CHAPTER NINE

SUMMARY AND CONCLUSION
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Breast cancer is one of the leading causes of death in women (Stephens et al., 2012). Several chemotherapeutic drugs have been identified to treat breast cancer, yet a convincing cure remains elusive. Therefore, there is continuing need for development of new anticancer drugs. India is one of the richest biodiversity centers with respect to medicinal plants. Such plants are utilized in traditional system of medicine for cancer treatment. Plant derived compounds have played an important role in the development of several clinically useful anticancer agents (Cragg et al., 2011). Sesquiterpene lactones are diverse group of natural products and considered as potential anticancer agents.

Apoptosis is a genetically regulated process of cell death. Cells undergoing apoptosis exhibit shrunken pyknotic nuclei, DNA fragmentation and membrane blebbing. Induction of apoptosis of cancer cells is recognized as a valuable tool for cancer treatment (Cotter, 2009). The agents that are capable of inducing selective apoptosis of cancer cells have received considerable attention in the development of novel cancer chemotherapeutic drugs (Ocker and Höpfner, 2012). The current study investigated the pharmacological activities of two selected indigenous medicinal plant extracts in a group of human breast cancer (MCF7, MDA MB 231) and cervical cancer cells (HeLa). A methodical evaluation of detailed cytotoxicity and cell proliferation effects in human cancer cells revealed that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* impart dose-dependent cytotoxicity and cell growth inhibitory activity. *Withania somnifera* ethanolic extract exhibited relatively potent cytotoxic effects in human cancer cells as compared to *Tinospora cordifolia*. Acridine orange/ethidium bromide assay using fluorescent microscopy revealed that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* induce apoptosis but not the necrosis in MCF7, MDA MB 231 and HeLa. Hoechst 33342 assay and DNA fragmentation assay corroborated hallmark properties of apoptosis such as membrane blebbing, nuclear condensation and DNA fragmentation in breast cancer cells. Activation of cell cycle plays a significant role in the regulation apoptosis and can occur at specific stages in cell cycle (Li et al., 2012). Further, cell cycle analysis using flow cytometry revealed that treatment of breast cancer cells with the *Withania somnifera* extract caused significant arrest of cells at G2/M phase, significantly increased Sub-G0 phase and diminished S phase, indicating mitotic arrest, induction of apoptosis and inhibition of DNA replication respectively. On the other hand, *Tinospora*
extract significantly increased Sub-G$_0$ phase and diminished S phase indicating induction of apoptosis and inhibition of DNA replication respectively. The current study also addressed the question of whether Withania somnifera and Tinospora cordifolia extract mediated suppression of cell viability and growth was selectively to cancer cells. Interestingly, it was found that extracts did not induce any apoptosis in human ‘normal’ epithelial cells (HaCaT, immortalized cells) at the concentration that was cytotoxic to the breast cancer cells (MCF7). These results suggested that extracts of Withania somnifera and Tinospora cordifolia possess cancer cell-specific cytotoxicity and apoptosis inducing properties which is a highly attractive trait for cancer chemotherapeutic agents.

Recent studies suggest that cancer is a stem cell disorder. Indeed a small population of cancer stem cells has been identified within several malignancies that both functionally and phenotypically resemble normal stem cells. Cancer stem cells have self-renewal and differentiation ability and are highly tumorigenic than the rest of the population (Greaves, 2011). An increasing body of evidence suggests that cancer stem cells are highly resistant to chemotherapy and often relapse in cancer is associated with the inability of current drugs to target these cancer stem cells (Clevers, 2011). Stem cells from multiple tissues have been identified based on their ability to efflux the lipophilic dye Hoechst 33342 using flow cytometry which forms the basis of side population assay (Goodell et al., 1996). Side population (SP) phenotype is mediated by the ABC family of drug transporter proteins (ABCB1, ABCG2 and ABCC1) that can actively pump several drugs, including chemotherapeutic drugs, out of cells (Zhou et al., 2002). Since stem cells show increased expression of ABC transporters, they also tend to exhibit the SP phenotype. Thus, SP assay has become a critical assay for the identification and isolation of stem cells (Willan and Farnie, 2011). SP has been found to be highly enriched in cancer stem cells. The present study investigated the existence of SP phenotype in different types of cancer cells by flow cytometry based SP assay, and thereafter tested the effects of the two plant extracts. Among the various cell types evaluated, it was found that human cervical cancer line (HeLa), human breast cancer lines (MCF7, MDA MB 231), human lung adenocarcinoma (A549), human colon carcinoma (CaCo2) and rat glioma (C6) cells harbor a detectable and well resolved side population phenotype distinct from bulk population of cells. SP phenotype in HeLa, CaCo2 and C6 cells were significantly inhibited in presence of verapamil, an ABCB1 inhibitor,
indicating an active efflux-based SP phenotype, mediated through the ABCB1 transporter. In contrast, side population SP phenotype in A549 and MCF7 cells were significantly inhibited in the presence of fumetrimorgin C, an ABCG2 inhibitor, indicating a predominantly ABCG2-mediated efflux of Hoechst 33342 in these cell types.

When the cancer cells were cultured with sub-lethal concentrations of doxorubicin, a currently available chemotherapeutic drug widely used for the treatment of breast cancer, the SP phenotype was significantly enriched. Further, cytotoxicity assays in flow sorted SP and non-SP cells revealed that doxorubicin caused significantly increased cell death in non-SP cells as compared to SP cells indicating that SP cells were resistant to doxorubicin. Therefore, doxorubicin is effective in eliminating the bulk of cancer cells (or non-SP). However, SP which identifies the cancer stem cells were found to be highly resistant to cell death induced by doxorubicin. This is consistent with the cancer stem cell hypothesis that predicts that current chemotherapeutic drugs reduce the burden of tumor, but leave behind the cancer stem cells population (Greaves, 2011). The current study also investigated SP inhibitory effects of Withania somnifera and Tinospora cordifolia extracts. We found that extracts of Tinospora cordifolia significantly inhibited SP by inducing SP-specific cell death. Hence, inhibition of the ‘side population’ in the human cancer cells upon treatment with extracts of Tinospora cordifolia draws substantial attention as a highly promising drug to specifically eliminate rare population of cancer stem cells. Bioactivity guided (based on anti-SP activity) fractionation of Tinospora cordifolia extract revealed that petroleum ether fraction and dichloromethane fractions imparted dose dependent cytotoxicity, effectively inhibited SP phenotype and multidrug resistant drug transporter (ABCB1, P-glycoprotein) in cancer cells. Thus, Tinospora cordifolia petroleum ether and dichloromethane fractions may contain the active constituent’s responsible for biological activity. We hence selected the Tinospora cordifolia petroleum ether and dichloromethane fractions for further fractionation and isolation of the active cytotoxic compounds.

The current study adopted the strategy of bioactivity or mechanism of action directed isolation and characterization of active compounds from the selected plant. In order to isolate and characterize the active compound, Tinospora cordifolia petroleum ether and dichloromethane fractions were further sub-fractioned into a total of 34 fractions (F1 to F34) by column chromatography and each fraction was screened for anticancer activity. Results
indicated that among the 34 sub-fractions, *Tinospora cordifolia* dichloromethane sub-fractions F4, F5, F6S, F7 and F8 significantly inhibited SP and ABCB1 (MRD1) drug transporter. These fractions were obtained by column chromatography of *Tinospora cordifolia* dichloromethane fraction by 10% step-wise gradient elution with a solvent system of petroleum ether - ethyl acetate - methanol. Thin layer chromatography of these sub-fractions F4, F5, F6S, F7 and F8 revealed that each sub-fraction contained more than a single spot which indicating a mixture compound. Therefore, most biologically active sub-fractions, F4 (TC-D3), F5 (TC-D4), F6S (TC-D5) were selected for the further isolation of anticancer compounds. Flash chromatography and subsequent purification by column chromatography led to the final purified compounds, TCD5-F2-C (TC-A), TCD5-F3-B (TC-B), TC-D4-A2 (TC-C) and TC-D3-A2 (TC-D). Chemical characterization and structural decipher of the compounds were carried out by NMR and Mass spectroscopy.

The current study investigated the detailed mechanism of action of the isolated compounds TC-A, TC-B, TC-C and TC-D for anticancer activity in a panel of human epithelial cancer cells with special emphases towards breast cancer. The agents that are capable of inducing cytotoxicity against cancer cells without harming the normal cells are likely to be of substantial interest for the development of newer anticancer drugs. We have found that TC-A, TC-B, TC-C and TC-D possess cancer cell specific cytotoxicity and growth inhibitory activities against human breast cancer cells. The compounds imparted less cytotoxic effects against human mammary epithelial cells (MCF10A, immortalized cells). However, the anticancer drug, doxorubicin possessed dose dependent inhibition of cell proliferation in both breast cancer and MCF10A cells. The ability of the compounds to induce apoptosis in cancer cells was investigated by flow cytometry based apoptosis assay and cell cycle analysis. Annexin V assay by flow cytometry revealed that the isolated compounds significantly induced apoptosis (both early apoptosis and late apoptosis) against human breast and lung cancer cells. The induction of apoptosis was quite comparable with anticancer drugs mitoxantrone or cisplatin. Cell cycle analysis corroborated that treatment with TC-A, TC-B, TC-C and TC-D resulted in the accumulation of cells in Sub-Go phase and diminished S-phase suggesting that induction of apoptosis and inhibition of DNA replication. Moreover, treatment with TC-A resulted in the accumulation of cells in G2/M phase which indicate G2/M arrest and hence inhibition of mitosis. However, TC-A, TC-B, TC-C and TC-D did not
imparted any significant apoptosis or changes in the cell cycle profile in MCF10A cells at concentrations that effectively inhibited cancer cell proliferation.

We have also investigated the pharmacological effects of the compounds, TC-A, TC-B, TC-C and TC-D on SP phenotype. It was found that these compounds significantly inhibited the SP phenotype against human breast cancer (MCF7, MDA MB 231), lung cancer (A549), and colon cancer (CaCo2) cells. The SP inhibitory activities of the compounds were comparable with the known cancer stem cell inhibitors such as salinomycin and parthenolide. In contrast, doxorubicin significantly enriched SP phenotype, as has been shown before. The SP analysis also revealed that SP cells bear the G$_0$ phase or quiescent phase of the cell cycle. It was also found that the compounds, TC-A, TC-B, TC-C and TC-D significantly inhibited G$_0$ or quiescent phase of the cell cycle. The cytotoxicity studies in FACS sorted both SP and non-SP cells indicated that TC-A, TC-B, TC-C and TC-D impart SP-specific cell death. In contrast, doxorubicin had little effect on SP cells.

Cell surface marker (CD44 and CD24) based analysis using flow cytometry provides a valuable technique for the isolation of putative breast cancer stem cells (Krishan et al., 2011). It has been reported that among the various possible cell marker combinations only the CD44$^{\text{high}}$/CD24$^{\text{low}}$ fraction of breast cancer cells demonstrated the ability to induce tumor formation in the mice and possessed cancer stem cell characteristics (Al-Hajj et al., 2003). CD44/CD24 analysis in breast cancer cells (MCF7, BT549 and MDA MB 231) after treatment with TC-A, TC-B, TC-C and TC-D revealed that compounds significantly inhibited CD44$^{\text{high}}$/CD24$^{\text{low}}$ population. The CD44$^{\text{high}}$/CD24$^{\text{low}}$ inhibitory effects of the compounds were comparable with the standard cancer stem cell inhibitors such as salinomycin and parthenolide. The cytotoxicity studies in FACS sorted CD44$^{\text{high}}$ CD24$^{\text{low}}$ cells corroborated that compounds TC-A, TC-B, TC-C and TC-D impart specific cell death in CD44$^{\text{high}}$/CD24$^{\text{low}}$ cells. However, mitoxantrone enriched CD44$^{\text{high}}$/CD24$^{\text{low}}$ population and possessed minimal cytotoxic effect on FACS sorted CD44$^{\text{high}}$ CD24$^{\text{low}}$ cells.

The cancer cell culture as ‘cancer spheres’ in three dimension is considered to be one of the strategies to isolate and enrich for cancer stem cells. It has been reported that cancer spheres have self renewal capability and express stemness markers (Ponti et al., 2005). The current study also investigated the ability of the compounds, TC-A, TC-B, TC-C and TC-D to inhibit breast cancer spheres under methyl cellulose culture. It was found that the isolated
compounds TC-A, TC-B, TC-C and TC-D significantly inhibited cancer sphere formation. The sphere inhibitory activity of the compounds was compared with standard drugs such as salinomycin and parthenolide. The chemotherapeutic drugs also inhibited cancer spheres in culture; however, the effect of sphere inhibition was less as compared to the isolated compounds.

Multidrug resistance (MDR) plays a major role in the poor outcome of cancer chemotherapy. Among the various mechanisms identified for the MDR in cancer, the role of ABC transporters, especially, ABCB1 (MDR1), ABCG2 (BCRP) and ABCC1 (MRP1) have drawn considerable attention (Gillet and Gottesman, 2010). Inhibitors of drug transporters have been used mostly in clinics to reverse transporter-mediated cellular resistance and to enhance the effectiveness of the treatment of drug-resistant cancer. The present study investigated the MDR modulating activity of the isolated compounds in breast cancer cells by flow cytometry based functional assays. Rhodamine 123 efflux assay in cervical cancer cells indicated that TC-A, TC-B, TC-C and TC-D inhibited ABCB1 or P-glycoprotein activity. Similarly, mitoxantrone efflux and Calcien AM efflux assays revealed that these compounds were able to inhibit ABCG2 and ABCC1-mediated drug transport. ABCB1, ABCG2 and ABCC1 inhibitory effects of the compounds were comparable with standard inhibitors of drug transporters such as verapamil, fumetrimorgin C and MK571, respectively.

A recent study led to the generation of immortalized and transformed NBLE cells by overexpression of SV40ER and hTERT into stem cell enriched mammosphere-derived single cells from normal primary breast tissue (Paranjape et al., 2011). The CD44+/CD24- fraction of NBLE cells were isolated by FACS sorting and cultured in DMEM-F12 with growth factors to derive a cancer stem cell line, NBLE-CD44+/CD24-. The derived cells possessed stemness property, reduced differentiation markers, retained the ability to differentiate in vitro and imparted tumorigenic potential. Thus, the properties exhibited by the NBLE-CD44+/CD24- cells with respect to self-renewal, differentiation and tumorigenicity makes them a unique model system to study the pharmacological effects of drugs which targets cancer stem cells (Paranjape et al., 2011). The present study investigated cancer stem cell specific effects of isolated compounds, TC-A, TC-B, TC-C and TC-D in cancer stem cells enriched cells (NBLE-CD44+/CD24-). The results revealed that TC-A, TC-B, TC-C and TC-D imparted dose dependent anti-proliferative effects, induced cell death and significantly
inhibited CD44$^{\text{high}}$ CD24$^{\text{low}}$ population. However, the currently available chemotherapeutic drug, mitoxantrone, was found to be less effective against NBLE-CD44$^{+}$/CD24$^{-}$ cells.

To summarize, bio-activity guided isolation of medicinal plant, Tinospora cordifolia, for anticancer activity yielded four compounds TC-A, TC-B, TC-C and TC-D. Elucidation of mechanisms of action revealed that these compounds inhibited cancer cell proliferation, imparted cytotoxicity against human breast cancer cells without harming the normal cells, inhibited cell cycle progression and induced apoptosis specifically in the cancer cells. The compounds also found to have cancer stem cell specific anticancer activity mediated via inhibition of side population, CD44$^{\text{high}}$ CD24$^{\text{low}}$ population, breast cancer spheres and cancer stem cell enriched cell line (NBLE-CD44$^{+}$/CD24$^{-}$). Moreover, these compounds imparted inhibitory effects on MDR transporters, ABCB1, ABCG2 and ABCC1 suggesting it may work as MDR modulators. Among all the four compounds investigated for anticancer activity, TC-B was found to have most potent activity. These compounds will however have to be subjected to preclinical evaluations (in vivo mouse models), clinical trials, and toxicological studies for future therapeutic use as a potential chemotherapeutic drug for the effective treatment of breast cancer.

9.2 REFERENCES


