Yang et al., 2007). HL60 cells treated with ESB showed annexin V positive cells at lower concentrations and both annexin V FITC and PI positive cells at higher concentrations which revealed that there is postapoptotic necrosis. ESS exhibited moderate increase in annexin V FITC binding of cells upto 18.55% at 100 µg/ml. AAE caused concentration dependent increase from 6.38% in control to 17.78% at 15 µg/ml. Ghosh et al. (2006) also utilized double labelling techniques using annexin-V and PI to distinguish between apoptotic and necrotic cells. Most of the cells were bound to Annexin-
V FITC but not to PI and very few with both indicating that the mode of cell death was apoptosis.

Following an apoptotic signal, mitochondria have a pivotal role in apoptosis by releasing apoptogenic factors. A mitochondria-dependent step involving mitochondrial outer membrane permeabilization (MOMP) is associated with most pro-apoptotic stimuli. This process is controlled by the pro- and anti-apoptotic members of the Bcl-2 family and results in the cytosolic release of cytochrome c that triggers caspase activation. It is followed by the formation of a ternary complex called the apoptosome that is composed of the adaptor protein Apaf-1, caspase-9 and cytochrome c. In the apoptosome, active initiator caspase-9 processes effector caspases such as caspase-3. Next, active caspase-3 cleaves several cell substrates, utilize PARP, a DNA repair enzyme, to induce apoptosis and cell death (Arnoult, 2006; Wang et al., 2008). Similarly Ma et al. (2008) in their experiment have shown the role of antiapoptotic Bcl-2 protein in initiating apoptosis. They observed that *Pinus massoniana* bark extract caused the decrease in Bcl-2 expression in HeLa cells, which further changed the polarization of mitochondrial membrane and released cytochrome c in cytosol, which further elevated the caspase activity. Kim et al. (2007) also reported that apoptosis induced by the *Ganoderma lucidum* and *Duchesnea chrysanth* extracts on human leukemia HL-60 cells was associated with mitochondrial membrane change, Bcl-2 down-regulation, Bax translocation, mitochondrial cytochrome c release and caspase-3 activation. Yu et al. (2006) also reasoned the cytochrome c release as the major cause of activation of caspase 9, which further increase the caspase 3 catalytic activity significantly. Further Singh et al. (2006) have also reported that down-regulation of anti-apoptotic protein Bcl-2 might be an important target to increase drug-sensitivity of cancer cells.

The impairment of mitochondrial function has also been considered to be a key event in the ROS-mediated apoptotic pathway (Wang et al., 2007). Carvalho et al. (2006) reported that violacein from *Chromobacterium violaceum*, mediated ROS production followed by activation of Caspase-3, release of cytochrome c, and calcium to cytosol in Caco-2 cells. Jeong et al. (2010) reported the disruption in mitochondrial membrane potential due to ROS generation. Mulberry fruit extracts caused a significant reduction in mitochondrial membrane potential, and its effect was prevented by the antioxidants. The report indicated that extracts induced apoptotic cell death through ROS-dependent
mitochondrial pathway involving MPT, cytochrome c release, and caspase activation in human glioma cells. Barraja \textit{et al.} (2008) have also associated the mitochondrial membrane depolarization with mitochondrial production of reactive oxygen species. ROS accumulation has been proposed to be involved in ESB-induced cell death. ROS levels were determined in HL60 cells after ESB treatment using a peroxide sensitive fluorescent probe, DCFH-DA, and a six-fold increase was evidenced in a period of 24 h. Nitric oxide production was also observed at 18 h. Depletion in Bcl-2 levels upto 72% was observed. The integrity of mitochondrial membranes of ESB-treated cells was examined by measuring their ability to retain Rh-123, a fluorescent dye which was used to indicate the loss of mitochondrial transmembrane potential (Kim \textit{et al.}, 2007). A significant decrease of MMP was observed in HL60 cells after treatment with 100 µg/ml ESB for 18 h. Further investigation indicated that caspases are involved since caspase-9 and -3 were activated. It was also observed in the present study that Ac-DEVD-CHO, a caspase-3 inhibitor, achieved complete inhibition as well, suggesting that caspase-3 is the major executioner caspase in ESB-induced HL60 apoptosis. In general, both the mitochondria-initiated intrinsic pathway and the death receptor-triggered extrinsic pathway can lead to caspase-3 activation (Li \textit{et al.}, 2007; Jeong \textit{et al.}, 2010). But in ESB treated cells, caspase-9 was significantly activated, which implicated the mitochondrial involvement since caspase-9 is the initiator caspase for the mitochondria-mediated intrinsic apoptotic pathway. Furthermore, as there was no significant activation of caspase-8, the receptor-mediated pathway may have not been triggered.

In ESS treated HL-60 cells, ROS generation was only 1.39 fold even after 24h treatment. It was noticed that there was change in mitochondrial membrane potential upto 59.09% at highest concentration and 3.45 fold increase in cytosolic cytochrome c. There was an increase of 3.0 and 1.8 fold of caspase 9 and caspase 3 respectively. It might be possible that ROS production did not play a lead part in inducing apoptosis in ESS treated HL-60 cells.

A significant 6.9 fold increase in ROS generation was observed in AAE treated HL-60 cells with a fair increase in caspase activity. Here ROS and caspases both seemed to take central part in induction of apoptosis. Nitric Oxide has been shown to possess both pro-apoptotic and anti-apoptotic functions, depending on the cell type and cellular redox state, as well as on the concentration and flux of Nitric Oxide itself (Chanvorachote \textit{et al.},
2006). When measuring the end products of Nitric Oxide metabolism, the Griess reaction is usually the preferred assay. It is the most sensitive and has the largest linear range for routine analysis of nitric oxide (Curtin et al., 2002). When the cells were treated with various concentration of ESB, Nitric Oxide production was moderately increased in concentration dependent manner. ESS caused 52% increase in Nitric Oxide production in HL-60 cells. Upto 25% increase in Nitric Oxide production was observed after treatment with AAE. Das et al. (2006) have reported that Nitric Oxide is a cytotoxic agent, involved as mediator in inflammatory disorders and because of its cytotoxicity, overproduction is deleterious to cells and can induce oxidative stress. Shen et al. (2002) reported that mitochondria are a source of Nitric Oxide, the production of which may affect energy metabolism that further triggers the collapse of mitochondrial membrane potential, affecting mitochondrial respiration and culminating in delayed cell death. Zunino et al. (2007) also linked overproduction of Nitric Oxide to disruption of mitochondrial membrane potential by increasing the concentration of superoxide anion and other ROS.

One of the most commonly used techniques for confirmation of apoptosis is identification of DNA ladders (Wyllie et al., 1980). A well documented characteristic of apoptosis is the fragmentation of DNA into multimers of approximately 200 base pairs. Effector caspase-3 activity cleaves and inactivates the inhibitor of caspase activated DNase, thus releasing active endonuclease(s) that translocate into the nucleus to initiate internucleosomal DNA fragmentation (Perchellet et al., 2004). Lee et al. (2005) confirmed using DNA fragmentation assay that cell death induced by myristicin was due to apoptosis. Tang et al. (2009) also determined the induction of apoptosis in HT-29 cells by DNA fragmentation analysis using classical DNA laddering on agarose gel electrophoresis.

In the present study, in order to elucidate whether ESB decreased cell survival by the induction of DNA fragmentation, genomic DNA was isolated from cells exposed to different concentrations of ESB from HL60 cells and electrophoresed. The ESB treatment of HL60 cells indicated internucleosomal DNA breakdown, leading to DNA fragmentation as expected for apoptotic cells. In ESS treated HL-60 cells, DNA fragmentation was observed at 75 µg/ml, which further became prominent at higher concentration of 100, 125 and 150 µg/ml. A typical characteristic ladder was observed in AAE treated HL-60 cells at 10 µg/ml after 12 h treatment.
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In conclusion, our results indicate that an increase of ROS is the initial essential event that results in the dissipation of mitochondrial membrane potential, releasing cytochrome c followed by activation of caspase-9 and -3 that commit the cells to the mitochondria-mediated intrinsic apoptotic pathway in ESB treated HL60 cells. ESS induced apoptosis in HL-60 cells in vitro in a dose-dependent manner. Nitric Oxide generation and mitochondrial dysfunction causing excess of cytochrome c release are the possible contributing factors of apoptosis. The present study also suggested that in case of AAE treated HL-60 cells, an increase in cytosolic cytochrome c in concentration dependent manner indicated involvement of intrinsic pathway of apoptosis. The loss of mitochondrial membrane potential, induction of caspase -3 and -9 was also observed further confirming the induction of apoptosis by AAE accompanied by ROS generation. Thus, anticancer and apoptotic potential of 95% alcoholic extract from E. suberosa and A. arvensis has promising avenues for targeting the mitochondrial target based novel anticancer therapeutics. It can be concluded that E. suberosa and A. arvensis could be explored as novel source of anticancer agent(s).