CHAPTER SEVEN
ELUCIDATION OF ANTICANCER MECHANISMS OF ISOLATED PHYTO-CONSTITUENTS
7.1 INTRODUCTION

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, a convincing cure remains elusive. India has a rich diversity of plant species that has been claimed to possess anticancer potential (Vaghasiya et al., 2011). Plant-derived compounds have played an important role in the development of several clinically useful anti-cancer agents. (Burnett et al., 2012). Bio-activity guided isolation of indigenous medicinal plant *Tinospora cordifolia* of the family Menispermacea has resulted in the isolation of four most active compounds, TCD5-F2-C (TC-A), TCD5-F3-B (TC-B), TC-D4-A2 (TC-C) and TC-D3-A2 (TC-D). 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 emphasis towards breast cancer.

Apoptosis defines a genetically encoded cell death program, which is morphologically and biochemically distinct from the necrosis (Wyllie, 2010). Induction of apoptosis in cancer cells is considered as the one of the strategy for the development of anticancer drugs (Portt et al., 2011). Flow cytometry is a most valuable methodology of choice to study the apoptotic cascade (Telford et al., 2011). First, the cytotoxicity and growth inhibitory effects of the isolated compounds were investigated in human breast cancer cells (MCF7, MDA MB 231). The induction of apoptosis by the isolated compounds were investigated in human breast cancer cells (MCF7, MDA MB 231, and BT549) and lung cancer cells (A549). The agents that are capable of inducing selective apoptosis of cancer cells without harming normal cells have received considerable interest in the development of novel cancer chemotherapeutic drugs. Hence, the current study also addressed the question of whether the isolated compounds impart any cytotoxicity and induce apoptosis against normal human breast epithelial cells (MCF10A).

Recent reports suggest that there exists a sub-population of cells in the tumor known as ‘cancer stem cells’ that alone can self-renew and thus have the ability to reconstitute new tumor upon transplantation (Croker and Allan, 2011). It has been shown that such cancer stem cells are highly resistant to chemotherapy (Greaves, 2011). It is hypothesized that frequent relapse of cancer is often due to the inability of current drugs to target these cancer stem cells (Nguyen et al., 2012). Flow cytometry has been used as a valuable research tool to isolate
cancer stem cells (Krishan et al., 2011). For example, side population (SP) assay based on the
differential efflux of Hoechst 33342 and cell surface marker based (CD44/CD24) assay has
been used to isolate the breast cancer stem cell population (Hadinagu et al., 2006, Federici et
al., 2011). Both the SP and CD44\textsuperscript{high}/CD24\textsuperscript{low} populations contain tumor initiating cells and
possess cancer stem cell characteristics (Clevers, 2011). The ability of the isolated compounds
to inhibit SP and CD44\textsuperscript{high}/CD24\textsuperscript{low} population was investigated in human breast cancer cells.
Moreover, specific cytotoxicity of isolated compounds in FACS sorted cancer stem cells (both
SP and CD44\textsuperscript{high}/CD24\textsuperscript{low} populations) was evaluated. The SP phenotype bears the G\textsubscript{0} phase
of the cancer cell cycle and therefore makes them resistant to chemotherapeutic drugs
(Golebiewska et al., 2011). The pharmacological effects of the isolated compounds against
quiescent phase (G\textsubscript{0} phase) of the cancer cell cycle were investigated by cell cycle analysis.
Cancer sphere culture has been considered as the one of the technique to purify and culture
breast cancer stem cells (Dontu and Wicha, 2005). The cultured ‘cancer spheres’ are enriched
for cancer stem cell characteristics (Magee et al., 2012). Therefore, the ability of isolated
compounds to inhibit putative breast cancer stem cells was investigated by cancer sphere
culture.

The major obstacle to successful cancer chemotherapy is the development of multidrug
resistance (MDR) to a variety of structurally unrelated cytotoxic drugs (Wind and Holen,
2011). One of the key mechanisms described for MDR in cancer is overexpression of ATP-
binding cassette super family of proteins especially ABCB1 (MDR1), ABCG2 (BCRP) and
ABCC1 (MRP1) (Donnenberg et al., 2011). Multi-parameter flow cytometry may be used for
the identification of tumor cell subpopulations based on their drug retention profiles with or
without the presence of an efflux blocker (Ioja et al., 2011). The drug efflux blockers typically
inhibit the efflux of a chemotherapeutic agent and thus increase cellular retention and
sensitivity. This rapid procedure can be used for the identification of tumor cells with drug-
resistant phenotype. A functional flow cytometry assay using rhodamine 123 as a fluorescent
marker has been used to study the inhibitory potential of ABCB1 or P-glycoprotein
modulators in the cancer cells (Forster et al., 2012). Similarly, mitoxantrone and calcein AM
have been used as fluorescent substrates to evaluate the inhibitory potential of ABCG2 and
ABCC1 respectively (Kim et al., 2012, Karunaratne and Audus, 2011). Therefore, the current
study also investigated the ability of the isolated compounds to inhibit MDR transporters, specifically ABCB1, ABCG2 and ABCC1 in human breast cancer cells.

7.2 MATERIALS AND METHODS

7.2.1 Drugs and chemicals

Doxorubicin, mitoxantrone, cisplatin, verapamil, fumetrimorgin C, MK571, rhodamine 123, calcien AM, Hoechst 33342, DMEM, DMEM-F12, Trypsin-EDTA, HBSS, MTT, propidium iodide, calcium chloride, sodium chloride, RNase A, HEPES buffer, methyl cellulose and proteinase K were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), B27, heparin, hEGF and hydrocortisone were purchased from Gibco, Invitrogen, USA. Annexin-V-FITC was purchased from Molecular probes, Invitrogen, USA. Antibody CD44 conjugated with FITC/PE and CD24 conjugated with PE/FITC were purchased from BD-Biosciences, USA. DMSO was purchased from Calbiochem.

7.2.2 Cell culture

MCF7 (Human breast carcinoma, ER+, tumorigenic and non-invasive), MDA MB 231 (Human breast carcinoma, ER-, tumorigenic and invasive), BT549 (Human breast carcinoma, ER-, tumorigenic and invasive), HeLa (Human cervical carcinoma, tumorigenic and invasive), A549 (Human lung adenocarcinoma, tumorigenic and invasive), CaCo2 (Human colon carcinoma, tumorigenic and invasive) were cultured in DMEM as described earlier (Section, 3.2.3, Section, 4.2.3). MCF10A (Human mammary epithelial cells, immortalized) were cultured in DMEM F12 with hEGF, hydrocortisone, insulin, B27, heparin, penicillin, streptomycin and fungizone.

7.2.3 Cytotoxicity assay

Briefly, MCF7, MDA MB 231 and MCF10A cells were treated with compound TC-A, TC-B, TC-C and TC-D. Each of the compounds was tested at four different concentrations, viz., 12.5 μg/ml, 25 μg/ml, 50 μg/ml and 100 μg/ml respectively for 48 hours. Doxorubicin was used as positive control. At the end of the treatment, MTT assay was performed as described earlier (Section, 3.2.4.2). The dose response curve was plotted with concentration of the compounds in the ordinate and percentage cell viability in the abscissa. IC_{50} (Concentration of the drug required to reduce the percentage cell viability to 50) were
obtained from the graph by nonlinear regression analysis as best curve-fit values. Statistically significant difference from the IC\textsubscript{50} values for the control cells exposed to vehicle control (DMSO) (p \textless 0.001) were determined by one way ANOVA using Dunnett's Multiple Comparison Test.

7.2.4 **Apoptosis assay**

Apoptosis assay was performed by Annexin V-FITC/PI staining by flow cytometry. Briefly, MCF7, MDA MB 231, A549 and MCF10A cells were seeded in a 6-well tissue culture plate (1 x 10\textsuperscript{5} cells/well) and cultured for 24 hours. Cells at the exponential growth phase were treated with compounds TC-A, TC-B, TC-C and TC-D at the IC\textsubscript{50} values (mentioned in the Table 7.1) for 48 hours. Mitoxantrone and cisplatin were used as positive controls (1.50 μM and 20 μM, respectively). At the end of the treatment, adherent cells were harvested by trypsinization and pooled with suspended dead cells. Thereafter, cells were centrifuged at 2000 rpm for 5 minutes at RT, washed with ice cold HBSS. The cells were then resuspended in 100μl of annexin binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl\textsubscript{2}, pH 7.4 in milli Q water). The cells were then stained with 5 μl of Annexin V-FITC and 10 μg of propidium iodide for 15 minutes in the dark at room temperature. After the incubation period, 400 μl of annexin binding buffer was added and kept in ice. The cells were immediately analyzed by flow cytometry (FACSCanto II, Becton Dickinson, USA). Annexin V-FITC and propidium iodide was excited by 488 nm laser and emission was detected at 530/30 nm and 585/42 nm respectively. The percentage number of early apoptotic cells, late apoptotic cells, dead cells (Necrotic cells) and live cells in untreated group and treated cells were analyzed by the software Summit v4.3 (Beckman Coulter, USA). Statistical analysis was performed by two way ANOVA using Bonferroni post test.

7.2.5 **Cell cycle analysis**

Briefly, MCF7, BT549, A549 and MCF10A cells were treated with compounds TC-A, TC-B, TC-C and TC-D at the IC\textsubscript{50} values (mentioned in the Table 7.1) for 48 hours. Mitoxantrone was used as positive control. At the end of treatment, cell cycle analysis was performed by propidium iodide based measurements of the DNA content of the cells by flow cytometry as described earlier (Section, 3.2.8). Data was analyzed by Summit v4.3 (Beckman Coulter, USA).
Coulter, USA) and statistical analysis was performed by two way ANOVA using Bonferroni post test.

7.2.6 Side population analysis

Briefly, MCF7, A549 and CaCo2 cells were also incubated with and without compounds TC-A, TC-B, TC-C, TC-D (50 μg/ml each) and fumetrimorgin C (10 μM) in the SP assay as described earlier. Similarly, MCF7, MDA MB 231 and A549 cells were treated with TC-A, TC-B, TC-C, TC-D (20 μg/ml each), salinomycin (2 μM), parthenolide (2 μM) and doxorubicin (0.40 μM) for 48 hours. At the end of the treatment period, SP analysis was carried out as described earlier (Section, 4.2.4). Statistical analysis was performed by one way ANOVA using Dunnett's Multiple Comparison Test.

7.2.7 Hoechst 33342 based cell cycle analysis

Briefly, MCF7 and A549 were stained with the Hoechst 33342 in DMEM containing 2% FBS and 10 mM HEPES in a circulating water bath at 37°C for 90 minutes. In control set, cells were incubated with compounds TC-A, TC-B, TC-C and TC-D (50 μg/ml each). DMSO was used as a vehicle control. At the end of incubation, cells were washed twice with ice-cold HBSS containing 2% FBS and re-suspended in 500 μl of ice cold HBSS. Cells were then immediately analyzed on a flow cytometer equipped with a UV laser (MoFlo, Beckman Coulter, USA). Hoechst 33342 was excited at 355 nm and emission was detected at 450/30 nm. DNA content in G1, G0, S and G2/M phases were determined based on Hoechst 33342 fluorescence. Data were analyzed by the software Summit v 4. 3 and statistical analysis was performed by two way ANOVA using Bonferroni post test.

7.2.8 FACS sorting of SP

For sorting experiments, MCF7 and A549 cells were cultured in 140 mm tissue culture dish as described above. SP assay was performed as described earlier (Section, 4.2.4). Both SP and non-SP were gated on Hoechst Blue (Y-axis) and Hoechst Red (X-axis) channel based on the inhibition with the FTC as live cells (PI negative cells). Both SP and NSP cells (5,000 cells each) were sorted into 96 well microtiter tissue culture plates under sterile conditions using MoFlo high speed cell sorter (Beckman Coulter, USA).
7.2.9 Cytotoxicity assay in SP

FACS sorted SP and NSP cells were cultured in DMEM for 12 hours. Defined concentrations of the compounds TC-A, TC-B, TC-C, TC-D, salinomycin, parthenolide, doxorubicin and mitoxantrone in culture media were freshly prepared by serial dilution to get final concentration of 0.01 μg/ml, 0.10 μg/ml, 1.0 μg/ml and 10 μg/ml respectively. Serial dilution was carried out in cell culture media in such a way that the final concentration of DMSO in the well did not exceed 0.5%. After 12 hours post incubation, both SP and NSP cells were treated with above mentioned concentrations of the compounds and drugs in triplicates for 48 hours. At the end of treatment, MTT assay was performed as described earlier (Section, 3.2.4.2).

7.2.10 CD44/CD24 analysis

Briefly, human breast cancer cells (MCF7, BT549 and MDA MB 231) were seeded in 60mm cell culture dish (5 x 10^5 cells). Cells in log phase were cultured with and without sub-lethal concentration of compounds TC-A, TC-B, TC-C, TC-D (20 μg/mL each), salinomycin (2 μM), parthenolide (2 μM) and mitoxantrone (0.20 μM) for 48 hours. DMSO was used as a vehicle control. At the end of the treatment period, cells were trypsinized and single cell suspension in HBSS (1 x 10^6 cells/ml) after a recovery period (60 minutes) was stained with 10μl of CD44 conjugated to FITC and 10 μl of CD24 conjugated to PE in ice for 30 minutes at the dark. At the end of incubation, cells were washed twice with ice-cold HBSS and resuspended in 500 μl of HBSS. Cells were analyzed on a flow cytometer (FACSCanto II, BD, USA). CD44-FITC and CD24-PE was excited with a 488 nm laser and emissions were detected at 530/30 nm BP and 585/42 nm. CD44-FITC and CD24-PE were plotted on y-axis and x-axis respectively. Fluorescence compensation was done to eliminate spectral overlap. The CD44<sup>high</sup> CD24<sup>low</sup> population was gated as blue color. Data were analyzed by Summit v 4.3 and statistical analysis was performed by one way ANOVA using Dunnett's Multiple Comparison Test.

7.2.11 FACS sorting of CD44<sup>high</sup> CD24<sup>low</sup> population

For sorting experiments, MDA MB 231 cells were cultured in 140 mm tissue culture dish and CD44/CD24 assay was performed as described above (Section, 4.2.4). The CD44<sup>high</sup> CD24<sup>low</sup> population was gated on the CD44-PE (y-axis) and CD24-FITC (x-axis) plot as blue
color. Similarly, the rest population (CD44\textsuperscript{low} CD24\textsuperscript{low}) was gated as green color. Both CD44\textsuperscript{high} CD24\textsuperscript{low} and CD44\textsuperscript{low} CD24\textsuperscript{low} populations (5,000 cells each) were sorted into 96 well microtiter tissue culture plates under sterile conditions using high speed cell sorter (MoFlo, Beckman Coulter, USA).

7.2.12 Cytotoxicity assay in CD44\textsuperscript{high} CD24\textsuperscript{low} cells

FACS sorted CD44\textsuperscript{high} CD24\textsuperscript{low} and CD44\textsuperscript{low} CD24\textsuperscript{low} cells were cultured in DMEM for 12 hours. Cells were then treated with TC-A, TC-B, TC-C and TC-D in triplicates for 48 hours. Each of the compounds was tested at three different concentrations, viz., 25 μg/ml, 50 μg/ml and 100 μg/ml respectively. Similarly, cells were also treated with salinomycin, parthenolide, and mitoxantrone at four different concentrations, viz., 0.01 μM, 0.10 μM, 1.0 μM and 10 μM respectively. Equal volume of DMSO was used as vehicle control. At the end of the treatment MTT assay was carried out as described earlier (Section, 3.2.4.2). Percentage cell viability in untreated control normalized to 100%. The dose response curve was plotted with concentration of the drug in the ordinate and percentage cell viability in the abscissa. IC\textsubscript{50} values of the compounds were obtained from the graph as the concentration which decreases percentage cell viability to 50.

7.2.13 Cancer sphere culture

MCF7 and MDA MB 231 cells were seeded (2 x 10\textsuperscript{5}) with methyl cellulose on bottom agar coated tissue culture plates. Media was replaced with DMEM containing TC-A, TC-B, TC-C, TC-D (50 μg/ml each), salinomycin (2 μM), parthenolide (2 μM), doxorubicin (0.8 μM) and mitoxantrone (0.4 μM) and further cultured in the presence of drugs for 7 days. DMSO was used as vehicle control. Cancer spheres were assessed under an inverted microscope regularly. The number of cancer spheres in untreated, vehicle control, TC-A, TC-B, TC-C, TC-D, salinomycin, parthenolide, doxorubicin and mitoxantrone group was enumerated. A graph was plotted with number of spheres in the y-axis and the treatment groups in x-axis. Level of inhibition and p-values were determined by one way ANOVA by using Dunnett's Multiple Comparison Test.
7.2.14 Rhodamine 123 efflux assay

ABCB1 (MDR1) inhibition studies were carried out by rhodamine 123 efflux assay using flow cytometry. Briefly, HeLa and A549 cells (1 x 10^6) were incubated with and without rhodamine 123 (0.50 μM) at 37°C, CO2 incubator for 30 minutes in DMEM with 2% FBS. After the centrifugation (1400 rpm for 4 minutes at 4°C) and removal of supernatant, cells were re-incubated in the presence or absence of TC-A, TC-B, TC-C and TC-D (concentration of 20 μg/ml each) without rhodamine 123 for an additional 30 minutes at 37°C. DMSO was used as vehicle control. Verapamil (50 μM) was used as a standard inhibitor of ABCB1 mediated drug transport. At the end of incubation, cells were washed twice with ice-cold HBSS and analyzed on a flow cytometer (FACSCanto II, Becton Dickinson, USA). Rhodamine 123 was excited at 488 nm and emission was detected at 530/30 nm band-pass filters. Analysis was gated to include single cells on the basis of FSC and SSC. Log fluorescence was displayed as single parameter overlay histograms. Mean Fluorescence Intensity (MFI) of rhodamine 123 was determined for untreated control and cells incubated with compounds TC-A, TC-B, TC-C, TC-D and verapamil. Relative fluorescence was used for the quantification or comparison between TC-A, TC-B, TC-C, TC-D and verapamil. Statistical analysis was performed by one way ANOVA using Dunnett Test.

7.2.15 Mitoxantrone efflux assay

ABCG2 (BCRP) inhibition studies were carried out by mitoxantrone efflux assay using flow cytometry. Briefly, MCF7 and A549 cells (1 x 10^6) were incubated with and without mitoxantrone (10 μM) at 37°C, CO2 incubator for 30 minutes. After the centrifugation and removal of supernatant, cells were re-incubated in the presence or absence of TC-A, TC-B, TC-C and TC-D (concentration of 20 μg/ml each) without mitoxantrone for an additional 30 minutes at 37°C. DMSO was used as vehicle control. FTC (10 μM) was used as a standard inhibitor of ABCG2 mediated drug transport. At the end of incubation, cells were washed twice with ice-cold HBSS and analyzed on a flow cytometer (FACSCanto II, Becton Dickinson, USA). Mitoxantrone was excited at 633 nm and emission was detected at 660/20 nm. Analysis was gated to include single cells on the basis of FSC and SSC. Log fluorescence was displayed as single parameter overlay histograms. Mean Fluorescence Intensity (MFI) of MXR was determined for untreated control and cells incubated with TC-A, TC-B, TC-C, TC-
D and FTC. Relative fluorescence was used for the quantification or comparison between TC-A, TC-B, TC-C, TC-D and FTC. Statistical analysis was performed by one way ANOVA using Dunnett Test.

7.2.16 Calcien AM efflux assay

ABCC1 (MRP1) inhibition studies were carried out by calcien AM efflux assay using flow cytometry. Briefly, MDA MB 231 and A549 cells in DMEM with 2% FBS were incubated with and without calcien AM (0.10 μM) at 37°C, CO₂ incubator for 30 minutes. After the centrifugation and removal of supernatant, cells were re-incubated in the presence or absence of TC-A, TC-B, TC-C and TC-D (concentration of 20 μg/ml each) without calcien AM for an additional 30 minutes at 37°C. DMSO was used as a vehicle control. MK571 (10 μM) was used as a standard inhibitor of ABCC1 mediated drug transport. At the end of incubation, cells were washed twice with ice-cold HBSS and analyzed on a flow cytometer (FACSCanto II, Becton Dickinson, USA). Calcien AM was excited at 488 nm and emission was detected at 530/30 nm. Mean Fluorescence Intensity (MFI) of calcien AM was determined for untreated control and cells incubated with TC-A, TC-B, TC-C, TC-D and MK571. Relative fluorescence was used for quantification or comparison between TC-A, TC-B, TC-C, TC-D and MK571. Statistical analysis was performed by one way ANOVA using Dunnett Test.

7.3 RESULTS

7.3.1 TC-A, TC-B, TC-C and TC-D inhibits breast cancer cell proliferation

The cytotoxicity and growth inhibitory effects of the TC-A, TC-B, TC-C and TC-D were investigated in MCF7, MDA MB 231 and MCF10A cells. Results indicated that compounds isolated from *Tinospora cordifolia* viz. TC-A, TC-B, TC-C, TC-D impart dose dependent inhibition of breast cell proliferation and TC-B was found to be the most active (Fig. 7.1 A, B). IC₅₀ values of TC-A, TC-B, TC-C and TC-D in MCF7 cells were found to be 15.71 ± 0.97 μg/ml, 11.78 ± 1.29 μg/ml, 20.21 ± 1.11 μg/ml and 25.17 ± 1.68 μg/ml respectively. Similarly, IC₅₀ values of TC-A, TC-B, TC-C and TC-D in MDA MB 231 cells were 31.79 ± 0.91 μg/ml, 18.69 ± 0.71 μg/ml, 24.57 ± 0.95 μg/ml and 32.34 ± 0.61 μg/ml respectively (Table 7.1). The positive control, doxorubicin imparted dose dependent inhibition of breast
cancer cell proliferation and the IC\textsubscript{50} values of doxorubicin in MCF7 and MDA MB 231 were 1.25 ± 0.05 μM and 0.70 ± 0.03 μM, respectively.

Our next question was whether TC-A, TC-B, TC-C and TC-D mediated suppression of cell viability and proliferation was selectively to cancer cells, and not to the normal cells. This would be a highly desirable trait for a potential therapeutic anti-cancer agent. We addressed this question by determining the cytotoxic and growth inhibitory effects of TC-A, TC-B, TC-C and TC-D against human mammary epithelial cells (MCF10A, immortalized). IC\textsubscript{50} values of TC-A, TC-B, TC-C and TC-D in MCF710A cells was found to be 97.86 ± 4.82 μg/ml, 101.90 ± 4.11μg/ml, 113.50 ± 4.32 μg/ml and 79.61 ± 3.87 μg/ml respectively (Fig. 7. 1 C). TC-A, TC-B, TC-C and TC-D showed increased IC\textsubscript{50} values in MCF10A cells as compared to breast cancer cells. These data suggested compounds TC-A, TC-B, TC-C and TC-D possess relatively less cytotoxic effects in human mammary epithelial cells. However, doxorubicin showed a dose dependent cytotoxicity in MCF10A cells with an IC\textsubscript{50} value of 6.26 ± 0.25 μM. Table 7. 1 shows a comparison profile of IC\textsubscript{50} values for TC-A, TC-B, TC-C, TC-D and doxorubicin in human breast cancer cells (MCF7 and MDA MB 231) and human mammary epithelial cells (MCF10A). The data represent ± SD of three independent experiments (n=3).

7.3.2 TC-A, TC-B, TC-C and TC-D induce apoptosis in breast cancer cells

In order to evaluate the ability of TC-A, TC-B, TC-C and TC-D to induce apoptosis, MCF7, MDA MB 231 and A549 cells were treated with TC-A, TC-B, TC-C and TC-D, at their IC\textsubscript{50} concentration (Table 7.1) for 48 hours. Cells were also treated with anticancer drugs (mitoxantrone and cisplatin) which are known to induce apoptosis in cancer cells. DMSO was used as a vehicle control. At the end of the treatment period, cells were harvested and stained with Annexin V-FITC/PI as described in materials and methods. Percentage number of live, apoptosis and dead (necrosis) were determined in untreated controls, solvent control, TC-A, TC-B, TC-C, TC-D, methotrexate and cisplatin treated groups. Results indicated that, in MDA MB 231 cells as compared to vehicle control, percentage number of apoptotic cells (both early apoptosis and late apoptosis) were significantly increased (**p<0.001) upon treatment with compounds TC-A, TC-B, TC-C and TC-D (Fig. 7.2). Similar finding was observed with MCF7 and A549 cells, respectively (Fig. 7.3 and Fig. 7.4). Anticancer drugs methotrexate and cisplatin showed a significant induction of apoptosis in cancer cells (**p<0.001). The ability of the compounds, TC-A, TC-B, TC-C and TC-D to induce
apoptosis in cancer cells were comparable with mitoxantrone and cisplatin when treated at their respective IC50 values. Similarly, apoptotic effects of TC-A, TC-B, TC-C and TC-D was evaluated in human mammary epithelial cells (MCF10A). In order to evaluate the apoptosis, MCF10A cells were treated with the compounds TC-A, TC-B, TC-C, TC-D, mitoxantrone and cisplatin (with the IC50 values of the respective compounds and drugs in MCF7) for 48 hours. Results indicated that compounds TC-A, TC-B, TC-C and TC-D do not induce apoptosis in MCF10A cells (Fig. 7.5). In contrast, mitoxantrone and cisplatin induces apoptosis (both early and late apoptosis, p<0.001). Table 7.2 and Table 7.3 shows percentage live, early apoptosis, late apoptosis and necrosis in MDA MB 231, A549, MCF7 and MCF10A cells upon treatment with TC-A, TC-B, TC-C, TC-D, mitoxantrone and cisplatin. Numerical data are means ± SD of three independent experiments (n=3). Statistical analysis was performed by two way ANOVA using Bonferroni post test (***p<0.001, **p<0.01, *p<0.05).

7.3.3 Cell cycle specific inhibitory effects of TC-A, TC-B, TC-C and TC-D

In order to evaluate the cell cycle specific pharmacological effects of compounds, MCF7, BT549 and A549 were cultured in the presence of TC-A, TC-B, TC-C, TC-D and mitoxantrone (at their IC50 concentration given Table 7.1) for 48 hours. At the end of the treatment period, cell cycle analysis was performed based on propidium iodide (PI) staining using flow cytometry. DNA content in G1/G0 phase, Synthetic (S) phase, G2/M phase and Sub-G0 phase was determined. An increased Sub-G0 phase indicates DNA fragmentation and apoptosis. Similarly, an elevated G1/G0 phase and G2/M phase indicate cell growth arrest and blockade of mitosis or cell division, respectively. A decrease in S-phase indicates inhibition of DNA replication. In MCF7 cells, compared with vehicle control, upon treatment with TC-A, TC-B, TC-C and TC-D result in a significant increase of Sub-G0 phase (**p<0.001) (Fig. 7.6). Similar results were observed in BT549 and A549 cells, respectively (Fig. 7.7 and Fig. 7.8). TC-A caused a significant increase of G2/M phase and Sub-G0 phase in both (**p<0.001) BT549 and A549 cells. Mitoxantrone showed significantly increased Sub G0 phase in MCF7, BT549 and A549 cells (**p<0.001). Table 7.4 depicts the cell cycle specific effects of TC-A, TC-B, TC-C, TC-D and mitoxantrone in BT549 and A549 cells. These data suggest that the compounds TC-A, TC-B, TC-C and TC-D induced apoptosis and inhibit DNA replication in breast cancer cells. Similarly, in order to evaluate cell cycle specific
pharmacological effects of TC-A, TC-B, TC-C and TC-D in normal cells, MCF10A cells were treated with the compounds (with IC\textsubscript{50} values of the respective compounds in MCF7) for 48 hours. Mitoxantrone was used as positive control. At the end of the treatment period, DNA content was determined based on propidium iodide staining using flow cytometry. Results revealed that TC-A, TC-B, TC-C and TC-D did not caused any significant change in Sub-G\textsubscript{0} phase, G\textsubscript{1}/G\textsubscript{0} phase, Synthetic (S) phase and G\textsubscript{2}/M phase as compared to vehicle control (Fig. 7.9). However, upon treatment with mitoxantrone, there was a significant increase in Sub-G\textsubscript{0} phase, indicating induction of apoptosis (***p<0.001). Table 7.5 depicts the cell cycle specific effects of TC-A, TC-B, TC-C, TC-D and mitoxantrone in MCF7 and MCF710A cells. Numerical data are means ± SD of three independent experiments (n=3). Statistical analysis was performed by two way ANOVA using Bonferroni post test (***p<0.001).

### 7.3.4 TC-A, TC-B, TC-C and TC-D inhibits SP phenotype

In order to understand the effects of compounds TC-A, TC-B, TC-C and TC-D on SP, MCF7, A549 and CaCo2 cells were incubated with TC-A, TC-B, TC-C and TC-D in a SP assay. Equal volume of DMSO was used as a vehicle control. Results indicated that SP in untreated MCF7 cells was 2.78 ± 0.27%. SP in TC-A, TC-B, TC-C and TC-D group was found to be 0.01 ± 0%, 0.00%, 0.02 ± 0.01% and 0.04 ± 0.01%, respectively (Fig. 7.10). Similarly, SP in untreated A549 cells was 8.57 ± 0.39%. When cells are incubated with TC-A, TC-B, TC-C and TC-D, SP was found to be 0.01 ± 0.01%, 0.00%, 0.05 ± 0.01% and 0.12 ± 0.03% respectively (Fig. 7.11). Similarly, SP in untreated CaCo2 cells was 1.95 ± 0.12%. SP in TC-A, TC-B, TC-C and TC-D group was found to be 0.01 ± 0%, 0.01 ± 0%, 0.06 ± 0.01% and 0.09 ± 0.01% respectively (Fig. 7.12). Vehicle control (DMSO) did not have any significant effect in SP. These results suggested that TC-A, TC-B, TC-C and TC-D significantly inhibit SP phenotype.

Next, we have investigated whether TC-A, TC-B, TC-C and TC-D possess any ability to inhibit SP in cell culture. In this direction, MCF7, MDA MB 231 and A549 cells were treated with sub-lethal concentrations of TC-A, TC-B, TC-C and TC-D for a period of 48 hours. Standard inhibitors of cancer stem cells (parthenolide and salinomycin) and anticancer drug (doxorubicin) were used as positive control. DMSO was used as solvent control. At the end of the treatment period, cells were harvested and SP assay was performed. Results indicated that SP in untreated MCF7 cells was 3.68 ± 0.26%. Upon treatment with TC-A, TC-B, TC-C and
TC-D, SP was found to be 0.01 ± 0%, 0.00%, 0.04 ± 0.01% and 0.48 ± 0.27% respectively. Similarly, upon treatment parthenolide, salinomycin and doxorubicin SP was found to be 1.96 ± 0.11%, 0.30 ± 0.20% and 13.54 ± 1.03% respectively (Fig. 7.13). SP in untreated MDA MB 231 cells was 0.12 ± 0.03%. Upon treatment with TC-A, TC-B, TC-C and TC-D, SP was found to be 0.01 ± 0%, 0.00%, 0.01 ± 0.0% and 0.01 ± 0% respectively. Similarly, upon treatment parthenolide, salinomycin and doxorubicin SP was found to be 0.06 ± 0.01%, 0.00%, and 0.31 ± 0.03% respectively (Fig. 7.14). SP in untreated A549 cells was 8.59 ± 0.34%. Upon treatment with compounds TC-A, TC-B, TC-C and TC-D, SP was found to be 0.01 ± 0%, 0.00%, 0.05 ± 0.01% and 0.07 ± 0.01% respectively. Similarly, upon treatment parthenolide, salinomycin and doxorubicin SP was found to be 2.84 ± 0.73%, 0.00% and 15.29 ± 0.97% respectively (Fig. 7.15). SP was completely eliminated in the presence of FTC, a standard inhibitor of ABC transporter (0.00%) which indicates valid SP. These results corroborate that TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited SP phenotype in human breast cancer cells (MCF7, MDA MB 231) and human lung cancer cells (A549). However, currently used anticancer drug, doxorubicin, significantly enrich SP. SP inhibitory effects of TC-A, TC-B, TC-C and TC-D were quite comparable with parthenolide and salinomycin. Fig. 7.16 show SP inhibitory effects of TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin and doxorubicin in MCF7, MDA MB 231 and A549 cells. All values are mean ± SD of three independent experiments, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test.

7.3.5 TC-A, TC-B, TC-C and TC-D inhibits quiescent (G₀) phase of cancer cell cycle

Hoechst 33342 binds with A-T regions of DNA hence the distinct regions of the SP profile also identify different phases of the cell cycle (G₀/G₁, S, G₂/M). SP bears in G₀ phase or quiescent phase of the cell cycle. In order to understand the effects of compounds TC-A, TC-B, TC-C and TC-D in the G₀ phase of the cell cycle (quiescent phase), MCF7 and A549 cells were stained with Hoechst 33342 in the presence or absence of TC-A, TC-B, TC-C and TC-D for 90 minutes at 37°C. At the end of the incubation period, DNA content in G₁ phase, G₀ phase, Synthetic (S) phase and G₂/M phase was determined based on Hoechst 33342 using flow cytometry. The G₀ phase in untreated MCF7 cells was found to be 4.92 ± 0.12. In the presence of TC-A, TC-B, TC-C and TC-D, G₀ phase was found to be 0.16 ± 0.01, 0.15 ± 0.02, 0.18 ± 0.03 and 0.22 ± 0.01 respectively (Fig. 7.17). Similarly, the G₀ phase in untreated A549
cells was found to be 26.53 ± 3.10. In the presence of TC-A, TC-B, TC-C and TC-D, G0 phase was found to be 0.58 ± 0.04, 0.34 ± 0.05, 0.67 ± 0.03 and 0.69 ± 0.05 respectively (Fig. 7.18). These data revealed that TC-A, TC-B, TC-C and TC-D significantly inhibits G0 phase of cell cycle in both A549 and MCF7 cells. DMSO did not have any significant effect in the G0 phase. All values are mean ± SD of three independent experiments, ***p<0.001, *p<0.05 determined by two way ANOVA by using Bonferroni post test.

7.3.6 TC-A, TC-B, TC-C and TC-D induce specific cell death in SP

Next, we have investigated whether TC-A, TC-B, TC-C and TC-D induces any specific cell death against side population cells as compared to non-side population or bulk of the cells. In this direction, cytotoxicity assay was performed in both FACS sorted SP and NSP cells. In sorting experiments, SP in A549 cells was found to be 19.79% and SP was completely eliminated (0.00%) in the presence of FTC indicating valid SP phenotype (Fig. 7.19 A). The purity of flow sorted SP cells was found to be 83.40% (Fig. 7.19 B). Gated population of SP and NSP cells were sorted into 96 well tissue culture microtiter plates by FACS sorter under sterile conditions. SP and NSP cells were treated with TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin and mitoxantrone for 48 hours and the percentage cell proliferation was determined by MTT assay.

Results indicated that TC-A, TC-B, TC-C and TC-D impart a dose dependent inhibition of cell proliferation against both SP and NSP cells. However, it was found that SP cells sensitize effectively to the compounds TC-A, TC-B, TC-C and TC-D than NSP (Fig. 7.20 A). The IC50 values of compounds TC-A, TC-B, TC-C, and TC-D against SP was found to be 11.47 ± 1.26, 8.60 ± 0.43, 12.49 ± 1.24 and 17.89 ± 1.19 respectively. Similarly, the IC50 values of compounds TC-A, TC-B, TC-C, and TC-D against NSP was found to be 26.44 ± 1.58, 24.58 ± 1.47, 23.43 ± 1.87 and 38.71 ± 3.09 respectively (Table 7.6). These results suggested that compounds TC-A, TC-B, TC-C, and TC-D possess a significant increase in cytotoxicity and inhibition of cell proliferation in SP as compared to NSP. Similarly, salinomycin and parthenolide impart significantly increased cytotoxicity and inhibition of cell proliferation in SP as compared to NSP. In contrast, mitoxantrone showed increased cytotoxicity on NSP as compared to SP (Fig. 7.20 B). The IC50 value of salinomycin and parthenolide against SP was found to be 4.85 ± 0.15 and 7.01 ± 0.41 respectively. Similarly, the IC50 values of parthenolide and salinomycin against NSP was found to be 11.61 ± 0.80 and 20.50 ± 1.43
respectively (Table 7.6). The IC$_{50}$ values of mitoxantrone against SP and NSP were found to be 9.06 ± 0.52 and 3.10 ± 0.12 respectively (Table 7.6).

The cytotoxic effects of compounds TC-A, TC-B, TC-C and TC-D against SP was investigated in MCF7 cells. In sorting experiments, SP in MCF7 cells was found to be 2.62% and SP was completely eliminated (0.00%) in the presence of FTC indicating valid SP phenotype (Fig. 7.21 A). The purity of flow sorted SP cells was found to be 96.25% (Fig. 7.21 B). Gated population of both SP and NSP cells were sorted by FACS and cultured with TC-A, TC-B, TC-C, TC-D, parthenolide, doxorubicin and mitoxantrone for 48 hours. Percentage cell viability was determined by MTT assay. Results indicated that TC-A, TC-B, TC-C, TC-D and parthenolide significantly increased cytotoxicity against SP as compared to NSP. In contrast, doxorubicin and mitoxantrone showed significantly increased cytotoxicity against NSP cells (Fig. 7.22). These results corroborated that compounds TC-A, TC-B, TC-C, and TC-D possess specific cell death in cancer stem cells (SP) and the cytotoxic effects was comparable with standard inhibitors such as salinomycin and parthenolide. However, cancer stem cells (SP) were resistant to the chemotherapeutic drugs, doxorubicin and mitoxantrone. Numerical data are means ± SD of three independent experiments (n=3). Statistically significant difference from IC$_{50}$ values for the control cells exposed to vehicle control (blank), ***p<0.001 determined by two way ANOVA using Bonferroni post test.

### 7.3.7 TC-A, TC-B, TC-C and TC-D inhibits CD44$^{\text{high}}$ CD24$^{\text{low}}$ population

Next, we have investigated the pharmacological effects of TC-A, TC-B, TC-C and TC-D in putative CD44$^{\text{high}}$ CD24$^{\text{low}}$ population of breast cancer cells. In this direction, human breast cancer cells (MCF7, BT549, MDA MB 231) were treated with TC-A, TC-B, TC-C, TC-D, salinomycin, parthenolide and mitoxantrone for 48 hours. At the end of the treatment, CD44/CD24 assay was performed to detect CD44$^{\text{high}}$ CD24$^{\text{low}}$ population. Results indicated that in MCF7 cells, CD44$^{\text{high}}$ CD24$^{\text{low}}$ population in untreated cells was 7.64 ± 0.78%. However, upon treatment with TC-A, TC-B, TC-C, TC-D, salinomycin, parthenolide and mitoxantrone, it was found to be 1.09 ± 0.13%, 0.40 ± 0.16%, 1.44 ± 0.31%, 3.49 ± 0.36%, 1.05 ± 0.18%, 1.16 ± 0.25% and 18.77 ± 2.02% respectively (Fig. 7.23). Similarly, CD44$^{\text{high}}$ CD24$^{\text{low}}$ population in untreated BT549 cells was 11.77 ± 1.63%. Upon treatment with TC-A, TC-B, TC-C TC-D, salinomycin, parthenolide and mitoxantrone, CD44$^{\text{high}}$ CD24$^{\text{low}}$ population was found to be 2.07 ± 2.39%, 0.87 ± 0.10%, 2.69 ± 0.45%, 3.90 ± 0.39%, 0.52 ±
0.10%, 0.14 ± 0.04% and 35.63 ± 3.55% respectively (Fig. 7.24). Similarly, CD44<sup>high</sup> CD24<sup>low</sup> population in untreated MDA MB 231 cells was 43.97 ± 2.44%. Upon treatment with TC-A, TC-B, TC-C, TC-D, salinomycin and parthenolide, CD44<sup>high</sup> CD24<sup>low</sup> population was found to be 8.60 ± 0.54%, 1.53 ± 0.33%, 8.92 ± 0.37%, 13.26 ± 0.78%, 0.09 ± 0.06% and 3.35 ± 1.05% respectively (Fig. 7.25). These results indicated that TC-A, TC-B, TC-C and TC-D significantly inhibited CD44<sup>high</sup> CD24<sup>low</sup> population in human breast cancer cells. The ability of these compounds to inhibit CD44<sup>high</sup> CD24<sup>low</sup> population was quite comparable with standard inhibitors of cancer stem cells such as salinomycin and parthenolide. However, chemotherapeutic drug, mitoxantrone enriched CD44<sup>high</sup> CD24<sup>low</sup> population. Interestingly, TC-B found to have highly active against CD44<sup>high</sup> CD24<sup>low</sup> population. A comparative data of CD44<sup>high</sup> CD24<sup>low</sup> population upon treatment with TC-A, TC-B, TC-C TC-D, salinomycin, parthenolide and mitoxantrone is depicted in Fig. 7.26. All values are mean ± SD of three independent experiments, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test.

7.3.8 **TC-A, TC-B, TC-C and TC-D induce specific cell death CD44<sup>high</sup> CD24<sup>low</sup> cells**

Next we have investigated that whether compounds TC-A, TC-B, TC-C and TC-D imparts any specific cell death against CD44<sup>high</sup> CD24<sup>low</sup> population as compared with bulk population. Therefore, cytotoxicity assay was performed in both FACS sorted CD44<sup>high</sup> CD24<sup>low</sup> population and bulk population (CD44<sup>-</sup> CD24<sup>-</sup>). In sorting experiments (MDA MB 231), CD44<sup>high</sup> CD24<sup>low</sup> and CD44-CD24- population were 21.46% and 15.33% respectively (Fig. 7.27 A). Gated R5 (blue color-21.46%) and R6 (green color-15.33%) populations indicates CD44<sup>high</sup> CD24<sup>low</sup> and CD44-CD24- respectively. The purity of flow sorted CD44<sup>high</sup> CD24<sup>low</sup> cells were found to be 76.64% (Fig. 7.27 B). Gated population of both CD44<sup>high</sup> CD24<sup>low</sup> and CD44-CD24- cells were sorted by the FACS sorter and treated with TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin and mitoxantrone for 48 hours. Percentage cell viability was determined by MTT assay. Results revealed that TC-A, TC-B, TC-C, TC-D possess a dose dependent inhibition of cell proliferation in both CD44<sup>high</sup> CD24<sup>low</sup> and CD44-CD24- cells. However, it was found that CD44<sup>high</sup> CD24<sup>low</sup> cells sensitize efficiently to the compounds TC-A, TC-B, TC-C and TC-D. Therefore, these compounds showed an enhanced cytotoxicity against CD44<sup>high</sup> CD24<sup>low</sup> cells as compared to CD44-CD24- cells (Fig. 7. 28 A and B). The IC<sub>50</sub> value of TC-A, TC-B, TC-C, and TC-D in CD44<sup>high</sup> CD24<sup>low</sup>
cells was 30.04 ± 1.47, 25.17 ± 1.26, 46.73 ± 3.17 and 64.94 ± 4.47, respectively. Similarly, the IC$_{50}$ value of TC-A, TC-B, TC-C, and TC-D in CD44- CD24- cells was 68.68 ± 3.13, 67.92 ± 2.98, 116.50 ± 6.38 and 139.50 ± 4.67, respectively (Table 7.7). Salinomycin and parthenolide showed significantly increased cytotoxicity and inhibition of cell proliferation against CD44 high CD24 low/- cells as compared to CD44- CD24- cells. In contrast, mitoxantrone showed less cytotoxic effects against CD44 high CD24 low/- cells (Fig. 7.28 C and Fig. 7.28 D). The IC$_{50}$ values of salinomycin, parthenolide and mitoxantrone against CD44 high CD24 low/- cells were 5.65 ± 0.41, 8.70 ± 0.58 and 10.05 ± 0.05, respectively. Similarly, the IC$_{50}$ values of salinomycin, parthenolide and mitoxantrone against CD44- CD24- cells were 9.68 ± 0.45, 16.61 ± 0.76 and 1.71 ± 0.05, respectively (Table 7.7). These results suggested that compounds TC-A, TC-B, TC-C, and TC-D impart a significantly increased cytotoxicity against CD44 high CD24 low/- cells as compared to the rest of the population. The level of inhibition of these compounds was comparable with standards such as salinomycin and parthenolide. It was also found that CD44 high CD24 low/- cells are resistant to the chemotherapeutic drug, mitoxantrone. Among the four compounds investigated against CD44 high CD24 low/- population, TC-B has highest activity. Numerical data are mean ± SD of three independent experiments (n=3). Statistically significant difference from IC$_{50}$ values for the control cells exposed to vehicle control (blank) ***p<0.001 determined by two way ANOVA using Bonferroni post test.

7.3.9 **TC-A, TC-B, TC-C and TC-D inhibits ‘breast cancer spheres’**

Cancer stem cells have the ability to grow under suspension culture or methyl cellulose as distinct spheres called ‘cancer spheres’. We have investigated the ability of TC-A, TC-B, TC-C and TC-D to inhibit cancer spheres in culture. In this direction, human breast cancer cells (MCF7 and MDA MB 231) were cultured in suspension under methyl cellulose with or without isolated compounds (TC-A, TC-B, TC-C, TC-D), standard cancer stem cell inhibitors (salinomycin and parthenolide) and chemotherapeutic drugs (doxorubicin and mitoxantrone). Results indicated there were a good number of intact spheres with distinct boundaries in untreated cells. Interestingly, when the cells are cultured in the presence of TC-A, TC-B, TC-C, TC-D, salinomycin and parthenolide, the number of spheres are significantly reduced (***p<0.001). The inhibition of the spheres meditated by compounds was quite comparable with salinomycin and parthenolide. Results also indicated that there was a significant
inhibition of sphere in the presence of doxorubicin and mitoxantrone (**p<0.01). However, the level of sphere inhibition was higher in the TC-A, TC-B, TC-C TC-D, salinomycin and parthenolide treated groups (Fig. 7.29). Vehicle control (DMSO) did not have any effect in sphere inhibition. Similar kind of results was obtained with MDA MB 231 cells grown under methyl cellulose culture (Fig. 7.30).

7.3.10 TC-A, TC-B, TC-C and TC-D inhibits ABCB1 (MDR1) drug transporter

Functional assay based on flow cytometry using rhodamine 123 as a fluorescent substrate has been used to study the ABCB1 or P-glycoprotein modulators in the cancer cells. When rhodamine 123 is diffused into the cell, ABCB1 actively pumps out the fluorochrome (Active efflux phase). If verapamil which is an inhibitor of ABCB1 presented in the same manner along with fluorescence marker, the fluorescence marker accumulates in the cell, resulting in a higher intensity of fluorescence. Therefore, compounds that impede the efflux of the rhodamine 123 can be thus quantitatively analyzed and compared with respect to their potency to inhibit the ABCB1 drug transporter.

In order to evaluate the effect of TC-A, TC-B, TC-C and TC-D on ABCB1 drug transporter activity, rhodamine 123 efflux assay was performed in HeLa cells. Verapamil, an inhibitor of ABCB1 (P-gp) was used as standard. Results indicated that the mean fluorescent intensity (R-MFI) of rhodamine 123 on efflux phase was 24.69 ± 2.88. Verapamil caused a significant increase in the rhodamine 123 retention in HeLa cells (306.10 ± 14.55) which indicate inhibition of ABCB1. R-MFI in the presence of TC-A, TC-B, TC-C and TC-D were 166.20 ± 9.58, 250.90 ± 11.70, 151.30 ± 11.10 and 140.00 ± 16.85 respectively (Fig. 7.31). A significant increase in the intracellular concentration of rhodamine 123 in the presence of TC-A, TC-B, TC-C and TC-D indicated the inhibition of ABCB1 pump. The efflux inhibition potency of the compounds was compared with that of standard, verapamil as relative mean fluorescence. Compounds TC-A, TC-B, TC-C and TC-D showed a significant inhibition which was 54.31%, 82.02%, 49.67% and 45.75% respectively of that observed with verapamil. These results corroborate that TC-A, TC-B, TC-C and TC-D inhibited ABCB1 drug transporter in human cancer cells. Among all the four compounds investigated, TC-B found to have highest activity. All values are mean ± SD of three independent experiments, **p<0.001, determined by one way ANOVA by using Dunnett Test.
7.3.11 TC-A, TC-B, TC-C and TC-D inhibits ABCG2 (BCRP) drug transporter

Functional assay based on flow cytometry using mitoxantrone as a fluorescent marker has been used to study the ABCG2 activity in the cancer cells. When mitoxantrone (MXR), fluorescence substrate for ABCG2 is diffused into the cell, ABCG2 actively pumps out the fluorochrome (Active efflux phase). If an ABCG2 inhibitor (fumetrimorgin C) presented in the same manner along with fluorescence marker, MXR accumulates in the cell, resulting in a higher intensity of fluorescence. Hence, compounds that block the efflux of MXR can be thus quantitatively analyzed and compared with respect to their potency to inhibit the ABCG2 efflux pump. In order to evaluate the ability of TC-A, TC-B, TC-C and TC-D to inhibit ABCG2 drug transporter, MXR efflux assay was carried out in MCF7 and A549 cells. Results indicated that in MCF7 cells mean fluorescent intensity of MXR (M-MFI) in efflux phase was 611.60 ± 38.24. FTC has caused a significant increase in MXR accumulation in MCF7 cells (1873 ± 136.60) which indicate inhibition of ABCG2. M-MFI in the presence of TC-A, TC-B, TC-C and TC-D were 909.20 ± 18.81, 1336.00 ± 127.40, 936.40 ± 32.93 and 858.50 ± 64.47 respectively (Fig. 7.32). A significant increase in the intracellular retention of MXR in the presence of TC-A, TC-B, TC-C and TC-D indicated the inhibition of ABCG2 mediated drug transport. The efflux inhibition potency of the compounds was compared with that of standard, FTC as relative mean fluorescence. Compounds TC-A, TC-B, TC-C and TC-D showed a significant inhibition which was 48.53%, 71.32%, 49.97% and 45.80% respectively of that observed with FTC. Similarly, in A549 cells mean fluorescent intensity of MXR (M-MFI) in efflux phase was 60.75 ± 4.10. The FTC has caused a significant increase in MXR accumulation in A549 cells (119.80 ± 7.26) which indicated the inhibition of ABCG2. M-MFI in the presence of TC-A, TC-B, TC-C and TC-D were 130.30 ± 4.58, 165.60 ± 12.22, 134.40 ± 10.87 and 126.70 ± 9.39 respectively (Fig. 7.33). The efflux inhibition potency of the compounds was compared with that of standard, FTC as relative mean fluorescence. Compounds TC-A, TC-B, TC-C and TC-D showed a significant inhibition which was 108.76%, 138.65%, 112.18% and 105.75%, respectively of that observed with FTC. Among all the four compounds investigated, TC-B found to have highest activity. All values are mean± SD of three independent experiments, ***p<0.001, determined by one way ANOVA by using Dunnett Test.
7.3.12 TC-A, TC-B, TC-C and TC-D inhibits ABCC1 (MRP1) drug transporter

Functional assay using calcein AM as a fluorescent marker has been used to study the ABCC1 inhibitory activity of the compounds in the cancer cells. When calcein AM, fluorescence substrate for ABCC1 drug transporter diffused into the cell, ABCC1 actively pumps out the fluorochrome (Active efflux phase). If an ABCC1 inhibitor (MK571) presented in the same manner along with fluorescence marker, the calcein AM accumulates in the cell, resulting in a higher intensity of fluorescence. Compounds that block the efflux of calcein AM can be thus quantitatively analyzed and compared with respect to their potency to inhibit the ABC1 drug efflux pump. The ability of the compounds TC-A, TC-B, TC-C and TC-D to inhibit ABCC1 mediated drug transport was studied using calcein AM efflux assay in MDA MB 231 and A549 cells. Results indicated that in MDA MB 231 cells, the mean fluorescent intensity of calcein AM (C-MFI) in efflux phase was 1176.60 ± 120.50. MK571 caused a significant increase in the calcein AM accumulation in MDA MB 231 cells (2151 ± 72.49) which indicate the inhibition of ABCC1. C-MFI in the presence of TC-A, TC-B, TC-C and TC-D were 1998 ± 124.80, 2253 ± 70.85, 2105 ± 159.40 and 1772 ± 112.60 respectively (Fig. 7.34). A significant increase in the intracellular retention of calcein AM in the presence of TC-A, TC-B, TC-C and TC-D indicated the inhibition of ABCC1 mediated drug transport. The efflux inhibition potency of the compounds was compared with that of standard, MK571 as relative mean fluorescence. Compounds TC-A, TC-B, TC-C and TC-D showed a significant inhibition which was 92.88%, 97.86%, 97.86% and 82.38% respectively of that observed with MK571. Similarly, in A549 cells, C-MFI in efflux phase was 1154 ± 199.70. MK571 caused a significant increase in the calcein AM accumulation in A549 cells (1590 ± 54.06) which indicate inhibition of ABCC1. MFI in the presence of TC-A, TC-B, TC-C and TC-D were 2234 ± 177.40, 2479 ± 115.90, 1940 ± 95.68 and 1589 ± 79.08 respectively (Fig. 7.35). The efflux inhibition potency of the compounds was compared with that of standard, MK571 as relative mean fluorescence. Compounds TC-A, TC-B, TC-C and TC-D showed a significant inhibition which was 140.50%, 155.91%, 122.01% and 99.93% respectively, of that observed with MK571. Thus, among all the four compounds investigated TC-B found to have highest activity. All values are mean ± SD of three independent experiments, ***p<0.001, determined by one way ANOVA by using Dunnett Test.
7.4 DISCUSSION

The present study demonstrated that compounds TC-A, TC-B, TC-C and TC-D possess cancer cell specific cytotoxicity and growth inhibitory activities against human breast cancer cells. Among the all the four compounds studied, compounds TC-B revealed the most potent activity against both MCF7 and MDA MB 231 cells. The chronological order of cytotoxicity and growth inhibitory activities of compounds were TC-B>TC-A>TC-C>TC-D. Human mammary epithelial cells (MCF10A) appear to be less susceptible to cytotoxicity by these compounds. At concentrations of 30 μg/ml, compounds TC-A, TC-B, TC-C and TC-D did not significantly affect the viability of the cells. Doxorubicin found to have dose dependent inhibition of cell proliferation in both cancer and normal breast cells. Breast cancer cells, MCF7 (estrogen dependent) and MDA MB 231 (estrogen independent) exhibited similar sensitivity to compounds, TC-A, TC-B, TC-C and TC-D, therefore, inhibition of cell proliferation was unlikely an antiestrogenic effect. The flow cytometric analysis indicated that treatment with compounds TC-A, TC-B, TC-C and TC-D resulted in the accumulation of cells in Sub-Go phase indicating apoptosis occurred. The effects of compounds on cell cycle distribution were examined using the concentrations that effectively inhibited the cell proliferation. The percentage of cells in S-phase was significantly reduced by all the four compounds in a concentration-dependent manner. TC-A induced the accumulation of cells in G2/M phase, suggesting G2/M arrest, and therefore cells were not able to process from G2/M into G1 phase. Mitoxantrone, conferred significant accumulation of Sub G0 phase hence by inducing apoptosis in cancer cells. The induction of apoptosis with TC-A, TC-B, TC-C and TC-D was comparable with that of mitoxantrone. However, compounds TC-A, TC-B, TC-C and TC-D did not show any significant changes in the cell cycle profile and apoptosis in human normal mammary epithelial cells at concentrations that effectively inhibited the cancer cell proliferation.

Induction of apoptosis was measured based on the Annexin V-FITC/Propidium iodide (PI) assay using flow cytometry. In normal viable cells, phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane. Typically, PS is translocated from the inner to the outer leaflet of the plasma membrane, exposing the PS to the external environment. Annexing-V conjugates binds to PS on apoptotic cell surfaces in the presence of calcium. Annexin V is conjugated with flurochrome, FITC which allows the quantification by flow
Propidium iodide is a cell-impermeant dead cell stain and in combination with Annexin V-FITC distinguishes dead or necrotic cells from apoptotic cells. Percentage number of Annexin V-FITC negative/PI negative (live cells), Annexin V-FITC positive/PI negative (early apoptosis), Annexin V-FITC positive/PI positive (late apoptosis) and Annexin V-FITC negative/PI positive (dead or necrosis) were determined by flow cytometry. Results revealed that TC-A, TC-B, TC-C and TC-D significantly induced apoptosis (both early apoptosis and late apoptosis) in human breast and lung cancer cells. The effect of apoptosis was comparable with mitoxantrone and cisplatin. However, TC-A, TC-B, TC-C and TC-D did not impart any significant apoptosis against human normal mammary epithelial cells at concentrations that effectively inhibited the cancer cell proliferation. These results suggest that, TC-A, TC-B, TC-C and TC-D inhibited cancer cell proliferation, possess cytotoxicity against human breast cancer cells, inhibit cell cycle progression and induced apoptosis specifically in cancer cells. Interestingly, the compounds possessed less cytotoxicity as they did not induce apoptosis against normal mammary epithelial cells at concentrations that effectively inhibited the cancer cell proliferation. This is a highly desirable trait for cancer chemotherapeutic agents.

An increasing body of evidence suggests that a rare, biologically unique population of cancer stem cells exists in most cancers and may be responsible for tumor initiation, progression, metastasis and relapse (Clevers, 2011). Chemotherapy and radiation therapy is capable of eliminating most of the differentiated bulk of the tumor cells; however, cancer stem cells are highly resistant to chemo and radiation therapy. It is hypothesized that often relapse in cancer has been due to the existence of such rare population cancer stem cells. The novel anticancer drugs should be designed to eliminate the bulk of cancer cells and rare cancer stem cells that are alone responsible for initiation and maintenance of tumor for an effective therapy in cancer (Clevers, 2011). Recent studies suggest that SP cells have the properties of cancer stem cells, such as self-renewal ability, initiate tumor in immunocompromised mice and are highly resistant to chemotherapeutic drugs (Willan and Farnie, 2011). ABC transporters responsible for the SP phenotype are implicated in drug resistance which leads to poor outcome in cancer chemotherapy (Gillet and Gottesman, 2010). Thus, targeting the SP should also offer an alternative option to approach drug resistance. Our previous studies revealed that human lung cancer (A549), breast cancer cells (MCF7, MDA MB 231) and colon cancer cells (CaCo2) contain a distinct population of side population
cells. We have investigated the pharmacological effects of the compounds isolated from the plant *Tinospora cordifolia*, TC-A, TC-B, TC-C and TC-D in the side population phenotype of A549, MCF7, MDA MB 231 and CaCo2 cells. Interestingly, it was found that all the four compounds significantly inhibit SP in all the cell types. The SP inhibition was prominent when cells are cultured in the presence of these compounds over a period of time. Among all the four compounds investigated, TC-B found to have most potent SP inhibitory activity. The known cancer stem cell inhibitors like salinomycin and parthenolide also significantly inhibit the SP in all the cell type investigated. However, chemotherapeutic drug, doxorubicin significantly enrich the SP phenotype. SP assay also identifies the cell cycle profile of the cells based on the Hoechst 33342 fluorescence. SP typically bears the G0 phase or quiescent phase of the cell cycle. The effects of the compounds, TC-A, TC-B, TC-C and TC-D on G0 phase of cycle revealed that these compounds significantly inhibits G0 phase. Since the most of the chemotherapy drugs evade from G0 phase, the inhibitory effects of the compounds on G0 phase draw considerable attention in cancer chemotherapy. Cytotoxicity studies in FACS sorted SP cells indicated that TC-A, TC-B, TC-C and TC-D impart SP or cancer stem cell-specific cell death in both breast (MCF7) and lung (A549) cancer cells. The IC50 of the compounds in SP cells was significantly lesser as compared to NSP cells which indicated a selective cell death in SP. The results were comparable with salinomycin and parthenolide. In contrast, doxorubicin exerts cell death predominantly on non-side population or bulk cells (evident from a significantly decreased IC50 on NSP) indicating SP cells are resistant to chemotherapeutic drug. The inhibition of SP phenotype against human cancer cells by the compounds isolated from the plant, TC-A, TC-B, TC-C and TC-D may be due to the selective cell death against cancer stem cells, blockade of ABC transporter activity, inhibition of the cell signaling pathways that regulate cancer stem cells (Hedgehog, Wnt/β-catenin, Notch, PTEN, SHH (sonic hedgehog) and BMI-1.

It has been reported that among the all possible cell marker combinations (CD24+CD44+, CD24-CD44-, CD24+CD44- and CD24-CD44+), only the CD44+CD24-/low fraction of cells demonstrated the ability to induce tumor formation in the mice and possessed cancer stem cell characteristics (Al-Hajj et al., 2003). This distinct population of CD44^{high}/CD24^{low} by flow cytometry represents breast cancer stem cells. Therefore, cell surface marker (CD44 and CD24) based analysis using flow cytometry provide a valuable technique for the isolation of
putative breast cancer stem cells. In the current study, CD44/CD24 analysis was carried out in breast cancer cell lines (MCF7, BT549 and MDA MB 231) after treatment with the compounds, TC-A, TC-B, TC-C and TC-D. The results corroborated that TC-A, TC-B, TC-C and TC-D significantly inhibits CD44^{high}/CD24^{low} population in all the cell types investigated. Parthenolide and salinomycin are reported to have specific cancer stem cell inhibitory activity in AML and solid tumors. In the current study, we found that parthenolide and salinomycin significantly inhibited CD44^{high}/CD24^{low} in the breast cancer cells. The cytotoxicity studies in FACS sorted CD44^{high}/CD24^{low} cells and the rest of the population suggested that compounds TC-A, TC-B, TC-C and TC-D impart specific cell death in CD44^{high}/CD24^{low} cells as compared to the rest of the population. CD44^{high}/CD24^{low} inhibitory potential of TC-A, TC-B, TC-C and TC-D was comparable with parthenolide or salinomycin. The current study also revealed that chemotherapeutic drug, mitoxantrone enriches the CD44^{high}/CD24^{low} population and less susceptible to FACS sorted CD44^{high}/CD24^{low} cells. The CD44^{high}/CD24^{low} specific inhibitory effects of TC-A, TC-B, TC-C and TC-D correlate with the SP-inhibitory activity.

The cancer cell culture as “cancer spheres” is considered as one of the strategies to isolate and enrich cancer stem cells (Ponti et al., 2005). The cancer spheres can be grown in methyl cellulose under suspension culture. The ability of the cells to form the distinct spheres correlates with stem cell characteristics (Ponti et al., 2005). It has been reported that cancer harbor self-renewal capacity, extensive proliferation and initiate tumor formation in nude mice. We have also investigated the ability of the compounds, TC-A, TC-B, TC-C and TC-D to inhibit breast cancer spheres. The cells were cultured in methyl cellulose in the presence of the isolated compounds, chemotherapeutic drugs (doxorubicin and mitoxantrone) and standard inhibitors of cancer stem cells (salinomycin and parthenolide). Interestingly, it was found that, isolated compounds significantly inhibit the number of spheres in culture as compared to untreated control. The sphere inhibitory activity of the compounds was comparable with standard drugs salinomycin and parthenolide. Chemotherapeutic drugs significantly inhibited the cancer spheres in culture; however, the effect of sphere inhibition was less as compared to the TC-A, TC-B, TC-C, TC-D, salinomycin and parthenolide. This may be due to the enrichment of the drug resistant population in the culture in the presence of chemotherapeutic drugs. The ability of the isolated compounds to inhibit the cancer spheres
may be due to the blockade of self renewal pathway such as Bmi-1, Notch, Wnt and Sonic hedgehog pathways.

Inhibitors of drug transporters, like MDR1, BCRP and MRP1 are the most useful tools to reverse transporter mediated cellular resistance to anticancer drugs and to enhance the effectiveness of the treatment of drug-resistant cancer (Gillet and Gottesman, 2010). In the present study, we have also investigated the MDR modulating activity of the isolated compounds in human epithelial 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 inhibit ABCB1 or p-glycoprotein activity. Similarly, the inhibitory effects on other drug transporters, ABCG2 and MRP1 were investigated by mitoxantrone and calcien AM based assay. TC-A, TC-B, TC-C and TC-D were able to inhibit ABCG2 in both breast and lung cancer cells. Moreover, it was also found that these compounds inhibit ABCC1 mediated drug transport in the breast and lung cancer cells. The inhibitory effects on ABCB1, ABCG2 and ABCC1 were comparable with standard inhibitors of drug transporters, like verapamil, fumetrimorgin C and MK571, respectively. Therefore the present study suggests that the compounds TC-A, TC-B, TC-C and TC-D could be considered as promising lead molecules as MDR chemosensitizers. The combination of these compounds along with chemotherapeutic drugs may result in the effective treatment of cancer as an effective and safe chemosensitizer by overcoming MDR in cancer.
Figure 7.1 Cytotoxicity TC-A, TC-B, TC-C and TC-D A in human breast cancer and mammary epithelial cells

Cytotoxicity of compounds isolated from *Tinospora cordifolia* viz. TC-A, TC-B, TC-C and TC-D A. Human breast cancer cells (MCF7). B. Human breast cancer cells (MDA MB 231). C. Human mammary epithelial cells (MCF10A). Cells were treated with specified concentrations of TC-A, TC-B, TC-C and TC-D for 48 hours and the percentage cell viability was determined by MTT assay. Percentage cell survival in untreated control cells was calculated as 100%. Each point represents the mean ± SD of three independent experiments performed in triplicate (n=3).
### Table 7.1 IC\textsubscript{50} values of TC-A, TC-B, TC-C and TC-D in breast cancer and mammary epithelial cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>IC\textsubscript{50} (μg/mL)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF7</td>
<td>MDA MB 231</td>
<td>MCF-10A</td>
</tr>
<tr>
<td>TC-A</td>
<td>15.71 ±0.97</td>
<td>31.79 ±0.91</td>
<td>97.86 ±4.82</td>
</tr>
<tr>
<td>TC-B</td>
<td>11.78 ±1.29</td>
<td>18.69 ±0.71</td>
<td>101.90 ±4.11</td>
</tr>
<tr>
<td>TC-C</td>
<td>20.21 ±1.11</td>
<td>24.57 ±0.95</td>
<td>113.50 ±4.32</td>
</tr>
<tr>
<td>TC-D</td>
<td>25.17 ±1.68</td>
<td>32.34 ±0.61</td>
<td>79.61 ±3.87</td>
</tr>
<tr>
<td>Doxorubicin*</td>
<td>1.25 ±0.05</td>
<td>0.70 ±0.03</td>
<td>6.26 ±0.25</td>
</tr>
</tbody>
</table>

IC\textsubscript{50} values of TC-A, TC-B, TC-C and TC-D in human breast cancer cells (MCF7 and MDA MB 231) and human mammary epithelial cells (MCF-10A). IC\textsubscript{50} (Concentration of the drug required to reduce the percentage cell viability to 50) was determined from the graph by non-linear regression analysis as best curve- fit values. Anticancer drug, doxorubicin was used as positive control. *The IC\textsubscript{50} of doxorubicin is represented as μM. Numerical data are mean ± SD of three independent experiments (n=3). Statistically significant difference from IC\textsubscript{50} values for the control cells exposed to vehicle control (DMSO) (p < 0.001).
Figure 7.2 TC-A, TC-B, TC-C and TC-D induces apoptosis in breast cancer cells (MDA MB 231)

MDA MB 231 cells were treated with specified concentrations of TC-A, TC-B, TC-C, TC-D, mitoxantrone and cisplatin for 48 hours. At the end of the treatment, live cells, early apoptosis, late apoptosis and necrosis were determined based on Annexin-V-FITC assay using flow cytometry. These figures are representative of three independent experiments.
Figure 7.3 TC-A, TC-B, TC-C and TC-D induces apoptosis in breast cancer cells (MCF7)

MCF7 cells were treated with specified concentrations of TC-A, TC-B, TC-C, TC-D mitoxantrone and cisplatin for 48 hours. At the end of the treatment, live cells, early apoptosis, late apoptosis and necrosis were determined based on Annexin-V-FITC assay using flow cytometry. These figures are representative of three independent experiments.
Figure 7.4 TC-A, TC-B, TC-C and TC-D induces apoptosis in lung cancer cells (A549)

A549 cells were treated with specified concentrations of TC-A, TC-B, TC-C, TC-D Mitoxantrone and cisplatin for 48 hours. At the end of the treatment, live cells, early apoptosis, late apoptosis and necrosis were determined based on Annexin-V-FITC assay using flow cytometry. These figures are representative of three independent experiments.
Figure 7.5 Apoptotic effects of TC-A, TC-B, TC-C and TC-D in mammary epithelial cells

MCF10A cells were treated with compounds and drugs with specified concentrations that were cytotoxic to breast cancer cells (MCF7). At the end of the treatment, live cells, early apoptosis, late apoptosis and necrosis were determined based on Annexin-V-FITC assay using flow cytometry. Compounds TC-A, TC-B, TC-C and TC-D do not induce apoptosis in MCF10A cells. In contrast, mitoxantrone and cisplatin induce apoptosis. These figures are representative of three independent experiments.
Table 7.2 Apoptotic effects of TC-A, TC-B, TC-C and TC-D in cancer cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA MB 231</th>
<th>A549</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early apoptosis</td>
<td>Late apoptosis</td>
</tr>
<tr>
<td>Untreated control</td>
<td>1.13±0.40</td>
<td>0.40±0.09</td>
</tr>
<tr>
<td>Vehicle control (DMSO)</td>
<td>1.33±0.21</td>
<td>0.21±0.10</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>12.69±1.43</td>
<td>24.84±1.94</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>9.64±0.75</td>
<td>8.40±0.46***</td>
</tr>
<tr>
<td>TC-A</td>
<td>8.09±0.86</td>
<td>7.78±0.88***</td>
</tr>
<tr>
<td>TC-B</td>
<td>8.55±0.87</td>
<td>8.15±1.34***</td>
</tr>
<tr>
<td>TC-C</td>
<td>8.26±0.46</td>
<td>6.49±0.61***</td>
</tr>
<tr>
<td>TC-D</td>
<td>6.32±1.18</td>
<td>9.21±0.72***</td>
</tr>
</tbody>
</table>

Apoptotic effects of TC-A, TC-B, TC-C, TC-D, mitoxantrone and cisplatin in human breast cancer cells (MDA MB 231) and lung cancer cells (A549). Percentage number of live cells, early apoptotic cells, late apoptotic cells and necrotic cells were determined based on Annexin-V-FITC assay using flow cytometry. Numerical data are mean ± SD of three independent experiments (n=3). Statistically significant difference from the values for the control cells exposed to vehicle control (blank). ***p<0.001, **p<0.01, *p<0.05 determined by two way ANOVA using Bonferroni post test.
### Table 7.3 Apoptotic effects of TC-A, TC-B, TC-C, TC-D in MCF7 and MCF10A cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>MCF7</th>
<th>MCF10A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early apoptosis</td>
<td>Late apoptosis</td>
</tr>
<tr>
<td>Untreated control</td>
<td>0.85±0.30</td>
<td>0.45±0.17</td>
</tr>
<tr>
<td>Vehicle control (DMSO)</td>
<td>0.45±0.15</td>
<td>1.36±0.62</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2.02±0.16*</td>
<td>3.65±0.20***</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>2.36±0.31*</td>
<td>3.46±0.14***</td>
</tr>
<tr>
<td>TC-A</td>
<td>4.13±0.66***</td>
<td>5.97±0.49***</td>
</tr>
<tr>
<td>TC-B</td>
<td>2.57±0.14*</td>
<td>4.95±0.26***</td>
</tr>
<tr>
<td>TC-C</td>
<td>2.38±0.20*</td>
<td>3.13±0.35***</td>
</tr>
<tr>
<td>TC-D</td>
<td>2.27±0.98*</td>
<td>3.28±0.21***</td>
</tr>
</tbody>
</table>

Apoptotic effects of TC-A, TC-B, TC-C, TC-D, mitoxantrone and cisplatin in human breast cancer cells (MCF7) and human mammary epithelial cells (MCF710A). Percentage number of live cells, early apoptotic cells, late apoptotic cells and necrotic cells were determined based on Annexin-V-FITC assay using flow cytometry. Numerical data are mean ± SD of three independent experiments (n=3). Statistically significant difference from the values for the control cells exposed to vehicle control (blank). ***p<0.001, **p<0.01, *p<0.05 determined by two way ANOVA using Bonferroni post test.
MCF7 cells were treated with TC-A, TC-B, TC-C, TC-D and mitoxantrone for 48 hours. At the end of the treatment, DNA content was analyzed based on propidium iodide staining using flow cytometry. The percentage number of DNA in G₀/G₁, S, G₂/M and sub G₀ (Apoptosis) phase was determined. These figures are representative of three independent experiments (n=3).
Figure 7.7 Cell cycle specific inhibitory effects of TC-A, TC-B, TC-C and TC-D in BT549 cells

BT549 cells were treated with TC-A, TC-B, TC-C, TC-D and mitoxantrone for 48 hours. At the end of the treatment, DNA content was analyzed based on propidium iodide staining using flow cytometry. The percentage number of DNA in G0/G1, S, G2M and sub G0 (Apoptosis) phase was determined. These figures are representative of three independent experiments (n=3).
Figure 7.8 Cell cycle specific inhibitory effects of TC-A, TC-B, TC-C and TC-D in A549 cells

A549 cells were treated with TC-A, TC-B, TC-C, TC-D and mitoxantrone for 48 hours. At the end of the treatment, DNA content was analyzed based on propidium iodide staining using flow cytometry. The percentage number of DNA in G₀/G₁, S, G₂M and sub G₀ (Apoptosis) phase was determined. These figures are representative of three independent experiments (n=3).
Figure 7.9 Cell cycle specific inhibitory effects of TC-A, TC-B, TC-C and TC-D in MCF10A cells

MCF10A cells were treated with TC-A, TC-B, TC-C, TC-D and mitoxantrone for 48 hours. At the end of the treatment, DNA content was analyzed based on propidium iodide staining using flow cytometry. The percentage number of DNA in G0/G1, S, G2M and sub G0 (Apoptosis) phase was determined. These figures are representative of three independent experiments (n=3).
Table 7.4 Cell cycle inhibitory effects of TC-A, TC-B, TC-C and C-D in cancer cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>BT549</th>
<th>A549</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sub G₀ phase</td>
<td>G₀/G₁ phase</td>
</tr>
<tr>
<td>Untreated control</td>
<td>1.14±0.40</td>
<td>63.16±21.87</td>
</tr>
<tr>
<td>Vehicle control (DMSO)</td>
<td>1.17±0.55</td>
<td>64.12±2.86</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>38.14±2.31***</td>
<td>41.53±0.60</td>
</tr>
<tr>
<td>TC-A</td>
<td>21.76±1.50***</td>
<td>15.23±2.71</td>
</tr>
<tr>
<td>TC-B</td>
<td>21.02±0.91***</td>
<td>41.93±1.62</td>
</tr>
<tr>
<td>TC-C</td>
<td>15.97±1.51***</td>
<td>41.97±1.40</td>
</tr>
<tr>
<td>TC-D</td>
<td>15.30±1.05***</td>
<td>42.37±1.91</td>
</tr>
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</table>

Cell cycle analysis in human breast (BT549) and lung (A549) cancer cells after treatment with TC-A, TC-B, TC-C, TC-D and mitoxantrone. DNA content was determined in G₀/G₁, S, G₂/M and sub G₀ (Apoptosis) phase. An increased Sub G₀ phase indicates that compounds induced apoptosis in cancer cells. An increased G₂/M phase indicates cell cycle arrest and prevents mitosis. Numerical data are mean ± SD of three independent experiments (n=3). Statistically significant difference from the values for the control cells exposed to vehicle control (blank). ***p<0.001, determined by two way ANOVA using Bonferroni post test.
Table 7.5 Cell cycle inhibitory effects of TC-A, TC-B, TC-C and C-D in breast cancer and normal mammary epithelial cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>MCF7</th>
<th>MCF10A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sub ( G_0 ) phase</td>
<td>( G_{o}/G_1 ) phase</td>
</tr>
<tr>
<td>Untreated control</td>
<td>0.07±0.02</td>
<td>62.53±2.40</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>0.11±0.05</td>
<td>63.75±1.80</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>20.67±2.49***</td>
<td>42.79±3.31</td>
</tr>
<tr>
<td>TC-A</td>
<td>34.09±2.30***</td>
<td>39.25±1.29</td>
</tr>
<tr>
<td>TC-B</td>
<td>41.09±2.02***</td>
<td>33.53±0.91</td>
</tr>
<tr>
<td>TC-C</td>
<td>22.15±1.78***</td>
<td>50.81±1.57</td>
</tr>
<tr>
<td>TC-D</td>
<td>19.82±1.31***</td>
<td>53.13±2.52</td>
</tr>
</tbody>
</table>

Cell cycle analysis in human breast cancer cells (MCF7) and human mammary epithelial cells (MCF10A) after treatment with TC-A, TC-B, TC-C, TC-D and mitoxantrone. DNA content was determined in \( G_0/G_1 \), S, \( G_2/M \) and sub \( G_0 \) (Apoptosis) phase. An increased Sub \( G_0 \) phase indicated that compounds induce apoptosis in cancer cells. An increased \( G_2/M \) phase indicates cell cycle arrest and prevents mitosis. Numerical data are mean ± SD of three independent experiments (\( n=3 \)). Statistically significant difference from the values for the control cells exposed to vehicle control (blank). ***\( p<0.001 \), **\( p<0.001 \), determined by two way ANOVA using Bonferroni post test.
Figure 7.10 Anti-SP activity of TC-A, TC-B, TC-C and TC-D in MCF7 cells

MCF7 cells were incubated with and without TC-A, TC-B, TC-C and TC-D in a SP assay. At the end of the incubation, cells were analyzed using flow cytometry to detect SP. TC-A, TC-B, TC-C and TC-D significantly inhibit SP. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, ns=not significant; determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.11 Anti-SP activity of TC-A, TC-B, TC-C and TC-D in A549 cells

A549 cells were incubated with and without TC-A, TC-B, TC-C and TC-D in a SP assay. At the end of the incubation, cells were analyzed using flow cytometry to detect SP. TC-A, TC-B, TC-C and TC-D significantly inhibits SP. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, ns=not significant; determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.12 Anti-SP activity of TC-A, TC-B, TC-C and TC-D in CaCo2 cells

Caco2 Cells were incubated with and without TC-A, TC-B, TC-C and TC-D in a SP assay. At the end of the incubation, cells were analyzed using flow cytometry to detect SP. TC-A, TC-B, TC-C and TC-D significantly inhibit SP. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, ns=not significant; determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.13 SP inhibitory effects of TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin and doxorubicin in MCF7 cells

Cells were treated with TC-A, TC-B, TC-C, TC-D, doxorubicin, parthenolide and salinomycin for 48 hours. At the end of the treatment, SP assay was performed. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited SP phenotype, whereas doxorubicin significantly enrich SP. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.14 SP inhibitory effects of TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin and doxorubicin in MDA MB 231 cells

Cells were treated with TC-A, TC-B, TC-C, TC-D, doxorubicin, parthenolide and salinomycin for 48 hours. At the end of the treatment, SP assay was performed. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited SP phenotype, whereas doxorubicin significantly enrich SP. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.15 SP inhibitory effects of TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin and doxorubicin in A549 cells

Cells were treated with TC-A, TC-B, TC-C, TC-D, doxorubicin, parthenolide and salinomycin for 48 hours. At the end of the treatment, SP assay was performed. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited SP phenotype, whereas doxorubicin significantly enrich SP. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.16 Comparative profile of anti-SP activity of TC-A, TC-B, TC-C and TC-D

SP inhibitory effects of TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin and doxorubicin. A. Human breast cancer cells (MCF7) B. Human breast cancer cells (MDA MB 231) C. Human lung cancer cells (A549). TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited SP phenotype, whereas doxorubicin significantly enrich SP. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.17 Effects of TC-A, TC-B, TC-C and TC-D in quiescent phase of cell cycle (MCF7)

DNA content was analyzed based on Hoechst 33342 staining using flow cytometry. TC-A, TC-B, TC-C and TC-D significantly inhibited G0 phase. All values are mean ± SD of three independent experiments (n=3, *p<0.05, determined by two way ANOVA by using Bonferroni post test).
Figure 7.18 Effects of TC-A, TC-B, TC-C and TC-D in quiescent phase of cell cycle (A549)

TC-A, TC-B, TC-C, TC-D inhibit G_0 (Quiescent phase) phase of cell cycle in human lung cancer cells (A549). DNA content was analyzed based on Hoechst 33342 staining using flow cytometry. TC-A, TC-B, TC-C and TC-D significantly inhibited G_0 phase. All values are mean ± SD of three independent experiments (n=3, *p<0.05, determined by two way ANOVA by using Bonferroni post test).
Figure 7.19 Isolation of SP by FACS sorting in A549

A. Isolation of side population (SP) and non-side population (NSP) from human lung cancer cells (A549) by FACS sorting. Gated R2 (blue color) and R3 (green color) populations indicating SP and NSP respectively. SP and NSP cells were sorted by the FACS sorter (MoFlo) into the 96 well plates. B. Purity of flow sorted SP was found to be 83.40%.
Figure 7.20 Cytotoxicity of TC-A, TC-B, TC-C and TC-D in SP of A549

A. Cytotoxicity of TC-A, TC-B, TC-C and TC-D in SP and NSP. B. Cytotoxicity of parthenolide, salinomycin and mitoxantrone in SP and NSP. SP and NSP from A549 cells were sorted by the FACS sorter and cultured with and without TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin and mitoxantrone for 48 hours. Cell viability was determined by MTT assay. The cell viability in untreated cells was normalized as 100%. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin shows increased cytotoxicity against SP cells as compared to NSP. In contrast, mitoxantrone show increased cytotoxicity against NSP. Each point represents the mean ± SD of three independent experiments performed in triplicate.
<table>
<thead>
<tr>
<th>Groups</th>
<th>$IC_{50}$ μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP</td>
</tr>
<tr>
<td>TC-A</td>
<td>11.47 ± 1.26</td>
</tr>
<tr>
<td>TC-B</td>
<td>8.603 ± 0.43</td>
</tr>
<tr>
<td>TC-C</td>
<td>12.49 ± 1.24</td>
</tr>
<tr>
<td>TC-D</td>
<td>17.89 ± 1.19</td>
</tr>
<tr>
<td>Salinomycin</td>
<td>4.85 ± 0.15</td>
</tr>
<tr>
<td>Parthenolide</td>
<td>7.01 ± 0.41</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>9.06 ± 0.52</td>
</tr>
</tbody>
</table>

$IC_{50}$ values of TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin and mitoxantrone in SP and NSP. $IC_{50}$ (Concentration of the drug required to reduce the percentage cell viability to 50) was obtained from the graph by non-linear regression analysis as best curve-fit values. Numerical data are means ± SD of three independent experiments (n=3). Statistically significant difference from $IC_{50}$ values for the control cells exposed to vehicle control (blank) ***p<0.001 determined by two way ANOVA using Bonferroni post test.
Figure 7.21 Isolation of SP by FACS sorting in MCF7

A. Isolation of SP and NSP from human breast cancer cells (MCF7) by FACS sorting. Gated R2 (blue color) and R3 (green color) populations indicates SP and NSP respectively. SP and NSP were sorted by the FACS sorter (MoFlo) into the 96 well plates. B. Purity of flow sorted SP and NSP cells. Purity of SP was found to be 96.25%.
Figure 7.22 Cytotoxicity of TC-A, TC-B, TC-C and TC-D in SP of MCF7

Cytotoxicity of TC-A, TC-B, TC-C, TC-D, doxorubicin, mitoxantrone and parthenolide in SP. SP and NSP from MCF7 cells were sorted by the FACS sorter (MoFlo) and treated with and without TC-A, TC-B, TC-C, TC-D, doxorubicin, mitoxantrone and parthenolide for 48 hours. Cell viability was determined by MTT assay. TC-A, TC-B, TC-C, TC-D, and parthenolide shows significantly increased cytotoxicity in SP as compared to NSP. In contrast, doxorubicin and mitoxantrone shows significantly increased cytotoxicity in NSP. All values are mean ± SD of three independent experiments. (n=3, ***p<0.001, determined by two way ANOVA using Bonferroni post test)
Figure 7.23 CD44/CD24 analysis after treatment with compounds in MCF7

MCF7 cells were treated with and without TC-A, TC-B, TC-C, TC-D, mitoxantrone, parthenolide and salinomycin for 48 hours. At the end of the treatment, CD44/CD24 assay was performed to detect CD44 high CD24 low population using flow cytometry. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited CD44 high CD24 low population. In contrast, mitoxantrone significantly enrich CD44 high CD24 low population. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.24 CD44/CD24 analysis after treatment with compounds in BT549

BT549 cells were treated with and without TC-A, TC-B, TC-C, TC-D, mitoxantrone, parthenolide and salinomycin for 48 hours. At the end of the treatment, CD44/CD24 assay was performed to detect CD44<sup>high</sup>CD24<sup>low</sup> population using flow cytometry. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited CD44<sup>high</sup>CD24<sup>low</sup> population. In contrast, mitoxantrone significantly enrich CD44<sup>high</sup>CD24<sup>low</sup> population. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.25 CD44/CD24 analysis after treatment with compounds in MDA MB 231

MDA MB 231 cells were treated with and without TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin for 48 hours. At the end of the treatment, CD44/CD24 assay was performed to detect CD44 high CD24 low population using flow cytometry. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited CD44 high CD24 low population. All values are mean ± SD of three independent experiments (n=3, **p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.26 Effects of TC-A, TC-B, TC-C and TC-D in CD44\textsuperscript{high} CD24\textsuperscript{low} population

TC-A, TC-B, TC-C and TC-D significantly inhibited CD44\textsuperscript{high} CD24\textsuperscript{low} population in human breast cancer cells. A. MCF7. B. BT549. C. MDA MB 231. Cells were treated with and without TC-A, TC-B, TC-C, TC-D, mitoxantrone, parthenolide and salinomycin for 48 hours. At the end of the treatment, CD44/CD24 assay was performed to detect CD44\textsuperscript{high} CD24\textsuperscript{low} population. The ability of TC-A, TC-B, TC-C and TC-D to inhibit CD44\textsuperscript{high} CD24\textsuperscript{low} population was quite comparable with as salinomycin and parthenolide. However, mitoxantrone enrich CD44\textsuperscript{high} CD24\textsuperscript{low} population. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.27 Isolation of CD44$^{\text{high}}$ CD24$^{\text{low/−}}$ population by FACS sorting

A. Isolation of CD44$^{\text{high}}$ CD24$^{\text{low/−}}$ population and rest of the population (CD44-CD24-) from human breast cancer cells (MDA MB 231) by FACS sorting. Gated R5 (blue color-21.46%) and R6 (green color-15.33%) populations indicates CD44$^{\text{high}}$ CD24$^{\text{low/−}}$ and CD44-CD24- respectively. CD44$^{\text{high}}$ CD24$^{\text{low/−}}$ cells were sorted by the FACS sorter (MoFlo)

B. Purity of sorted CD44$^{\text{high}}$ CD24$^{\text{low/−}}$ cells was 76.64%.

Both CD44 high CD24 low/- and CD44-CD24- cells from MDA MB 231 were sorted by the FACS sorter and treated with and without TC-A, TC-B, TC-C, TC-D, mitoxantrone, parthenolide and salinomycin for 48 hours. Cell viability was determined by MTT assay. The cell viability in untreated cells was normalized as 100%. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin show an increased cytotoxicity in CD44 high CD24 low/- cells as compared to CD44-CD24- cells. In contrast, mitoxantrone show increased cytotoxicity in CD44- CD24-cells. Each point represents the mean ± SD of three independent experiments performed in triplicate.
<table>
<thead>
<tr>
<th>Group</th>
<th>IC$_{50}$ μg/ml</th>
<th>CD44</th>
<th>CD24</th>
<th>CD44-CD24-</th>
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<tr>
<td></td>
<td></td>
<td>high</td>
<td>low/-</td>
<td></td>
</tr>
<tr>
<td>TC-A</td>
<td>30.04±1.47</td>
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<tr>
<td>TC-B</td>
<td>25.17±1.26</td>
<td>67.92±2.98</td>
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<td>TC-C</td>
<td>46.73±3.17</td>
<td>116.50±6.38</td>
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<td>TC-D</td>
<td>64.94±4.47</td>
<td>139.50±4.67</td>
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<tr>
<td>Salinomycin</td>
<td>5.65±0.41</td>
<td>9.68±0.45</td>
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<tr>
<td>Parthenolide</td>
<td>8.70±0.58</td>
<td>16.61±0.76</td>
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<tr>
<td>Mitoxantrone</td>
<td>10.05±0.05</td>
<td>1.71±0.05</td>
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<td></td>
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</tbody>
</table>

IC$_{50}$ values of TC-A, TC-B, TC-C, TC-D, mitoxantrone, parthenolide and salinomycin in CD44$^{\text{high}}$ CD24$^{\text{low/-}}$ and CD44-CD24$^{-}$ populations. IC$_{50}$ (Concentration of the drug required to reduce the percentage cell viability to 50) was obtained from the graph by non-linear regression analysis as best curve-fit values. Numerical data are means ± SD of three independent experiments (n=3). Statistically significant difference from IC$_{50}$ values for the control cells exposed to vehicle control (blank) ***p<0.001 determined by two way ANOVA using Bonferroni post test.
**Figure 7.29 Effects of TC-A, TC-B, TC-C and TC-D in breast cancer spheres (MCF7)**

MCF7 cells were grown in methyl cellulose with and without TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin, doxorubicin and mitoxantrone for 7 days. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited number of spheres. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, **p<0.01 determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
MDA MB 231 cells were grown in methyl cellulose with and without TC-A, TC-B, TC-C, TC-D, parthenolide, salinomycin, doxorubicin and mitoxantrone for 7 days. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited number of spheres. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, **p<0.01 determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 7.31 MDR1 inhibitory effects of TC-A, TC-B, TC-C and TC-D

TC-A, TC-B, TC-C and TC-D inhibited ABCB1 (MDR1) drug transporter in human cervical cancer cells (HeLa) cells. Rhodamine 123 efflux assay was performed with and without TC-A, TC-B, TC-C and TC-D using flow cytometry. Modulation of drug efflux was compared with standard inhibitor of ABCB1 transporter, verapamil. TC-A, TC-B, TC-C and TC-D significantly increased rhodamine 123 mean fluorescence intensity which indicates enhanced drug retention owing to the inhibition of ABCB1 mediated drug transport. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett Test).
Figure 7.32 ABCG2 inhibitory effects of TC-A, TC-B, TC-C and TC-D (MCF7)

TC-A, TC-B, TC-C and TC-D inhibited ABCG2 (BCRP) mediated drug transport in human breast cancer cells (MCF7). Mitoxantrone efflux assay was performed with and without TC-A, TC-B, TC-C and TC-D using flow cytometry. Modulation of drug efflux was compared with standard inhibitor of ABCG2 transporter, fumetrimorgin C (FTC). TC-A, TC-B, TC-C and TC-D significantly increased mitoxantrone mean fluorescence intensity which indicates enhanced drug retention owing to inhibition of ABCG2 mediated drug transport. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, **p<0.01, *p<0.05 determined by one way ANOVA by using Dunnett Test).
Figure 7.33  ABCG2 inhibitory effects of of TC-A, TC-B, TC-C and TC-D (A549)

TC-A, TC-B, TC-C and TC-D inhibited ABCG2 (BCRP) drug transporter in human lung cancer cells (A549). Mitoxantrone efflux assay was performed with and without TC-A, TC-B, TC-C and TC-D using flow cytometry. Modulation of drug efflux was compared with standard inhibitor of ABCG2 transporter, fumetrimorgin C (FTC). TC-A, TC-B, TC-C and TC-D significantly increased mitoxantrone mean fluorescence intensity which indicates enhanced drug retention owing to inhibition of ABCG2 mediated drug transport. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett Test).
Figure 7.34 Abcc1 inhibitory effects of TC-A, TC-B, TC-C, and TC-D (MDA MB 231)

TC-A, TC-B, TC-C, and TC-D inhibited ABCC1 (MRP1) mediated drug transport in human breast cancer cells (MDA MB 231). Calcein AM efflux assay was performed with and without TC-A, TC-B, TC-C, and TC-D using flow cytometry. Modulation of drug efflux was compared with standard inhibitor of ABCC1 transporter, MK571. TC-A, TC-B, TC-C, and TC-D significantly increased calcein AM mean fluorescence intensity which indicates the inhibition of ABCC1 mediated drug transport. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett Test).
Figure 7.35 ABCC1 inhibitory effects of TC-A, TC-B, TC-C and TC-D (A549)

TC-A, TC-B, TC-C and TC-D inhibited ABCC1 (MRP1) mediated drug transport in human lung cancer cells (A549). Calcein AM efflux assay was performed with and without TC-A, TC-B, TC-C and TC-D using flow cytometry. Modulation of drug efflux was compared with standard inhibitor of ABCC1 transporter, MK571. TC-A, TC-B, TC-C and TC-D significantly increased calcein AM mean fluorescence intensity which indicates the inhibition of ABCC1 mediated drug transport. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, **p<0.01 determined by one way ANOVA by using Dunnett Test).
7.5 REFERENCES


Hadnagy, A., Gaboury, L., Beaulieu, R. & Balicki, D. 2006. SP analysis may be used to identify cancer stem cell populations. Experimental Cell Research, 312, 3701-3710.


