CHAPTER THREE
CYTOTOXICITY AND APOPTOSIS ACTIVITIES OF WITHANIA SOMNIFERA AND TINOSPORA CORDIFOLIA EXTRACTS
3.1 INTRODUCTION

Various studies on breast cancer published from India reflect the disease profile and treatment characteristics unique to the urban rich and the middle class patients (Kuraparthy et al., 2007). Recent reports indicate that breast cancer is emerging as prevalent cancer amongst women, surpassing cervical cancer in India (Kuraparthy et al., 2007). Chemotherapy is a major modality of breast cancer treatment. Chemotherapy aims at treatment of cancer by destroying the rapidly proliferating cancer cells. It is also called a systemic treatment as the drug enters through the bloodstream, travels throughout the body and kills cancer cells at their sites (Pluen et al., 2001). Intensive chemotherapy regimens using cytotoxic agents do effectively kill certain malignancies, especially of hematopoietic origin and some solid cancers. However, malignant tumors are often resistant to chemotherapy and even develop acquired chemoresistance or show multi-drug resistance as a consequence of the previous treatment (Gottesman, 2002). Among the cellular mechanisms proposed to mediate multidrug resistance, over expression of a family of plasma membrane efflux transporters, ATP-binding cassette (ABC) transporters, has received extensive investigation. It is observed that the over expression of these ABC transporters, predominantly by ABCB1 (MDR1), ABCC1 (MRP1) and ABCG2 (BCRP) (Shukla et al., 2008) in cancer cells limits the intracellular accumulation of anticancer drugs for efficient activity through the active extrusion of the cytotoxic drugs. Classical multidrug resistance to plant based hydrophobic compounds has been shown to be due to the elevated expression of cell-membrane transporters, which result in an increased efflux of the cytotoxic drugs from the cancer cells, thus lowering their intracellular concentrations (Nobili et al., 2011).

Plants are being used as indigenous cure in folklore or traditional system of medicine for treatment of various kinds of illness including cancer (Pandey et al., 2011). Recently, a greater emphasis has been given towards the research on complementary and alternative medicine that deals with cancer management (Sawadogo et al., 2012). In traditional medicine, plants are being used for healing purposes and are effective as they contain biologically active principles which are non toxic (Duffy et al., 2012). With an understanding of cell biology, mechanism based bioassays have become increasingly important and bio-activity guided phytochemical investigation has resulted in the isolation and characterization of several new molecules possessing interesting medicinal properties (Pan et al., 2012). Ayurveda, a
traditional sect of Indian system of medicine mainly based on plant drugs had been successful since very early times for the prevention or suppressing ailments (Liu, 2011). Plants represent the principal therapy in traditional medicine since time immemorial (Ogilvie, 2003). Early documentation about the use of medicinal plants has been mentioned in Discorides and Ayurveda (Spitzer, 2011). Epidemiological studies suggest that consumption of diets containing fruits and vegetables which are the major sources of phytochemicals and micronutrients reduce the risk of developing cancer (Davis and Milner, 2010). Certain products from plants are known to induce apoptosis in neoplastic cells but not in normal cells (Sharif et al., 2012).

Apoptosis is a highly organized cell death process characterized by loss of plasma membrane phospholipid asymmetry, enzymatic cleavage of the DNA into oligonucleosomal fragments, and segmentation of the cells into membrane-bound apoptotic bodies (Cotter, 2009). Apoptosis has been recognized to play an important role in the maintenance of tissue homeostasis by the selective elimination of excessive cells (Schmitt, 2003). Induction of apoptosis of cancer cells is recognized as a valuable tool for cancer treatment. The agents that are capable of inducing selective apoptosis of cancer cells are receiving considerable attention in the development of cancer chemotherapeutic drugs for cancer.

The current study investigated, two indigenous medicinal plants, *Withania somnifera* of the family Solanaceae and *Tinospora cordifolia* of the family Menispermaceae. The selection of plants were based on valuable information obtained from Ayurveda on anticancer properties, detailed ethno-botanical review, results obtained from the preliminary anticancer screening and contain potential anticancer agents like sesquiterpene lactones. The pharmacological activities of the selected plants, *Withania somnifera* and *Tinospora cordifolia* have been investigated by various *in vitro* and *in vivo* experiments. However, detailed investigations on anticancer properties of the two selected medicinal plants are unknown. In this direction, we have studied the detailed anticancer activities of *Withania somnifera* and *Tinospora cordifolia* in human breast cancer cells using a cell proliferation assay, fluorescent microscopy based assays of apoptosis, DNA fragmentation assay and flow cytometry based cell cycle analysis. Agents that are proficient to induce apoptosis in cancer cells without harming normal cells have considerable attention on development of novel anticancer drugs (Cotter, 2009). Therefore, the current study also investigated the cancer cell
specific activity of *Withania somnifera* and *Tinospora cordifolia* in comparison with human normal epithelial cells.

3.2 MATERIALS AND METHODS

3.2.1 Identification, collection and preparation of crude extracts of selected medicinal plants

The stem part of *Tinospora cordifolia* (Wild.) Hook. F. & Thomas of the family Menispermaceae and the roots of *Withania somnifera* Dunal of the family Solanaceae were collected from the Udupi district, Karnataka (India) in the month of January 2009 and were authenticated by Dr. Gopala Krishna Bhatt, Taxonomist, Professor and Head, Department of the botany, Poorna Prajna College, Udupi, Karnataka. The authenticated specimen was kept in the Herbarium of Manipal College of pharmaceutical sciences, Manipal. Shade dried and coarsely powdered plant (2 kg) was extracted in a soxhlet apparatus for 72 hours using absolute ethanol as solvent. The crude ethanolic extracts were concentrated in a rotary evaporator under reduced pressure and stored in a desiccator and dried completely. Similarly, for the preparation of aqueous extracts of selected medicinal plants, shade dried and coarsely powdered plant materials were extracted with double distilled water in a glass beaker by repeated warming at 50-60°C for 72 hours. Each extract was then filtered through whatman filter paper. Crude aqueous extracts were concentrated in a rotary evaporator under reduced pressure and stored in a desiccator and dried completely. The crude ethanolic and aqueous extracts were further dried by lyophilization. For the biological studies, crude ethanolic extracts were dissolved in DMSO (Calbiochem) at 20 mg/ml and aqueous extracts were dissolved in sterile MilliQ water at 10 mg/ml.

3.2.2 Drugs and chemicals

Doxorubicin hydrochloride solution, Dulbecco’s modified eagle medium (DMEM), Trypsin-EDTA, Hank’s Balanced Salt Solution (HBSS), Thiazolyl Blue Tetrazolium Bromide (MTT), Acridine orange, Ethidium bromide, Hoechst 33342 (Bisbenzimide Trihydrochloride), Propidium iodide, RNase A, Agarose, HEPES buffer, Proteinase K, Nonidet P40, Tris-HCl, were purchased Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco, Invitrogen, USA. Dimethyl Sulfoxide
(DMSO) was purchased from Calbiochem. All other fine chemicals were obtained from Qualigens fine chemicals (Mumbai, India).

3.2.3 Cell culture

MCF7 (human breast carcinoma, ER+, tumorigenic and non-invasive), MDA MB 231 (human breast carcinoma, ER-, tumorigenic and invasive) HeLa (Human cervical carcinoma, tumorigenic and invasive) and HaCaT (Human keratinocyte, immortalized) were cultured in DMEM with high glucose supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified 5% CO2 incubator at 37°C. Cells were cultured in healthy condition and exponentially growing cells were taken for the experiments.

3.2.4 Cytotoxicity assay (Methyl tetrazolium-MTT assay)

3.2.4.1 Determination of optimal cell number for assay

In order to determine optimal cell number required for the assay, serial dilutions of the MCF7, MDA MB 231 and HaCaT cells (2,000, 4,000, 6,000, 8,000, 12,000, 14,000, 16,000 and 18,000 cells/100 μl) were made in cell culture media and seeded in 96 well microtiter tissue culture plates. Cells were cultured, in a humidified 5% CO2 incubator at 37°C for 24 hours. At the end of the incubation period, 20 μl of MTT solution (Stock concentration, 5.00 mg/ml in HBSS) was added to each well and incubated for 4 hours under the same conditions. Thereafter, medium containing MTT was gently replaced by 200 μl DMSO to dissolve formazan crystals and the absorbance values were measured by a microtiter plate reader (Biotek ELx800 - MS) at 540 nm with a reference wavelength of 630 nm. A graph was plotted with the number of cells in X-axis and absorbance at 570/630 nm in Y-axis. Optimal cell densities of cell lines corresponding to absorbance values of 0.9 to 1.0 in the assay were selected for each of the cell lines such as MCF7, MDA MB 231 HeLa, and HaCaT to facilitate measurement of both stimulation and inhibition of cell proliferation within the linear range.

3.2.4.2 Evaluation of cytotoxicity

Briefly, specified cell types were trypsinized and resuspended in the culture medium to get a defined cell number for MCF7 (14,000/100 μl), MDA MB 231 (16,000/100 μl), HeLa (6,000/100 μl) and HaCaT (12,000/100 μl) in a 96-well microtiter tissue culture plate and
cultured in a humidified 5% CO₂ incubator at 37°C for 24 hours. Defined concentrations of the extracts in culture media were freshly prepared by serial dilution to get final concentration of 12.5, 25, 50, 100 and 200 μg/ml (for *Tinospora cordifolia* ethanolic extract); 10, 20, 40, 60 and 100 μg/ml (for *Withania somnifera* ethanolic extract); 25, 50, 100 and 200 μg/ml (for *Tinospora cordifolia* and *Withania somnifera* aqueous extracts). 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 0.50% (v/v). After 24 hours of incubation, cells were treated with above mentioned concentrations of *Tinospora cordifolia* and *Withania somnifera* extracts in triplicates for 48 hours. Doxorubicin hydrochloride, an anticancer drug was used as a positive control. Equal volume of DMSO was used as a vehicle control. At the end of treatment, 20 μl of MTT (Stock was made in HBSS at 5.00 mg/ml) reagent was added to each well and incubated for further 4 hours. Thereafter, the culture medium was removed and formazan crystals were dissolved in 200μl of DMSO. The plates were read in a 96 well microplate reader (Biotek-ELx-800) at a wavelength of 570 nm with a reference wavelength of 630nm. Percentage cell viability (Y-axis) was calculated from O.D values and plotted against concentration in μg/ml (X-axis). The absorbance values of both test and the control (untreated cells) were used for calculating the percentage cell viability (% Cell viability = O.D of Test/O.D of Control x 100). Cell viability in untreated control was normalized to 100%. The dose response curve was plotted with concentration of the drug in the ordinate and percentage cell viability in the abscissa. IC₅₀ values of the extracts were obtained from the graph as the concentration which decreases percentage cell viability to 50. IC₅₀ values for *Tinospora cordifolia* ethanolic and aqueous extracts; *Withania somnifera* ethanolic and aqueous extracts and doxorubicin hydrochloride were determined by nonlinear regression (curve fit) analysis by plotting log (inhibitors) vs. normalized response using software, Graph pad prism 5.

### 3.2.5 Acridine orange-ethidium bromide assay

MCF7, MDA MB 231, HeLa and HaCaT cells were seeded in a 6-well tissue culture plate at an optimal cell number and cultured for 24 hours. Cells at the exponential growth phase were treated with *Tinospora cordifolia* ethanolic extract, *Withania somnifera* ethanolic extract and doxorubicin (positive control) at the IC₅₀ values (mentioned in Table 3.1) for 48 hours. Untreated cells and DMSO treated cells were considered as untreated control and solvent control 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 HBSS twice. Cells were then stained with a mixture of acridine orange and ethidium bromide (2 μg/ml) for 10 minutes in a 37°C CO₂ incubator. At the end of staining, cells were washed with ice-cold HBSS twice and the pellet was re-suspended in 100 μl of HBSS. Thereafter, cells were mounted on a glass slide with cover slip and observed in an inverted fluorescent microscope equipped with 450-490 nm excitation source and 520/570 nm filters of emission wavelengths. Morphological characteristics of both necrosis and apoptosis were observed in both 20X and 40X magnifications. Number of live cells (even nucleated green colored), early apoptotic cells (green colored condensed or fragmented nuclei), late apoptotic cells (orange-red colored condensed or fragmented nuclei) and necrotic cells (even nucleated orange-red colored) were determined.

3.2.6 Hoechst 33342 assay

MCF7, MDA MB 231, HeLa and HaCaT cells were seeded in a 6-well tissue culture plate at an optimal cell number and cultured for 24 hours. Cells at the exponential growth phase were treated with *Tinospora cordifolia* ethanolic extract, *Withania somnifera* ethanolic extract and doxorubicin (positive control) at the IC₅₀ values (mentioned in Table 3.1) for 48 hours. Untreated cells and DMSO treated cells were considered as untreated control and solvent control respectively. At the end of 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 HBSS twice. Thereafter, the cell was stained with Hoechst 33342 (2 μg/ml) 10 minutes at 37°C. At the end of staining, cells were washed twice with ice-cold HBSS and the pellet was resuspended in a minimum volume of HBSS. Cells were mounted on a glass slide with a coverslip and observed under inverted fluorescent microscope equipped with 350 nm excitation source and emission filter of 450 nm wavelength. Morphological characteristics of apoptosis such as, condensed nuclei, fragmented nuclei and membrane blebbing were observed in both 20X and 40X magnifications.

3.2.7 DNA fragmentation assay

MCF7, MDA MB 231, HeLa and HaCaT cells were seeded in a 6-well tissue culture plate at an optimal cell number and cultured for 24 hours. Cells at the exponential growth phase
were treated with *Tinospora cordifolia* ethanolic extract, *Withania somnifera* ethanolic extract and doxorubicin (positive control) at the IC$_{50}$ values (mentioned in Table 3.1) for 48 hours. Untreated cells and DMSO treated cells were considered as untreated control and solvent control respectively. At the end of treatment, adherent cells were harvested by trypsinization and pooled with suspended dead cells. At the end of treatment, adherent cells were lysed with 300 μl of lysis buffer cocktail (1.00 mL stock which contains 10% NP-40 [100 μl] + 200 mM EDTA [40 μl] + 0.2 M Tris-HCl [50 μl; pH 7.5] + 0.50 mg proteinase K + 810 μl miliQ water). The lysate was then pooled with floating dead cells. The cell lysate was incubated at 56$^0$C for 1 hour and further incubated with RNase (100 μg/ml) at 56$^0$ C for 1 hour. At the end of the process, samples were diluted with 30% glycerol and ran in agarose gel (1.50% Agrarose in 1X TBE buffer). Electrophoresis was carried by using Bio-rad electrophoresis unit out at 60 V, 400mA for 90 min using 1X TBE buffer.

### 3.2.8 Cell cycle analysis by flow cytometry

Cell cycle analysis was performed by propidium iodide based measurements of the DNA content of the cells by flow cytometry. Briefly, MCF7, MDA MB 231, HeLa and HaCaT cells were seeded in a 6-well tissue culture plate at an optimal cell number and cultured for 24 hours. Cells at the exponential growth phase were treated with *Tinospora cordifolia* ethanolic extract, *Withania somnifera* ethanolic extract and doxorubicin (positive control) at the IC$_{50}$ values (mentioned in Table 3.1) for 48 hours. Untreated cells and DMSO treated cells were considered as untreated control and solvent control respectively. At the end of 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 HBSS twice and thereafter fixed with 70% ice cold ethanol and stored at -20$^0$C. The cells were then centrifuged at 3000 rpm for 5 minutes at RT and removed ethanol completely. Cells were further washed with HBSS twice and re-suspended in 400 μl of HBSS. Thereafter, RNase A (100 μg/ml) was added and incubated for 3 hours in water bath set at 56$^0$C. Propidium iodide (20 μg/ml) was then added and incubated at RT for 15 minutes and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA). Propidium iodide was excited at 488nm solid state laser and emission wavelength was detected at 585/42 nm (FL-2). The percentage number of DNA in G$_0$/G$_1$, S phase, G$_2$/M phase and sub-G$_0$ phase in untreated cells and treated cells were analyzed by software Summit v4.3 (Beckman Coulter, USA). Effects of
Tinospora cordifolia ethanolic extract, Withania somnifera ethanolic extract and doxorubicin in each and every phase of the cell cycle were determined.

3.3 RESULTS

3.3.1 Tinospora cordifolia and Withania somnifera extracts inhibits breast cancer cell proliferation

Cytotoxic and growth inhibitory effects of the selected plant extracts on human cancer cells were studied by MTT assay. Optimal cell densities corresponding to absorbance values of 0.9 to 1.0 in MTT assay was selected for each of the cell lines to facilitate measurement of both stimulation and inhibition of cell proliferation within the linear range (Fig. 3.1). The optimal cell number to be seeded for a cytotoxicity assay in MCF7, MDA MB 231, HeLa, and HaCaT determined from the plot and was found to be 14,000, 16,000, 6,000 and 12,000 cells/100 μl respectively. In order to evaluate the cytotoxic effects of the ethanolic and aqueous extracts of Tinospora cordifolia and Withania somnifera, MCF7, MDA MB 231, HeLa and HaCaT cells were treated with specified concentrations of the extracts for 48 hours. Doxorubicin was used as a positive control. The ethanolic extracts of Tinospora cordifolia and Withania somnifera showed cytotoxic and dose dependent inhibitory effects on cell proliferation of human breast cancer cells (MCF7 and MDA MB 231) and cervical cancer cells (HeLa). IC50 values of Tinospora cordifolia ethanolic extract in MCF7, MDA MB 231 and HeLa cells were found to be 84.40 ± 2.68 μg/ml, 66.39 ± 3.08 μg/ml and 155.30 ± 6.48 μg/ml, respectively (Fig. 3.2). Similarly, IC50 values of Withania somnifera ethanolic extract in MCF7, MDA MB 231 and HeLa cells were found to be 22.33 ± 1.45 μg/ml, 31.99 ± 1.64 μg/ml and 30.12 ± 1.68 μg/ml, respectively (Fig. 3.4). Aqueous extracts of Tinospora cordifolia and Withania somnifera did not show any concentration dependent cytotoxicity or growth inhibitory activity in any of the cell lines (IC50 >300.0 μg/ml) (Fig. 3.3 and Fig. 3.5). Thus, among the crude extracts screened for cytotoxicity and inhibition of cancer cell proliferation, both Tinospora cordifolia and Withania somnifera ethanolic extracts demonstrated dose dependent anticancer activity against human breast cancer cells (MCF7, MDA MB 231) and cervical cancer cells (HeLa). The positive control, doxorubicin imparted cytotoxic and dose dependent inhibition of cell proliferation and the IC50 values of
doxorubicin on MCF7, MDA MB 231 and HeLa cells was found to be $1.25 \pm 0.05 \, \mu M$, $0.70 \pm 0.03 \, \mu M$ and $2.12 \pm 0.14 \, \mu M$, respectively (Fig. 3.6).

The next question was whether *Tinospora cordifolia* and *Withania somnifera* extract-mediated suppression of cell viability and growth was selectively to cancer cells, and not to normal cells. This would be a highly desirable trait for a potential therapeuetic anti-cancer agent. This question was addressed by determining the cytotoxic and growth inhibitory effects of bio-active *Tinospora cordifolia* and *Withania somnifera* ethanolic extracts on human normal epithelial cells (HaCaT, an immortalized keratinocyte cell line). Results indicated that ethanolic extract of *Tinospora cordifolia* possess cytotoxic and cell growth inhibitory effects with an IC$_{50}$ value of $194.10 \pm 12.09 \, \mu g/ml$ (Fig. 3.2). Similarly, the ethanolic extract of *Withania somnifera* possesses cytotoxic and cell growth inhibitory effects with an IC$_{50}$ value of $75.40 \pm 4.33 \, \mu g/ml$ (Fig. 3.4). However, cell viability was not significantly affected by *Withania somnifera* and *Tinospora cordifolia* ethanolic extract treatment at the concentrations that were cytotoxic to MCF7, MDA MB 231 and HeLa cells. Moreover, an increased IC$_{50}$ value for *Withania somnifera* and *Tinospora cordifolia* ethanolic extracts against HaCaT cells as compared to MCF7, MDA MB 231 and HeLa cells indicate less cytotoxic effects. The well known anticancer drug, doxorubicin has a dose dependent cytotoxicity in HaCaT with an IC$_{50}$ value of $0.42 \pm 0.01 \, \mu M$ (Fig. 3.6). Table 3.1 show a comparison profile of IC$_{50}$ values for *Withania somnifera*, *Tinospora cordifolia* extracts and the standard, doxorubicin in human breast cancer cells (MCF7, MDA MB 435), human cervical cancer cells (HeLa) and human normal epithelial cells (HaCaT). These data suggested that *Withania somnifera* and *Tinospora cordifolia* ethanolic extracts do impart cancer cell-specific cytotoxic effects on human breast and cervical cancer cells. The data represent ± SD of three independent experiments (n=3).

3.3.2 *Withania somnifera* and *Tinospora cordifolia* ethanolic extracts induce apoptosis in human breast cancer cells

3.3.2.1 *Acridine orange-ethidium bromide assay*

In order to distinguish, if the cytotoxic effects of ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* was due to apoptosis or necrosis, human breast cancer cells (MCF7, MDA MB 231) and cervical cancer cells (HeLa) were treated with ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* at their IC$_{50}$ concentration as shown in Table
3.1 for 48 hours. Doxorubicin hydrochloride and DMSO were used in the study design as positive control and vehicle control respectively. At the end of the treatment, cells were harvested and stained with a mixture of acridine orange and ethidium bromide and observed under a fluorescent light microscope. Acridine orange can permeate cells and stain the nuclei green (indicating live cells), while ethidium bromide which stains the nuclei orange-red, can be taken up only by cells that have lost cytoplasmic membrane integrity, and hence indicate dead cells. In addition, while uniformly stained green nuclei indicate live cells; green colored condensed/fragmented nuclei indicate early apoptosis. Similarly, uniformly stained orange-red colored cells indicate necrosis, while orange-red colored condensed/fragmented nuclei indicate late apoptosis. As shown in the Fig. 3.7; untreated cells and vehicle control illustrate uniform green colored nuclei indicating live cells, while cells treated with ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* revealed condensed and fragmented green or orange-red nuclei indicating apoptosis. The apoptotic phenotype was quite comparable with anticancer drug, doxorubicin. These data suggest that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* induced apoptosis but not necrosis in cancer cells. All the experiments were performed three times independently (n=3).

3.3.2.2 **Hoechst 33342 assay**

In order to confirm if the cytotoxic effects of ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* was due to induction of apoptosis, human breast cancer cells (MCF7, MDA MB 231) and cervical cancer cells (HeLa) were treated with extracts and the positive control, doxorubicin at their IC\textsubscript{50} concentration as shown in Table 3.1 for 48 hours. DMSO was used as vehicle control. At the end of the treatment, cells were harvested and stained with DNA binding fluorescent dye Hoechst 33342 to spot the number of apoptotic cells by fluorescent microscopy. Hall mark properties of apoptosis such as condensed nuclei, DNA fragmentation and shrinkage was observed in MCF7, MDA MB231 and HeLa cells upon treatment with the ethanolic extracts of *Withania somnifera*, *Tinospora cordifolia* and doxorubicin (Fig. 3.8). Apoptotic phenotype of extract treated cells was compared with the doxorubicin treated cells. These results confirm that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* induced apoptosis in human breast cancer cells (MCF7, MDA MB 231) and cervical cancer cells (HeLa). All the experiments were performed three times independently (n=3).
3.3.2.3 DNA fragmentation assay

Fluorescent microscopy based nuclear staining studies indicate that *Withania somnifera* and *Tinospora cordifolia* ethanolic extracts induced apoptosis and not necrosis in human cancer cells. DNA fragmentation is a hallmark property of apoptosis. In order to validate the apoptosis, human breast cancer cells (MCF7, MDA MB 231) and cervical cancer cells (HeLa) were treated with ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* and anticancer drug, doxorubicin hydrochloride at their IC$_{50}$ concentration as shown in Table 3.1 for 48 hours. Doxorubicin hydrochloride and DMSO were used as positive control and vehicle control respectively. At the end of the treatment, cells were harvested; genomic DNA was isolated and subjected to agarose gel electrophoresis to detect DNA ladder formation, a hallmark property of apoptosis. Results showed that formation of DNA fragments in MCF7, MDA MB 231 and HeLa cells upon treatment with ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia*. DNA ladder phenotype was prominent in cells treated with doxorubicin and was quite comparable with ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia*. The genomic DNA was intact without any ladder pattern in untreated control and vehicle control groups (Fig. 3.9). These results corroborated that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* induced apoptosis in human breast cancer cells (MCF7, MDA MB 231) and cervical cancer cells (HeLa). All the experiments were performed three times independently (n=3).

3.3.3 Cell cycle specific inhibitory activity of ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* on human breast cancer cells

In order to evaluate the cell cycle specific pharmacological effects of ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia*, human breast cancer cells (MCF7, MDA MB 231) and cervical cancer cells (HeLa) were cultured in the presence or absence of extracts at their IC$_{50}$ concentration for 48 hours. Cells were also treated with doxorubicin and vehicle control (DMSO). At the end of the treatment period, cells were harvested, fixed with ice-cold 70% ethanol and stained with propidium iodide which binds to both DNA and RNA. However, RNA was removed by treatment with RNAse and DNA content was measured by flow cytometry as described in materials and methods. Percentage DNA content in G$_1$/G$_0$ phase, Synthetic (S) phase, G$_2$/M phase and Sub-G$_0$ phase were determined. As depicted in
Fig. 3.10, untreated and vehicle control treated cells showed a normal cell cycle progression. However, upon treatment with the ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* cause a significant inhibition of cell cycle progression in human cancer cells. An increased Sub-G0 phase indicates DNA fragmentation and apoptosis. Similarly, an elevated G1/G0 phase and G2/M phase indicate cell cycle arrest and blockade of mitosis or cell division respectively. A decrease in S-phase indicates inhibition of DNA replication. *Withania somnifera* extract treatment resulted in the clear increase of G2/M phase and Sub-G0 phase as compared with the untreated control. Similarly, *Tinospora cordifolia* extract treatment caused an increase of Sub-G0 phase. Cells treated with doxorubicin showed an arrest of cells in G2/M phase and elevated Sub G0 phase. The cell cycle specific effects of ethanolic extracts of *Withania somnifera, Tinospora cordifolia* and doxorubicin on G1/G0 phase, Synthetic (S) phase, G2/M phase and Sub-G0 phase were compared in Table 3.2. These data suggested that treatment of human cancer cells with the extract of *Withania somnifera* results in apoptosis, inhibition of DNA replication and mitosis. Similarly, *Tinospora cordifolia* extract caused induction of apoptosis and inhibition of DNA replication of human cancer cells.

### 3.3.4 Apoptotic effects of ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* in human normal epithelial cells

Comparison of cytotoxic and growth inhibitory activities of *Withania somnifera* and *Tinospora cordifolia* ethanolic extracts on human cancer cells (MCF7, MDA MB 231 and HeLa) and human normal epithelial cells (HaCaT) by MTT assay revealed that extracts imparted a cancer cell-specific cytotoxicity. In order to understand whether *Withania somnifera* and *Tinospora cordifolia* ethanolic extracts induce any apoptosis in normal epithelial cells at the concentrations that were cytotoxic to human breast cancer cells; HaCaT cells were treated with ethanolic extract of *Withania somnifera, Tinospora cordifolia* and doxorubicin at 22 μg/ml, 84 μg/ml, 1.25 μM respectively (IC50 values of the respective drugs in breast cancer cells, MCF7). At the end of the treatment, cells were harvested and fluorescent microscopy based Hoechst 33342 assay was performed to detect apoptosis. Results suggested that ethanolic extract of *Withania somnifera, Tinospora cordifolia* did not show any significant nuclear condensation or DNA fragmentation in HaCaT cells. However, doxorubicin treated cells demonstrated hall mark properties of apoptosis (Fig. 3.11). All the experiments were performed three times independently (n=3).
Similarly, in order to evaluate changes in cell cycle and apoptosis, HaCaT cells were treated with ethanolic extract of *Withania somnifera*, *Tinospora cordifolia* and doxorubicin at 22 μg/ml, 84 μg/mL, 1.25 μM respectively (IC₅₀ values of the respective drugs in MCF7). At the end of the treatment period, cells were harvested, fixed with ice-cold 70% ethanol and stained with propidium iodide and DNA content was measured by flow cytometry. Percentage DNA content in G₁/G₀ phase, Synthetic (S) phase, G₂/M phase and Sub-G₀ phase were determined. As depicted in the Fig. 3.12; untreated and vehicle control treated cells showed a normal cell cycle progression. Upon treatment with ethanolic extract of *Withania somnifera*, *Tinospora cordifolia* there was no any significant change in Sub-G₀ phase, G₁/G₀ phase, Synthetic (S) phase and G₂/M phase. However, upon treatment with doxorubicin, there was a significant increase in Sub-G₀ phase, indicating apoptosis. A comparative analysis of percentage DNA content in Sub-G₀, G₁/G₀, Synthetic (S) and G₂/M phase after treatment with ethanolic extract of *Withania somnifera*, *Tinospora cordifolia* and doxorubicin in MCF7 and HaCaT cells is shown in Table 3.3. These results demonstrated that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* possess cancer cell specific cytotoxicity and apoptosis. This is a highly attractive trait for cancer chemotherapeutic agents.

### 3.4 DISCUSSION

The current study investigated pharmacological activities of two selected indigenous medicinal plant extracts in human breast cancer (MCF7, MDA MB 231) and cervical cancer cells (HeLa). A methodical evaluation of detailed cytotoxicity and cell proliferation effects in human cancer cells reveals that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* harbors dose-dependent cytotoxicity and cell growth inhibitory activity. As per National Cancer institute (NCI, USA) anti-cancer screening in panel of human cell lines, an IC₅₀ value of less than 100 μg/ml for medicinal plant extracts were considered as potential anticancer agents and recommend for further isolation and characterization of bio-active molecules. The data suggest that *Withania somnifera* ethanolic extract imparted very potent cytotoxic and growth inhibitory activities in selected human cancer cells with IC₅₀ values less than 40 μg/ml. Similarly, *Tinospora cordifolia* ethanolic extract also exhibited potent cytotoxic and growth inhibitory activities with IC₅₀ values less than 90 μg/ml. Therefore, ethanolic extracts of both *Withania somnifera* and *Tinospora cordifolia* may be considered for further isolation and characterization of bio-active molecules.
The current study also investigated that whether ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* induces apoptosis in human breast cancer (MCF7, MDA MB 231) and cervical cancer cells (HeLa). Acridine orange/ethidium bromide assay by fluorescent microscopy revealed that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* induced apoptosis but not the necrosis in human cancer cells. Hoechst 33342 assay and DNA fragmentation assay revealed the hallmark properties of apoptosis such as membrane blebbing, nuclear condensation and DNA fragmentation in both human breast and cervical cancer cells upon treatment with ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia*. These findings corroborated that the ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* induced apoptosis in human breast and cervical cancer cells.

Cancer chemotherapeutic drugs are also classified based on their specificity on mammalian cell cycle as cell cycle-specific and cell cycle non-specific drugs. Cell cycle specific drugs has drawn considerable attention as they act on specific cancer cell cycle checkpoints (Go/G1 phase, S phase, G2/M phase) and inhibit cancer cell proliferation (Go/G1 phase arrest), or DNA replication (Diminished S phase) or mitosis (Go/G1 phase arrest). We have also investigated the cell cycle specific pharmacological effects of *Withania somnifera* and *Tinospora cordifolia* ethanolic extracts in human breast cancer (MCF7, MDA MB 231) and cervical cancer cells (HeLa) by propidium iodide (PI) based cell cycle analysis using flow cytometry. Propidium iodide binds to both DNA and RNA, DNA content in different phases of cell cycle (Go/G1 phase, S phase, G2/M phase and sub-G0) were measured based on fluorescent intensity of propidium iodide. Elevated sub-G0 phase indicate DNA fragmentation or apoptosis. The data suggest that treatment of human breast cancer and cervical cancer cells with the *Withania somnifera* ethanolic extract resulted in significant arrest of cells at G2/M phase (G2/M arrest) and prevent mitosis to occur. It also significantly increased Sub-G0 phase indicating induction of apoptosis and diminishes S phase indicating inhibition of DNA replication. On the other hand, treatment of human breast cancer and cervical cancer cells with the *Tinospora cordifolia* ethanolic extract significantly elevates Sub-G0 phase indicating induction of apoptosis and diminishes S phase indicating inhibition of DNA replication. These results revealed the cancer cell cycle-specific activities of ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* in human breast cancer and cervical cancer cells.
The agents that are capable of inducing selective apoptosis of cancer cells have received considerable interest in the development of novel cancer chemotherapeutic drugs. Hence, the current study also addressed the question of whether *Tinospora cordifolia* and *Withania somnifera* extract-mediated suppression of cell viability and growth was selectively to cancer cells, which is a highly desirable trait for potential cancer preventive and therapeutic agents. Cytotoxic and growth inhibitory study of *Tinospora cordifolia* and *Withania somnifera* ethanolic extracts in human normal epithelial cells (HaCaT, an immortalized keratinocyte cell line) indicated that ethanolic extract of *Withania somnifera* and *Tinospora cordifolia* possess considerably less cytotoxic and cell growth inhibitory activity. Similarly, the potential of *Withania somnifera* and *Tinospora cordifolia* ethanolic extracts to induce apoptosis selectively in cancer cells was investigated by treating human normal epithelial cells with the extracts at the concentrations which were cytotoxic to the human breast cancer cells. Induction of apoptosis and effects on various phases of cancer cell cycle (G₀/G₁ phase, S phase, G₂/M phase and sub-G₀) were measured by classical Hoechst 33342 assay and cell cycle analysis using flow cytometry respectively. More interestingly, the results revealed that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* does not induce any apoptosis in human normal epithelial cells. These results suggest that ethanolic extracts of *Withania somnifera* and *Tinospora cordifolia* possess cancer cell specific cytotoxicity and apoptosis which is a highly attractive trait for cancer chemotherapeutic agents.
Figure 3.1 Determination of optimal cell number to be seeded for MTT assay

Optimal cell number to be seeded was determined in MCF7, MDA MB 231, HeLa and HaCaT cells. Each point represents the mean ± S.D triplicate samples in a representative experiment.

Figure 3.2 Cytotoxicity of *Tinospora cordifolia* ethanolic extract

Cytotoxic effects in human breast cancer cells (MCF7 and MDA MB 231), human cervical cancer cells (HeLa) and human normal epithelial cells (HaCaT) after treatment with *Tinospora cordifolia* ethanolic extract. Each point represents the mean ± S.D of three independent experiments performed in triplicate (n=3).
Figure 3.3 Cytotoxicity of *Tinospora cordifolia* aqueous extract

Cytotoxic effects in human breast cancer cells (MCF7 and MDA MB 231), human cervical cancer cells (HeLa) and human normal epithelial cells (HaCaT) after treatment with *Tinospora cordifolia* aqueous extract. Each point represents the mean ± S.D of three independent experiments performed in triplicate (n=3).

Figure 3.4 Cytotoxicity of *Withania somnifera* ethanolic extract

Cytotoxic effects in human breast cancer cells (MCF7 and MDA MB 231), human cervical cancer cells (HeLa) and human normal epithelial cells (HaCaT) after treatment with *Withania somnifera* ethanolic extract. Each point represents the mean ± SD of three independent experiments performed in triplicate (n=3).
Figure 3.5 Cytotoxicity of *Withania somnifera* aqueous extract

Cytotoxic effects in human breast cancer cells (MCF7 and MDA MB 231), human cervical cancer cells (HeLa) and human normal epithelial cells (HaCaT) after treatment with *Withania somnifera* aqueous extract. Each point represents the mean ± SD of three independent experiments performed in triplicate (n=3).

Figure 3.6 Cytotoxicity of Doxorubicin

Cytotoxic effects in human breast cancer cells (MCF7 and MDA MB 231), cervical cancer cells (HeLa) and human normal epithelial cells (HaCaT) after treatment with doxorubicin. Each point represents the mean ± SD of three independent experiments performed in triplicate (n=3).
Table 3.1 IC\textsubscript{50} values of \textit{Withania somnifera} and \textit{Tinospora cordifolia} extracts

<table>
<thead>
<tr>
<th>Groups</th>
<th>IC\textsubscript{50} (μg/ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MCF7</td>
</tr>
<tr>
<td>\textit{Tinospora cordifolia}</td>
<td></td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td>84.40±2.68</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>430.90±13.44</td>
</tr>
<tr>
<td>\textit{Withania somnifera}</td>
<td></td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td>22.33±1.45</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>388.10±17.58</td>
</tr>
<tr>
<td>Doxorubicin *</td>
<td>1.25±0.05</td>
</tr>
</tbody>
</table>

IC\textsubscript{50} (Concentration of the drug required to reduce the percentage cell viability to 50 percentage) were obtained from the graph by non-linear regression analysis as best curve- fit values. IC\textsubscript{50} values of \textit{Withania somnifera} and \textit{Tinospora cordifolia} extracts and standard anticancer drug, doxorubicin were compared with human breast cancer cells (MCF7, MDA MB 435); human cervical cancer cells (HeLa) and human normal epithelial cells (HaCaT). *The IC\textsubscript{50} of doxorubicin is represented as μM. Numerical data are means ± S.D of three independent experiments (n=3). Statistically significant difference from IC\textsubscript{50} values for the control cells exposed to vehicle control (blank) (p < 0.001).
Figure 3.7 Acridine orange-ethidium bromide assay to discriminate necrosis or apoptosis

Green colored nuclei indicate live cells. A condensed or fragmented nucleus with green or orange-red color indicates apoptosis and even nucleated red color indicates necrosis. Ethanolic extracts of both *Withania somnifera* and *Tinospora cordifolia* induced apoptosis but not necrosis in cancer cells. These figures are representative of three independent experiments (n=3).
Figure 3.8 Hoechst 33342 assay for the detection of apoptosis

Condensed and fragmented nuclei upon treatment with *Withania somnifera* and *Tinospora cordifolia* extracts indicated hallmark properties of apoptosis in breast cancer cells. These figures are representative of three independent experiments (n=3).
DNA fragmentation assay in human breast cancer cells (MCF7, MDA MB 231) and cervical cancer cells (HeLa) indicated that *Withania somnifera* and *Tinospora cordifolia* induced DNA ladder formation, a hallmark property of apoptosis. A: Doxorubicin B: *Withania somnifera* ethanolic extract, C: *Tinospora cordifolia* ethanolic extract, D: Untreated control, E: Vehicle control (DMSO), M: DNA ladder (1kb). These figures are representative of three independent experiments (n=3).
Figure 3.10 Cell cycle analysis after treatment with *Withania somnifera* and *Tinospora cordifolia* extracts

DNA content was analyzed based on propidium iodide (PI) staining using flow cytometry. The percentage number of DNA content in G0/G1, S, G2/M and sub G0 (Apoptosis) phases were determined. These figures are representative of three independent experiments (n=3).
Cell cycle analysis in cancer cells after treatment with *Withania somnifera* extract, *Tinospora cordifolia* extract and doxorubicin. DNA content was determined in G₀/G₁, S, G₂M and sub G₀ (Apoptosis) phase based on propidium iodide (PI) staining using flow cytometry. An increased Sub G₀ phase indicating that extracts induced apoptosis in cancer cells. An increased G₂M phase indicates cell cycle arrest and prevents mitosis. Numerical data are means ± S.D 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.
Figure 3.11 Hoechst 33342 assay to detect apoptosis in human normal epithelial cells

Human normal epithelial cells (HaCaT) were treated with *Withania somnifera* extract, *Tinospora cordifolia* extract and doxorubicin at the concentration which was cytotoxic to the human breast cancer cells (MCF7) for 48 hours. Condensed and fragmented nuclei upon treatment with doxorubicin indicate apoptosis induced. However, extracts did not induced any significant apoptosis. These figures are representative of three independent experiments (n=3).

Figure 3.12 Cell cycle analysis in human normal epithelial cells after treatment with *Withania somnifera* and *Tinospora cordifolia* extracts

HaCaT cells were treated with *Withania somnifera* extract, *Tinospora cordifolia* extract and doxorubicin at the concentration which was cytotoxic to the human breast cancer cells (MCF7) for 48 hours. The percentage number of DNA in G0/G1, S, G2M and Sub G0 (apoptosis) phase was determined based on propidium iodide (PI) using flow cytometry. Doxorubicin significantly increased Sub G0 phase indicating induction of apoptosis. However, extracts did not imparted any cell cycle specific changes. These figures are representative of three independent experiments (n=3).
Table 3.3 Cell cycle specific effects of *Withania somnifera* and *Tinospora cordifolia* extracts in normal epithelial cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>MCF7</th>
<th>HaCaT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sub G₀ phase</td>
<td>G₀/G₁ phase</td>
</tr>
<tr>
<td>Untreated control</td>
<td>0.70±0.04</td>
<td>60.76±4.34</td>
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<tr>
<td>Vehicle control (DMSO)</td>
<td>1.90±0.10</td>
<td>57.38±5.38</td>
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<tr>
<td>Doxorubicin</td>
<td>30.46±2.07</td>
<td>34.90±3.24</td>
</tr>
<tr>
<td><em>Withania somnifera</em> extract</td>
<td>33.65±1.61</td>
<td>30.30±4.25</td>
</tr>
<tr>
<td><em>Tinospora cordifolia</em> extract</td>
<td>33.54±1.60</td>
<td>43.14±3.30</td>
</tr>
</tbody>
</table>

Cell cycle analysis in human breast cancer cells (MCF7) and normal epithelial cells (HaCaT) after treatment with *Withania somnifera* extract, *Tinospora cordifolia* extract and doxorubicin. HaCaT cells were treated at the concentrations which was cytotoxic to human breast cancer cells (MCF7). DNA content was determined in G₀/G₁, S, G₂M and sub G₀ (apoptosis) phase. An increased Sub G₀ phase indicated that extracts induces apoptosis specifically in cancer cells. However, currently used anticancer drug, doxorubicin induced apoptosis in both cancer and normal cells. All values are mean ± S.D of three independent experiments, p<0.001, determined by two way ANOVA using Bonferroni post test.
3.5 REFERENCES

