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Original Research

Cytotoxic and apoptotic activities of extracts of *Withania somnifera* and *Tinospora cordifolia* in human breast cancer cells

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Summary. *Withania somnifera* (WS) and *Tinospora cordifolia* (TC) have been used in the traditional system of medicine in India (Ayurveda) for the treatment of cancer. The current study investigated the cytotoxic and apoptotic effects of extracts of WS and TC on human breast cancer cells (MCF7 and MDA MB 231). MTT-based assays revealed dose-dependent cytotoxic effects of the ethanolic extracts of WS and TC in human breast cancer cells, while the aqueous extracts failed to induce significant cytotoxicity. Hoechst 33342 staining and DNA fragmentation assays revealed hallmark properties of apoptosis such as membrane blebbing, nuclear condensation, and DNA fragmentation. The ethanolic extracts of both WS and TC also increased the sub-G_0 content, further confirming induction of apoptosis, while WS extracts additionally caused cell cycle arrest in the G_2/M phase. Further, the current study also evaluated the cytotoxic effects of WS and TC extracts on human immortalized but, 'non-cancerous' cell line (HaCaT). Significantly, the extracts failed to show cytotoxicity or apoptosis in HaCaT cells at the concentration that was cytotoxic to breast cancer cells, indicating less cytotoxic effects of WS and TC against human 'non-cancerous' cells. Thus, our study reveals potential anti-cancer activities of the ethanolic extracts of TC and WS against human breast cancer cells.
Industrial relevance. The uses of WS and TC in traditional system of medicine for the management and treatment of cancer have drawn considerable attention. Varieties of pytochemicals and herbal formulations have been developed from plant sources, leading to the scientific interest in the discovery of anticancer agents from crude plant extracts. Medicinal plant extracts have played a significant role in the development of several clinically useful anti-cancer agents. Herbal formulations of the crude extracts could be useful for the treatment of breast cancer with less toxic effects against 'non-cancerous' cells. The identification of biologically active crude extracts possessing anti-apoptotic activities against breast cancer cells shows their potential for drug discovery.
Key words. *Withania somnifera; Tinospora cordifolia;* Cytotoxicity; Apoptosis; Human breast cancer cells.

INTRODUCTION

Cancer is the second leading cause of death following heart diseases, accounting for 23% of all deaths. Breast is the most leading sites of cancer in females (Cancer Facts and Figures, 2012). In India, breast cancer accounts for 19-34% of all cancer cases among women, and in recent years, has taken over cervical cancer as leading site of cancer (Development of cancer atlas of India, ICMR, 2005). Recently, a greater emphasis has been given towards the research on complementary and alternative medicine that deals with cancer management (Premalatha & Rajgopal 2005). In fact, plant-derived compounds have played an important role in the development of several clinically useful anti-cancer agents (Cragg & Newman 2005). *Withania somnifera* (WS) and *Tinospora cordifolia* (TC) have been used in the traditional system of medicine in India (Ayurveda) for the treatment of cancer (Premalatha & Rajgopal 2005; Williamson 2002; Dhar et al., 1968).

WS belongs to the family Solanaceae is an evergreen shrub, wildly grows and also cultivated for medicinal use in many parts of India (Kulkarni & Dhir 2008). Its efficacy in many ailments has been confirmed by various in *vitro* and *in vivo* pharmacological experiments (Uddin et al., 2012).WS has anti-inflammatory, anti-stress, radiosensitizer, antioxidant, immunomodulatory, and hemopoetic properties (Tripathi et al., 2011). It is often prescribed during convalescence, rheumatism, and insomnia; the plant has a rejuvenativing effect on the body (Uddin et al., 2012). TC is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae (Krishna et al., 2009). It is an important medicinal plant cultivated throughout the Indian subcontinent for the treatment of several diseases including cancer (Premalatha & Rajgopal 2005; Thippeswamy & Salimath 2007; Panchabhai et al., 2008; Mittal & Singh 2009). TC stem extract is a stomachic, diuretic, enriches the blood, and cures jaundice (Panchabhai et al., 2008). Dry barks of TC have anti-spasmodic, antipyretic, anti-allergic, anti-inflammatory, immuno-modulatory, antioxidant activities, and widely used in the treatment of diabetes mellitus (Singh et al., 2003; Sharma et al., 2012; Upadhyay et al., 2010). Administration of either alcoholic or aqueous extract of TC decreased the blood glucose level and increased glucose tolerance in rodents (Selvaraj et al., 2012).

In the context of cancer, WS root extracts have been shown to prevent DMBA-induced squamous cell carcinoma in swiss albino mice (Prakash et al., 2002), dose dependent inhibition on tumor growth and increased survival rate in Ehrlich ascites

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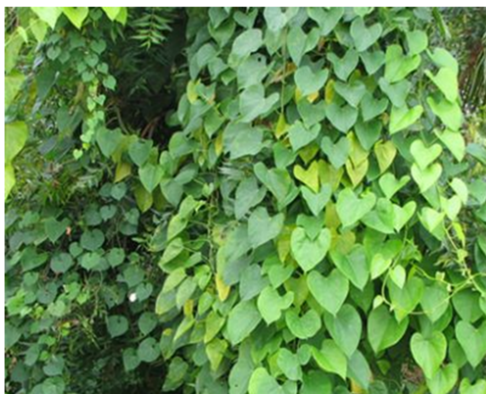
carcinoma mouse model (Sharada et al., 1996), inhibit forestomach and skin carcinogenesis in mice (Padmavathi et al., 2005), and possess anti-angiogenic effects against human laryngeal carcinoma (Mathur et al., 2006). Withaferin A isolated from the WS root extract showed significant antitumor and radiosensitising effects in experimental tumors *in vivo*, without any systemic toxicity (Devi PU 1996). Traditionally, extracts of TC have been applied locally to treat various tumors (Dash & Kashyap 1987) and clinically used against throat cancer in humans (Chauhan 1995). More recently, extracts of TC have been reported to inhibit skin carcinogenesis (Chaudhary et al., 2008) and experimental metastasis in mouse model (Leyon & Kuttan 2004), to induce cytotoxic effects in cultured HeLa cells (Jagetia et al., 1998; Jagetia and Rao 2006 a), and anti-neoplastic effects in Ehrlich ascites carcinoma bearing mice (Jagetia and Rao 2006 b). However, detailed investigations of anti-cancer effects of both WS and TC, and its mechanisms of action, in human breast cancer cells remains unexplored.

Induction of apoptosis in cancer cells is recognized as a valuable tool for breast cancer treatment (Cotter 2009). Therefore, the current study investigated the cytotoxic and apoptotic effects of WS and TC extracts against human breast cancer cell lines such as MCF7 and MDA MB 231. Cervical cancer cell line (HeLa) was used as experimental control in the study since previously TC extract was shown to have cytotoxic effects against HeLa (Jagetia et al., 1998; Jagetia & Rao, 2006 a). Agents that are proficient to induce apoptosis in cancer cells without harming normal cells have drawn considerable attention for the development of novel anticancer drugs (Cotter 2009). Hence, the current study also investigated the effects of WS and TC extracts on human immortalized but 'non-cancerous' cell line (HaCaT).

A

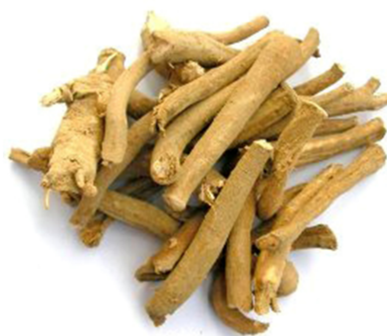


B

Tinospora cordifolia (Wild.) Hook.F. & Thomas of the family Menispermaceae. **A**: Whole plant, **B**: Stem part of the plant.
Courtesy: Google images

A

B



Withania somnifera Dunal of the family Solanaceae. **A**: Whole plant, **B**: Roots of the plant.
Courtesy: Google images

MATERIALS AND METHODS

Plant materials. The roots of *Withania somnifera* Dunal of the family Solanaceae and the stem part of *Tinospora cordifolia* (Wild.) Hook. F. & Thomas of the family Menispermaceae were collected from the Udupi district, Karnataka (India). The taxonomic identification was carried out by Dr. Gopala Krishna Bhatt (Professor and Head, Department of Botany, Poorna Prajna College, Udupi, Karnataka). A voucher specimen was deposited in the herbarium of Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, India.

Preparation of plant extracts. Dried and coarsely powdered plant materials (2 kg) were extracted in a Soxhlet apparatus for 72 h using absolute ethanol as solvent (for ethanolic extracts). The aqueous extracts were prepared using warm double distilled water (50-60°C) for 72 h. Both extracts were filtered and filtrates were concentrated using rotary evaporator *in vacuo*, and completely dried by lyophilization. The yields of WS ethanolic and aqueous extracts were 7.12% and 3.30%, respectively. Similarly, the yields of TC ethanolic and aqueous extracts were 4.94% and 2.15%, respectively. For the biological studies, ethanolic extracts were dissolved in DMSO and aqueous extracts were dissolved in sterile MilliQ water.

Drugs and chemicals. Doxorubicin, Dulbecco's modified eagle medium (DMEM), Trypsin-EDTA, Hank's Balanced Salt Solution (HBSS), Thiazolyl Blue Tetrazolium Bromide (MTT), Acridine orange (AO), Ethidium bromide (EB), Hoechst 33342, Propidium iodide (PI), RNase A, Agarose, HEPES buffer, Proteinase K, Nonidet P-40, and 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.

Cell culture. MCF7 (human breast carcinoma, ER+, tumorigenic, and non-invasive), MDA MB 231 (human breast carcinoma, triple negative, tumorigenic, and invasive), HeLa (human cervical carcinoma, tumorigenic, and invasive) and HaCaT (human immortalized, but 'non-cancerous') were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin, in a humidified 5% CO_2 incubator at 37°C. Cells were cultured in healthy condition and exponentially growing cells were used for experiments.

Evaluation of cytotoxicity by MTT assay. For cytotoxicity assays, MCF7 (14000/100 µl), MDA MB 231(16000/100 µl), HeLa (6000/100 µl), and HaCaT (12000/100 µl) cells were seeded in 96 well microtiter tissue culture plates for 24 h (Optimal cell number for each cell line was determined by seeding varying numbers and performing MTT assay for 24 h. Optimal cell densities for each cell line corresponding to absorbance values of 0.9 to 1.0 in the linear range was selected). Defined concentrations of the extracts in culture media were freshly prepared by serial dilution to get final concentrations of 10, 20, 40, 60, and 100 µg/mL (for WS ethanolic extract); 12.5, 25, 50, 100, and 200 µg/mL (for TC ethanolic extract); 25, 50, 100, and 200 µg/mL (for WS and TC aqueous extracts). After 24 h seeding of cells in 96 well plate, cells were treated with above mentioned concentrations of extracts in triplicates for 48 h. Doxorubicin was used as a positive control. At the end of treatment, 20 µl of MTT solution (5.00 mg/mL in PBS) was added to each well and further incubated for 4 h. Thereafter, medium containing MTT was gently replaced by 200 µl DMSO and the absorbance values were measured by a microtiter plate reader (Biotek ELx800 - MS) at 540 nm with a reference wavelength of 630 nm. The final concentration of DMSO in any of the wells did not exceed 0.5% (v/v), and thus, this concentration of DMSO was used as vehicle control. Dose response curves were generated by plotting percentage cell viability (calculated as % Cell viability = O.D of Test/O.D of Control x 100) in y-axis against concentration in µg/mL in the x-axis. Cell viability in untreated control was normalized to 100. IC_{50} values of the extracts were obtained from the graph as the concentration which decreases percentage cell viability to 50%.

Acridine orange-ethidium bromide and Hoechst 33342 staining for apoptosis detection. 4 x 10^5 cells seeded in a 6-well tissue culture plate for 24 h were treated with WS ethanolic extract, TC ethanolic extract, and doxorubicin (used as positive control) corresponding to their IC_{50} values (Table 1) for 48 h. At the end of the treatment, adherent cells were harvested by trypsinization and pooled with floating cells. Thereafter, cells were centrifuged at 2000 rpm for 5 min at RT, washed with HBSS twice. Cells were then stained with a mixture of AO and EB (2 µg/mL) for 10 min in a 37°C CO_2 incubator. Both AO and EB intercalate with DNA, and preferentially stain the cell nuclei. AO is cell-permeant, allowing for visualization of nuclear structure in living cells. EB is excluded from living cells. When cells die and their plasma membrane ruptures, ethidium bromide reaches the nucleus and stains orange red. Therefore, in this assay, uniformly stained green nuclei indicate live cells and uniformly stained orange-red nuclei indicate necrosis. Whereas, green or orange-red nuclei with condensed chromatin/fragmented DNA indicates apoptotic cells. Similarly, cells treated with drugs were also stained with Hoechst 33342 (2 µg/mL) for 10 min at 37°C. At the end of staining, cells were washed with ice-cold HBSS twice and the pellet was re-suspended in 100 µL of HBSS. The cells were mounted on a glass slide with cover slip and viewed under a fluorescent microscope (Leica, Germany). Live cells show uniform faint blue staining while the condensed nuclei of apoptotic cells show bright fluorescence.

DNA fragmentation assay. Cells were treated with WS ethanolic extract, TC ethanolic extract, and doxorubicin for 48 h as described above. At the end of treatment, both adherent cells and floating cells were harvested and incubated with 300 µl of lysis buffer cocktail (10% NP-40 + 200 mM EDTA + 0.2 M Tris-HCl + 0.50 mg/mL proteinase K) for an h and further incubated with RNAse (100 µg/mL) at 56^0 C for 1 h. At the end of the process, samples were mixed with loading dye (xylene cyanol in 30% glycerol) and resolved in agarose gel (1.50% Agrarose in 1X TBE buffer). Electrophoresis was carried out at 60 V for 90 min using 1X TBE buffer.

Cell cycle analysis by flow cytometry. Cell cycle analysis was performed by propidium iodide (PI) based measurements of the DNA content of the cells by flow cytometry. Briefly, cells were treated with WS ethanolic extract, TC ethanolic extract, and doxorubicin for 48 h as described above. At the end of treatment, adherent cells were harvested by trypsinization and pooled with floating cells. Thereafter, cells were centrifuged at 2000 rpm for 5 min at RT, washed with HBSS twice and thereafter fixed with 70% ice cold ethanol and stored at -20°C overnight. The cells were then centrifuged at 3000 rpm for 5 min at RT, washed with HBSS twice and re-suspended in 400 µl of HBSS. Thereafter, RNase A (100 µg/mL) was added and incubated for 3 h in water bath set at 56^0C. PI (20 µg/mL) was then added and incubated at RT for 15 min. DNA content was analyzed by flow cytometry (MoFlo, Beckman Coulter, USA).

RESULTS

Ethanolic extracts of WS and TC show cytotoxicity against human breast cancers with less effect in human immortalized, but 'non-cancerous' cells. Cytotoxic effects of the ethanolic and aqueous extracts of WS and TC were evaluated on MCF7, MDA MB 231 (breast cancer cells), HeLa (cervical cancer cells used as a control), and HaCaT (human immortalized, but 'non-cancerous' cells) by MTT assay. Results indicated that ethanolic extracts of both WS and TC possessed dose-dependent cytotoxic effects against human breast cancer cells (Fig. 1 A and B). Aqueous extracts of WS and TC did not show any concentration dependent cytotoxicity in any of the cell lines tested (Fig. 1 C and D). The IC_{50} values for WS extracts, TC extracts, and doxorubicin (positive control) is shown in Table 1.

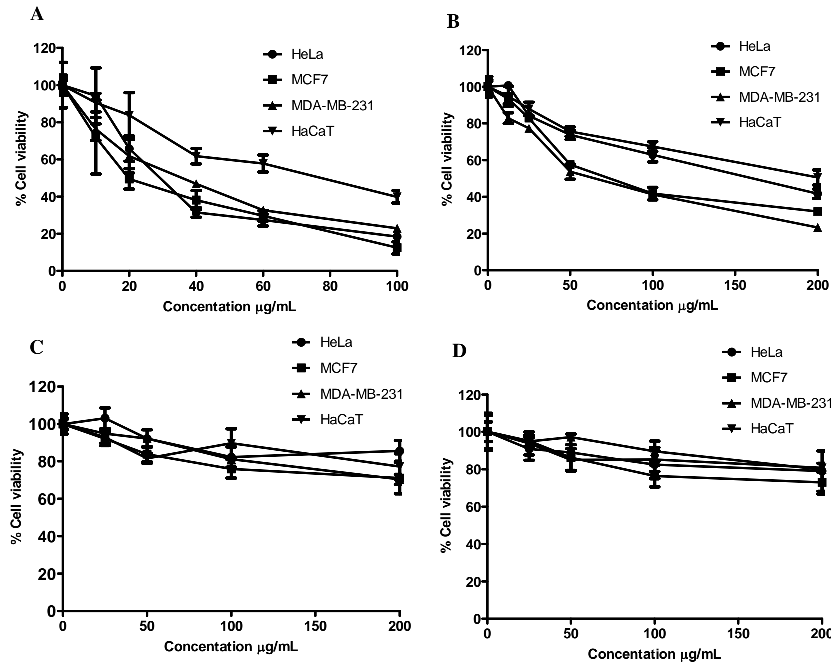


Figure 1. Cytotoxic effects of *Withania somnifera* (WS) and *Tinospora cordifolia* (TC) extracts on human breast cancer cell lines (MCF7 and MDA MB 231), human cervical cancer cell line (HeLa), and human immortalized, but 'non-cancerous' cell line (HaCaT).
WS ethanolic extract (A), TC ethanolic extract (B), WS aqueous extract (C), and TC aqueous extract (D). Each point represents the mean ± S.D of three independent experiments performed in triplicates.

Table 1. Cytotoxicity of extracts of *Withania somnifera* (WS) and *Tinospora cordifolia* (TC) on human breast cancer cell lines (MCF7 and MDA MB 231), human cervical cancer cell line (HeLa), and human immortalized, but 'non-cancerous' cell line (HaCaT).

Groups	IC_{50} (µg/mL)			
	MCF7	**MDA MB 231**	**HeLa**	**HaCaT**
WS Ethanolic extract	22.33±1.45	31.99±1.64	30.12±1.68	75.40±4.33
WS Aqueous extract	388.10±17.58	471.40±7.64	873.20±44.80	604.50±28.77
TC Ethanolic extract	84.40±2.68	66.39±3.08	155.30±6.48	194.10±12.09
TC Aqueous extract	430.90±13.44	830.70±38.54	589.80±24.56	647.50±40.34
Doxorubicin *	1.25±0.05	0.70±0.03	2.12±0.14	0.42±0.01

IC_{50} (Concentration of the drug required to reduce the percentage cell viability to 50) were obtained from the graph (Figure 1) by non-linear regression analysis as best curve-fit values. *IC_{50} of doxorubicin is represented as µM. Numerical data are means S.D. of three independent experiments.

It is evident from the results in the Table 1 that the IC_{50} values for the ethanolic extracts of WS and TC in HaCaT were found to be 2-3 fold higher compared to those for MCF7 and MDA MB 231 cells. Further, cell viability of HaCaT was not significantly affected at the concentrations that were cytotoxic to MCF7 (22.33 ± 1.45 µg/mL for WS and 84.40 ± 2.68 µg/mL for TC) and MDA MB 231 (31.99 ± 1.64 µg/mL for WS and 66.39 ± 3.08 µg/mL for TC). The increased IC_{50} values

of ethanolic extracts of WS and TC against HaCaT, as compared to MCF7 and MDA MB 231, indicates less cytotoxic effects of the extracts on 'non-cancerous' cells compared to breast cancer cells. Thus, the ethanolic, but not aqueous extracts, of both WS and TC show cytotoxicity against human breast cancer cells with less effect against 'non-cancerous' cells. Hence, further studies were undertaken with the ethanolic plant extracts.

Ethanolic extracts of WS and TC induce apoptosis in human breast cancer cells. We next investigated whether the cytotoxic effects of the ethanolic extracts of WS and TC are due to apoptosis or necrosis. For this, the cancer cell lines were treated with ethanolic extracts at their determined IC_{50} concentration (Table 1) for 48 h and subjected to staining with acridine orange and ethidium bromide to score for apoptotic and necrotic cells. Uniformly green nuclei with normal morphology were observed in DMSO (vehicle control) treated cells indicating viable cells (Fig. 2 A). Doxorubicin used as positive control, showed both green and orange-red colored condensed/fragmented nuclei indicating apoptosis (Fig. 2 A). Similarly, treatment with ethanolic extracts of WS and TC also resulted in bright green, early apoptotic cells with nuclear condensation as well as orange-red, late apoptotic cells with fragmented chromatin and apoptotic bodies (Fig. 2 A). We failed to detect uniformly stained orange-red nuclei characteristic of necrosis in all the treatment. Thus, these results indicated that WS and TC ethanolic extracts show cytotoxicity effect against breast cancer cells by inducing apoptosis and not necrosis. Additionally, Hoechst 33342 staining assay using fluorescent microscopy also revealed the hall mark properties of apoptosis such as condensed and fragmented nuclei upon treatment with WS and TC ethanolic extracts (Fig. 2 B), further confirming the induction of apoptosis by the plant extracts. DNA fragmentation assay revealed that similar to doxorubicin used as positive control, treatment with the ethanolic extracts of WS and TC also induced DNA ladder formation (Fig. 2 C). Together, these results corroborated that the ethanolic extracts of WS and TC induced apoptosis in human breast cancer cells.

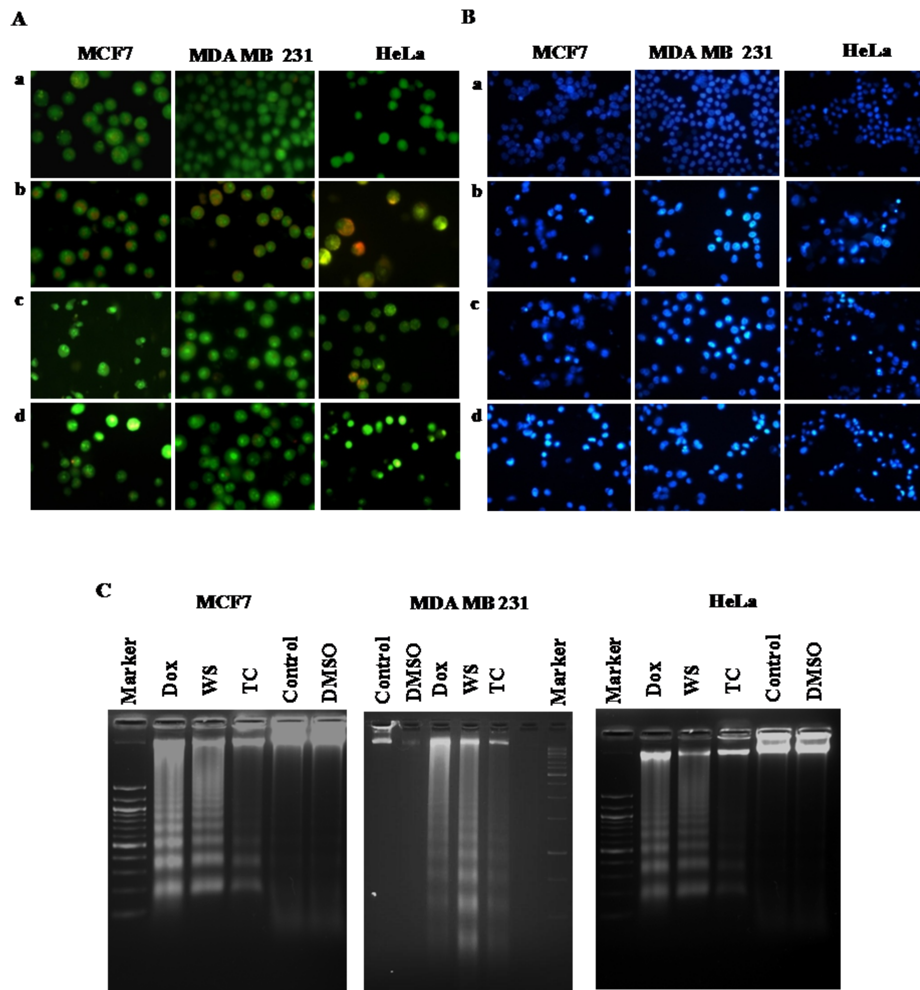


Figure 2. Apoptotic effects of ethanolic extracts of *Withania somnifera* (WS) and *Tinospora cordifolia* (TC) against human breast cancer cell lines.

Cells were treated with DMSO (a), doxorubicin (b), WS ethanolic extract (c), and TC ethanolic extract (d) for 48 h. **A.** Acridine orange/ethidium bromide (AO/EB) staining to distinguish between necrosis and apoptosis. Uniformly stained green colored nuclei indicate live cells; condensed or fragmented nucleus with green or orange-red color indicates apoptosis. **B.** Hoechst 33342 staining to detect apoptosis. Condensed and fragmented nuclei upon treatment with WS and TC ethanolic extracts indicate hall mark properties of apoptosis. **C.** DNA fragmentation assay for the detection of apoptosis. WS and TC ethanolic extracts induced DNA ladder formation, a hallmark property of apoptosis. The data are representative of three independent experiments.

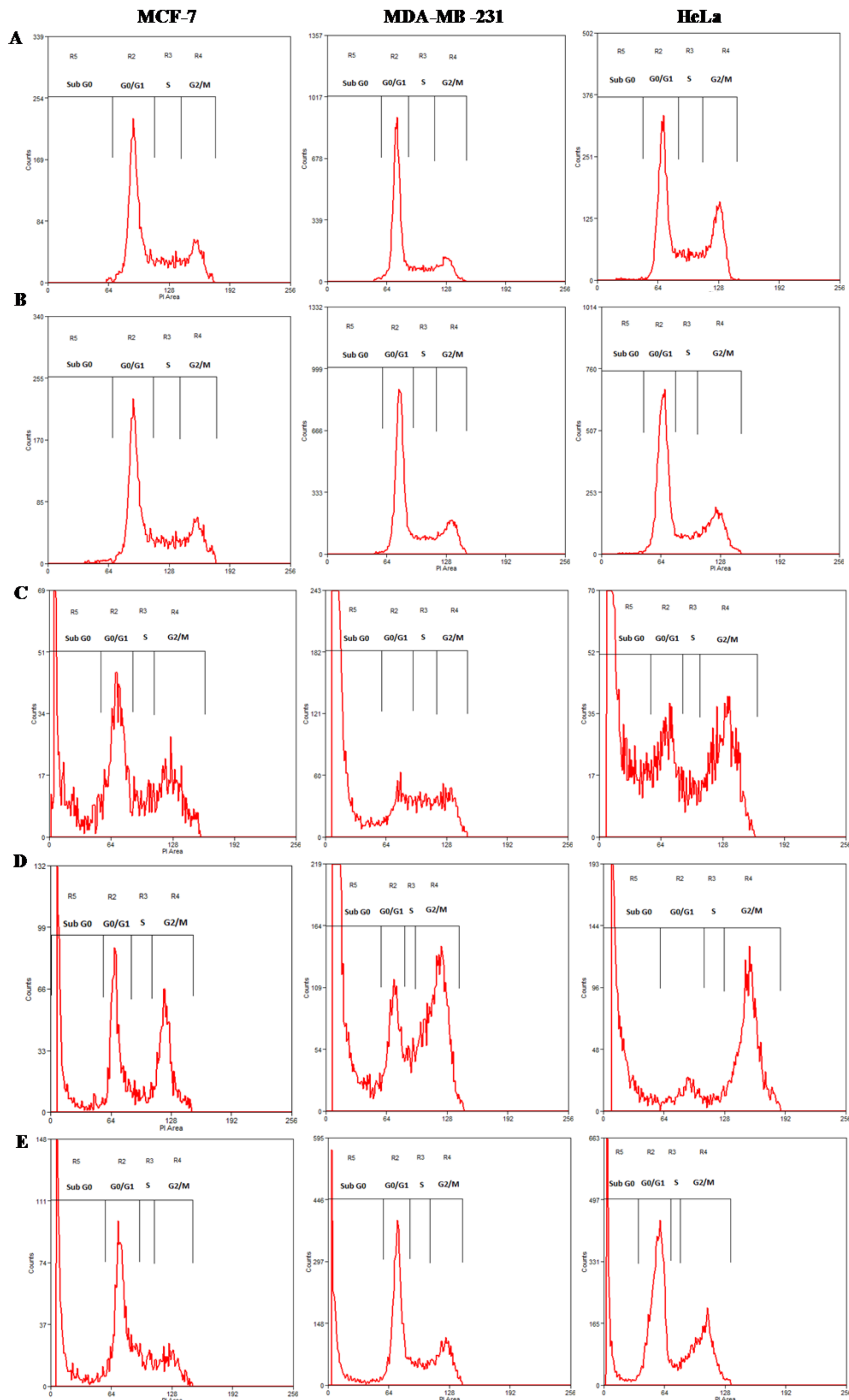


Figure 3. Effects of ethanolic extracts of *Withania somnifera* (WS) and *Tinospora cordifolia* (TC) on cancer cell cycle progression. Cells were treated without (A), or with DMSO (B), doxorubicin (C), WS ethanolic extract (D), and TC ethanolic extract (E) for 48 h; stained with propidium iodide (PI) and analyzed by flow cytometry. Percentage DNA content in G_0/G_1, S, G_2M and sub G_0 were calculated. The data are representative of three independent experiments.

Effects of ethanolic extracts of WS and TC on cell cycle. In order to evaluate the effects on cancer cell cycle, cells were treated with ethanolic extracts of WS and TC at their IC_{50} concentration (Table 1) for 48 h. At the end of the treatment, DNA content in G_0/G_1 phase, Synthetic (S) phase, and G_2/M phase were determined using flow cytometry. In the same experiment, a measure of sub-G_0 DNA content additionally reveals apoptosis. As shown in Fig. 3 A and B, untreated and DMSO (vehicle control) treated cells showed a normal cell cycle profile with hardly any sub-G_0 DNA. Doxorubicin used as positive control showed a significant G_2/M arrest and significantly increased sub-G_0 DNA content (Fig. 3 C). Treatment with WS ethanolic extract also caused a significant increase in G_2/M phase (indicating cell cycle arrest and blockade of mitosis). In addition, it showed an increase in the sub-G_0 region, indicative of apoptosis (Fig. 3 D). Treatment with TC ethanolic extract failed to show a specific block in cell cycle, however, triggered apoptosis as reflected by an increase in sub-G_0 DNA content (Fig. 3 E). Table 2 summarizes the effects of WS and TC ethanolic extracts on G_0/G_1 phase, Synthetic (S) phase, G_2/M phase and sub-G_0 phase. Thus, these results revealed that the ethanolic extracts of TC and WS induced apoptosis in breast cancer cell lines, while the WS extracts additionally caused G_2/M arrest.

Table 2. Effects of ethanolic extracts of *Withania somnifera* (WS) and *Tinospora cordifolia* (TC) extracts on human breast cancer cell cycle.

Groups	**MCF7**				**MDA MB 231**			
	Sub G_0 phase	**G_o/G_1 phase**	**S phase**	**G_2/M phase**	**Sub G_0 phase**	**G_o/G_1 phase**	**S phase**	**G_2/M phase**
Control	0.70±0.04	60.76±4.34	16.92±1.72	22.64±1.80	1.06±0.08	64.75±5.60	16.00±0.96	19.50±1.50
DMSO	1.90±0.10	57.38±5.38	16.76±2.13	25.20±2.45	0.62±0.10	62.72±4.80	14.37±0.77	23.57±2.56
Doxorubicin	30.46±2.07 ***	34.90±3.24	10.73±0.98	25.14±2.96	62.14±4.80 ***	14.17±1.23	11.96±1.23	12.83±0.76
WS ethanolic extract	26.93±1.61 ***	31.60±4.25	7.67±0.12	34.71±3.23 ***	48.28±3.78 ***	15.06±1.73	5.14±0.05	32.06±3.45 **
TC ethanolic extract	33.54±1.60 ***	43.14±3.30	8.67±0.85	15.77±1.45	23.86±2.78 ***	43.40±2.80	11.43±0.85	22.62±1.56

Numerical data are means ± S.D of three independent experiments (Figure 3). The data indicates statistically significant difference from the values for the control cells exposed to vehicle control (***P<0.001, **P<0.01); determined by two way ANOVA using Bonferroni posttests.

Apoptotic effects of ethanolic extracts of WS and TC in human immortalized, but 'non-cancerous' cell line. We further evaluated the effects of the ethanolic extracts of WS and TC on the induction of apoptosis in human immortalized, but 'non-cancerous' cell line (HaCaT) at the concentrations that were cytotoxic to human breast cancer cells. Briefly, HaCaT cells were treated with WS ethanolic extract (22.0 µg/mL), TC ethanolic extract (84.0 µg/mL), and doxorubicin (1.25 µM) for 48 h. At the end of the treatment, cells were harvested and subjected to Hoechst staining assay as well as cell cycle analyses for measuring apoptosis. Results indicated that as before, doxorubicin showed nuclear condensation indicating the induction of apoptosis (Fig. 4 A). However, WS and TC ethanolic extracts failed to induce apoptosis in HaCaT cells (Fig. 4 A) at these concentrations. Similarly, cell cycle analysis showed an increased sub-G_0 content in doxorubicin treated cells (Fig. 4 B), but treatment with WS and TC ethanolic extracts did not significantly alter the sub-G_0 content and no effect was detected on the cell cycle profile (Fig. 4 B). Table 3 summarizes the effects of WS and TC ethanolic extracts on sub-G_0 content, G_0/G_1 phase, Synthetic (S) phase, and G_2/M phase in both MCF7 and HaCaT. Together, these results revealed that the ethanolic extracts of WC and TS extracts failed to induce apoptosis in 'non-cancerous' cells at the concentrations that were cytotoxic to human breast cancer cells.

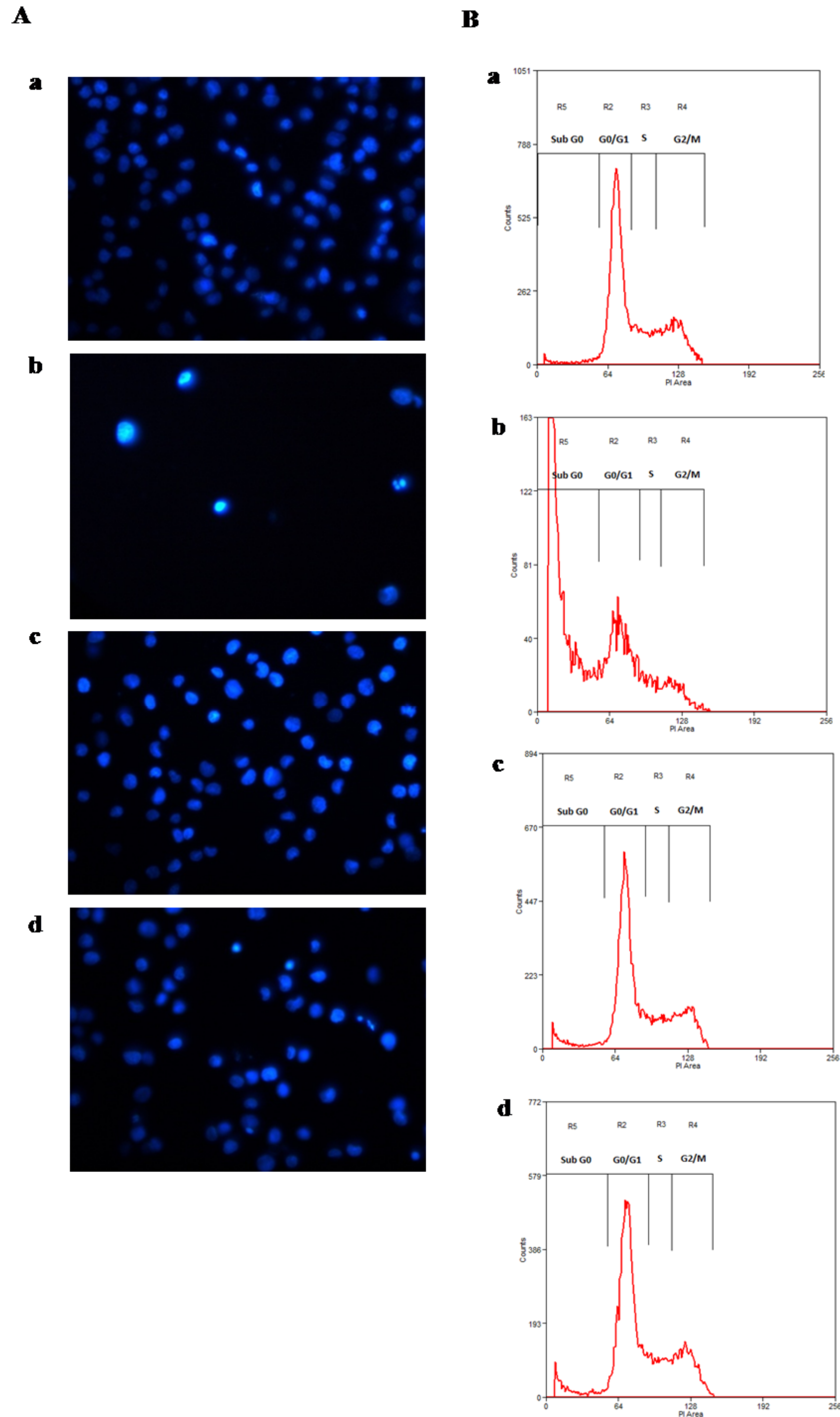


Figure 4. Effects of ethanolic extracts of *Withania somnifera* (WS) and *Tinospora cordifolia* (TC) on human immortalized, but 'non-cancerous' cell line (HaCaT).

Cells were treated with DMSO (a), doxorubicin, 1.25 µM (b), WS ethanolic extract, 22.0 µg/mL (c), and TC ethanolic extract, 84.0 µg/mL (d) at the concentrations that were cytotoxic to human breast cancer cells (MCF7) for 48 h. **A**. Hoechst 333442 assay using fluorescence microscopy revealed condensed and fragmented nuclei upon treatment with doxorubicin indicate apoptosis. However, ethanolic extracts of WS and TC failed to induce apoptosis. **B**. Cell cycle analysis by flow cytometry revealed that WS and TC ethanolic extracts did not significantly alter the sub-G_0 content and no effect was detected on the cell cycle profile. The data are representative of three independent experiments.

Table 3. Effects of ethanolic extracts of *Withania somnifera* (WS) and *Tinospora cordifolia* (TC) on the cell cycle progression in human immortalized, but 'non-cancerous' cell line (HaCaT) in comparison with human breast cancer cells (MCF7).

Groups	MCF7				HaCaT			
	Sub G_0 phase	G_o/G_1 phase	S phase	G_2/M phase	Sub G_0 phase	G_o/G_1 phase	S phase	G_2/M phase
DMSO	1.90±0.10	57.38±5.38	16.76±2.13	25.20±2.45	3.63±0.05	54.87±3.22	16.68±0.96	26.44±1.40
Doxorubicin	30.46±2.07 ***	34.90±3.24	10.73±0.98	25.14±2.96	63.19±3.54 ***	24.12±1.45	6.31±0.20	7.35±0.63
WS ethanolic extract	26.93±1.61 ***	31.60±4.25	7.67±0.12	34.71±3.23 ***	6.24±0.72	58.76±2.90	14.43±0.56	21.47±1.70
TC ethanolic extract	33.54±1.60 ***	43.14±3.30	8.67±0.85	15.77±1.45	6.56±0.65	58.63±3.11	15.07±0.88	21.24±2.80

Numerical data are means ± S.D of three independent experiments (Figure 4 B). The data indicates statistically significant difference from the values for the control cells exposed to vehicle control (***P<0.001); determined by two way ANOVA using Bonferroni posttests.

DISCUSSION

The current study investigated the anti-cancer activities of extracts of indigenous medicinal plants, *Withania somnifera* (WS) and *Tinospora cordifolia* (TC), against human breast cancer cells (MCF7 and MDA MB 231). Cervical cancer cell line (HeLa) was used as experimental control in this study. A methodical evaluation of cytotoxicity effects revealed that the ethanolic extracts of both WS and TC showed dose-dependent cytotoxicity activity against MCF7, MDA MB 231, and HeLa cells. However, aqueous extracts of both WS and TC did not possess any cytotoxic effect. The IC_{50} values of ethanolic extracts of WS and TC were found to be less than 100 µg/mL indicating potent cytotoxic effects in breast cancer cells and further potential of these extracts for the isolation of biologically active phytochemicals. WS ethanolic extract was more active (IC_{50} <40 µg/mL) as compared to TC ethanolic extract (IC_{50} <90 µg/mL).

Most anti-cancer drugs are designed to eliminate rapidly proliferating cancerous cells, and therefore, they typically show cytotoxicity and induce apoptosis in cancer cells (Kaufmann & Earnshaw 2000). 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). The current study also investigated the induction of apoptosis in breast cancer cells upon treatment with ethanolic extracts of WS and TC. Acridine orange-ethidium bromide assay and Hoechst 33342 assay by fluorescent microscopy revealed that the ethanolic extracts of WS and TC induced apoptosis, but not necrosis, in breast cancer cells. DNA fragmentation is a hall mark property of apoptosis (Bortner et al., 1995) and DNA fragmentation assay further corroborated the ethanolic extracts of WS and TC induced apoptosis in breast cancer cells.

Several anti-cancer drugs are known to cause their effects by blocking cell cycle (Stewart et al., 2003). Cancer cell cycle specific drugs has drawn considerable attention as they act on specific cancer cell cycle checkpoints (G_o/G_1 phase, S phase, G_2/M phase) and inhibit cancer cell proliferation (G_o/G_1 phase arrest), or DNA replication (diminished S phase) or mitosis (G_2/M phase arrest). Additionally, Sub-G_o DNA content in cells indicates apoptosis (Nunez 2001). We investigated the cell cycle specific pharmacological effects of WS and TC ethanolic extracts against human breast cancer cells by propidium iodide based cell cycle analysis using flow cytometry. The data revealed that WS ethanolic extract caused a significant arrest of cells at G_2/M phase (G_2/M arrest) and increased sub-G_0 phase indicating induction of apoptosis. These data suggest that WS ethanolic extract act by cell cycle-specific mechanism inducing mitotic arrest and apoptosis in breast cancer cells. However, TC ethanolic extracts caused significantly increased sub-G_0 phase indicating induction of apoptosis but without altering the cell cycle.

Anti-cancer drugs with minimal side effects on normal cells are highly desirable for therapeutic purposes (Buolamwini 1999). Hence, the current study also addressed the question of whether WS and TC ethanolic extract-mediated suppression of cell viability was selectively to cancer cells. Cytotoxicity study against human immortalized, but 'non-cancerous' cell line (HaCaT) indicated that the ethanolic extracts of WS and TC possess less cytotoxic activity against 'non-cancerous' cells. Agents that are capable of inducing selective apoptosis of cancer cells, without causing much harm to normal cells, have received considerable interest in the development of novel cancer chemotherapeutic drugs (Cotter 2009). We found that concentrations of ethanolic extracts of WS and TC that were cytotoxic to human breast cancer MCF7 and MDA MB 231 cells were failed to induce apoptosis in HaCaT cells. In contrast, doxorubicin, a conventionally used anticancer drug was found to have cytototoxic and apoptotic effects in 'non-cancerous' as well as in cancer cells at the same concentrations. These results suggest that ethanolic extracts of WS and TC possess anti-cancer activities in human breast cancer cells with less cytotoxic effects against human 'non-cancerous' cells. Thus, these extracts can be evaluated further for potential anti-cancer properties and isolation of bio-active phytochemicals.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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