Potential Role of *Withania somnifera* on Human Breast Cancer

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Abstract: Background: One of the most versatile plants used in the traditional Indian medicine system (Ayurveda) is *Withania somnifera* (Ashwaghandha). Western research supports its polypharmaceutical use as antioxidant, immune-modulating, antistress, a geriatric tonic and in traditional remedies for inflammations and improve overall physical and mental health. The aim of this study is to evaluate the effect of *Withania somnifera* on breast cancer cell line. .**Material & Methods**: The McF7 cells treated by different doses of W.S. root extract. The viability and cytotoxicity were measured by Trypane blue and MTT assay. The flow cytometery was used for cell cycle analysis and the follow up of morphological change was observed by inverted microscope. The DNA fragmentation test was done. **Results & Discussion:** The present study has demonstrated that W.S. can inhibit the proliferation of cancer cells *in vitro*. .. The results showed that W.S inhibited the proliferation of MCF7 at high concentration rich to 50% with IC_{50} 0.86%. The flow cytometeric analysis showed changes in cell cycle phases in treated cells with IC_{50} dose compared with control untreated cells. The morphology of cells is changed according to dose uptake. DNA damaged was measured by DNA fragmentation test and results showed sever damage of treated cells when compared with control untreated cells. From these results we concluded that W.S can inhibit the proliferation and have cytotoxic effect on human cancer cells through its selective bioactivity.
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1. Introduction

Cancer has become an important topic in medicine since it is a major cause of death after myocardial infarction **(Gruddy , 1991)**. Cancer of the breast is one of the most common human neoplasm's, accounting for one quarter of all cancers in females **(Bombardieri , et al, 2008)**. It is the leading cause of cancer related death for women aged between 35 and 55 years worldwide. One in nine women will suffer from breast cancer each year **(Hickey, et al, 2009)**. In the modern era of breast cancer treatment, rapid progress has led to a broad spectrum of therapeutic strategies for improving clinical outcomes **(Min-Ho et al, 2011)**. The mainstay of breast cancer treatment is surgery when the tumor is localized, followed by chemotherapy (when indicated), radiotherapy and adjuvant hormonal therapy for ER positive tumors (with tamoxifen or an aromatase inhibitor). Management of breast cancer is undertaken by a multidisciplinary team based on national and international guidelines. Depending on clinical criteria (age, type of cancer, size, metastasis) patients are roughly divided to high risk and low risk cases, with each risk category following different rules for therapy **(Kelly et al, 2008)**. Herbal medicines are plant-derived products which have been used as traditional folk medicine and food additives **(Miller, et al, 2008)**. Recently their medicinal properties are under extensive investigation and become a major part of complementary and alternative medicines (CAMs) **(Esmonde & Long , 2008)**. Their potency for treating different diseases has been reported including cancer, allergy and diabetes **(Längler , et al, 2008)**. One of the most versatile plants used in the traditional Indian medicine system (Ayurveda) is *Withania somnifera* (Ashwaghandha) **(Jayaprakasam, et al, 2003)**. *Withania somnifera* (L.) Dunal (common name– Ashwaghandha, family–Solanaceae) is an Indian medicinal plant that grows as an evergreen shrub in dry parts of India. It is classified in the ancient Indian system of medicine (Ayurveda) as a rasayana, a group of plant derived drugs that improve overall physical and mental health and put off diseases by rejuvenating the body in incapacitated conditions. It is highly reputed as 'Indian ginseng' and is a member of generally regarded as safe (GRAS) plants **(Mishra et al, 2000)**. It is used as a geriatric tonic and in traditional remedies for inflammations, conjunctivitis and tuberculosis. The root extract of W. somnifera has been shown to have health promoting effects such as anti-stress, anti-arthiritic, anti-inflammatory, analgesic, anti-pyretic, anti-oxidant and immunomodulatory properties (**al-Hindawi *et al.*, 1992 ; Davis etal, 1997; Agarwal *et al.*, 1999; Namasivayam, 1999; Scartezzini and Speroni, 2000; Dhuley, 2000;; Mishra *et al.*, 2000; Prakash *et al.*, 2002; Kuttan, 2002 and Gupta *et al.*, 2003)**

Methanolic root extracts of W. somnifera includes a variety of withanolides and was shown to induce nitric oxide synthase expression that could

account, at least in part, for its immunostimulant properties **(Iuvone *et al.*, 2003).** In this study we try to evaluate the role of W.S on breast cancer.

2. Material and Methods:

Cell line:

Human breast cancer cell line (MCF7) preserved and passage in NCI, Cairo, Egypt laboratory. Cells were cultivated in RPMI-1640 culture medium containing 10% fetal bovine serum, and penicillin / streptomycin at 37°C in a 5% Co_2 incubator.

Extract preparation:

Withania Somnifera **(**Egyptian Ashwaghandha**),** roots were harvested from Rafah, El-Arish, North Sinai, Egypt in September 2008.

Dry powder of Ashwaghandha roots was prepared by suspending 10 g of dry powder in 100 ml of distilled water and stirring it overnight at 45±5 °C, followed by filtration under sterile conditions. The filtrate thus obtained was treated as 100% W.S. It was stored at –20 °C in 1 ml aliquots until further use.

Trypane blue count:

Cells were cultured in 24-well plates and incubated for 24hrs. Cells treated with different concentrations of tested compound then incubated for 24hr and the viability was examined using trypan blue dye.

%Viability=(No. of viable cells/ total no. of cells) X 100

Percent related to control= Treated/ Control X100

Microscopic examination:

Cells were cultured in 6-well plates and incubated for 24hrs then Ashwaghandha extract was added and incubated for 24hrs. The treated cells were examined under inverted microscope and morphological changes were observed. Cells were photographed using digital camera.

MTT assay:

Cells were inoculated onto 96-well plates at the density of $0.5X10^5$ cells/well for 24hrs, then treated with various concentrations of extract (0.6, 0.7, 0.8, 0.9 and 1%), and incubated for 24hrs. then 10ul/well MTT reagent was added to each well, and cells were incubated for an additional 2-4 hrs at 37°C. the supernatant was aspirated and 100ul of DMSO was added to the wells to dissolve any precipitate present. The absorbance was then measured at 570nm by ELISA reader.

Percentage of relative viability was calculated using the following equation:

[Absorbance of treated cells / Absorbance of control cells)] X 100

Then the half maximal inhibitory concentration IC_{50} was calculated by the prism program.

Microscopic Examination:

DNA Fragmentation assay:

MCF7 cells were cultured in T-25 flasks at 75% confluence they were treated with different extract concentrations (0, 0.5, 1, 5 and 10 %) and incubated for 48hrs then cells were harvested with trypsin and their DNA was extracted and loaded in agarose gel and allowed to run.

Cell cycle analysis:

MCF7 cells (about 60% confluent) were treated with indicated doses of extract for 24 h. After the treatments, cells were harvested with trypsin and prepared for cell cycle analysis. Cells were collected in 1.5 ml tube, washed with cold PBS and were then fixed with 70% ethanol at 4 °C. The fixed cells were centrifuged (2000 rpm for 10 min), washed with cold PBS twice and then re-suspended in 0.25 ml PBS. The cell suspensions were stained with 10 ul of 1 mg/ml Propidium Iodide (PI) for 30 min in dark. To avoid false DNA-PI staining, RNA was removed by adding 5 ul of 1 mg/ml RNase- A at 37 °C for 1 h in the cells suspension before PI staining. After staining with PI, the cells were applied for cycle analysis using Coulter Epics XLTM Flow Cytometer (Beckman).

3. Results:

Trypan blue exclusion test:

Table (1): Effect of W.S on MCF7 cells, count after 24 hr. Count of cells using Trypane blue dye.

Conc. (%)	Dead	%Viability	Percent related to control
0	4.5	95.5	-
0.6	15	85	89
0.7	19.5	80.5	84.29
0.8	30	70	73.29
0.9	45	55	57.59
1	54.5	47.5	49.73

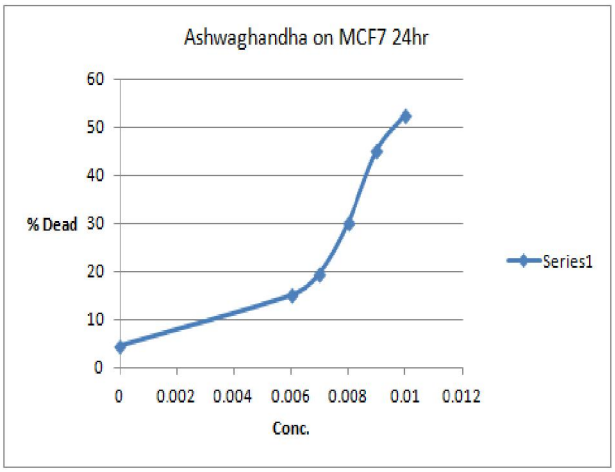

Figure (1): Curve showing relation between percent of dead cells and concentration of W.S extract.

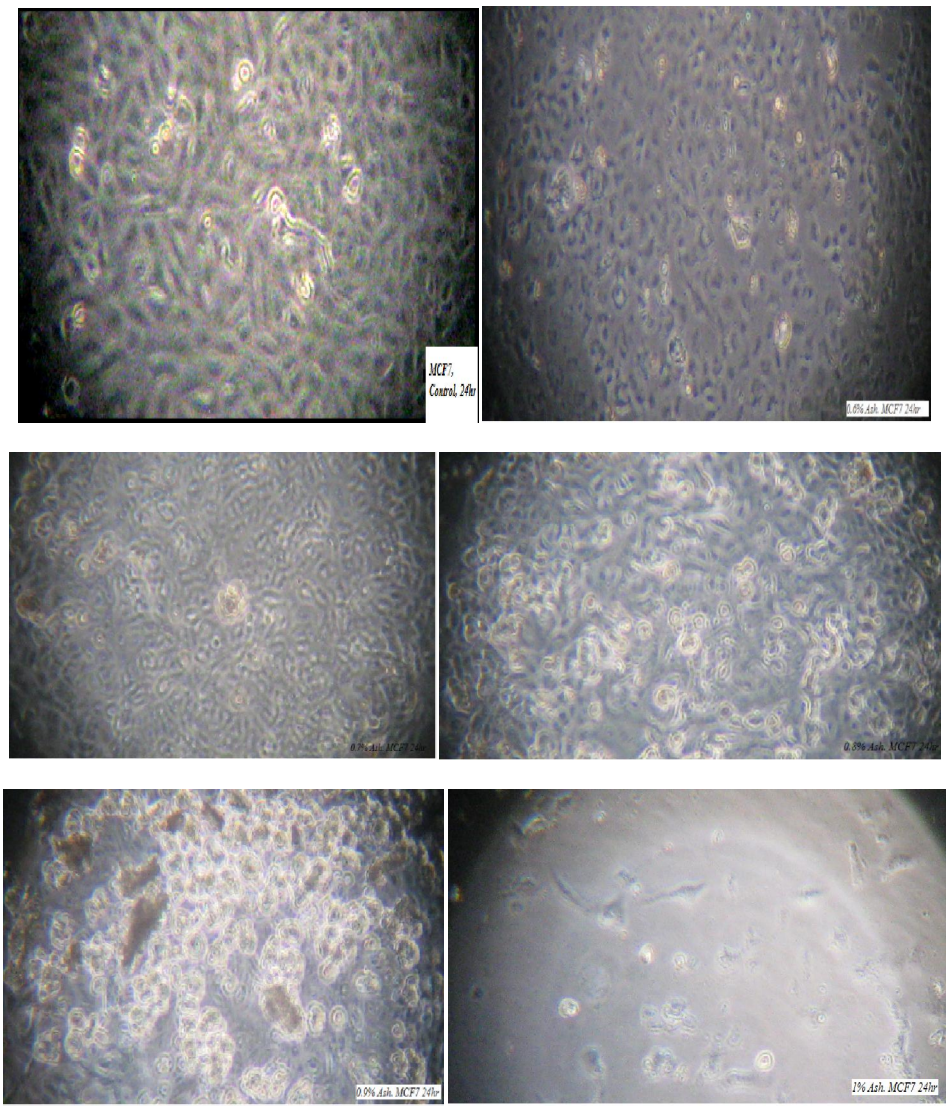

Figure (2): Photos showing morphological changes caused by W.S root water extract on MCF7 cells after 24hrs incubation.

Table (2): Notes observed during microscopic examination of effect of W.S on MCF7 after 24hr incubation.

Concentration	Observation
Control	Over growth of cells.
0.6%	Cells are slightly affected, there is no over-sheet
0.7%	Morphology of cells is highly affected and changed, cells seems to be killed by apoptosis. Cells are very small, destructed membrane, and irregular shape of cells.
0.8%	Cells are completely detached and killed.
0.9%	More than 60% of cells are completely detached and killed (floated cells) and attached ones their membranes are highly destructed.
1%	Almost all cells were degenerated and undergo apoptosis

MTT assay:

Table (3): Absorbance of W.S on MCF7, showing IC50 value. Calculated by prism program.

Conc.%	Abs.
0	1.661
0.6	1.798
0.7	1.469
0.8	0.948
0.9	0.8
1	1.001
IC50	0.86%

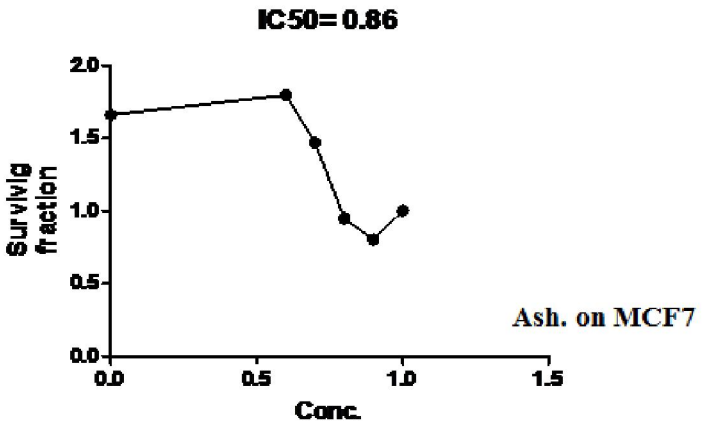

Figure (3): Curve showing IC50 value of W.S on MCF7 cells.

DNA Fragmentation:

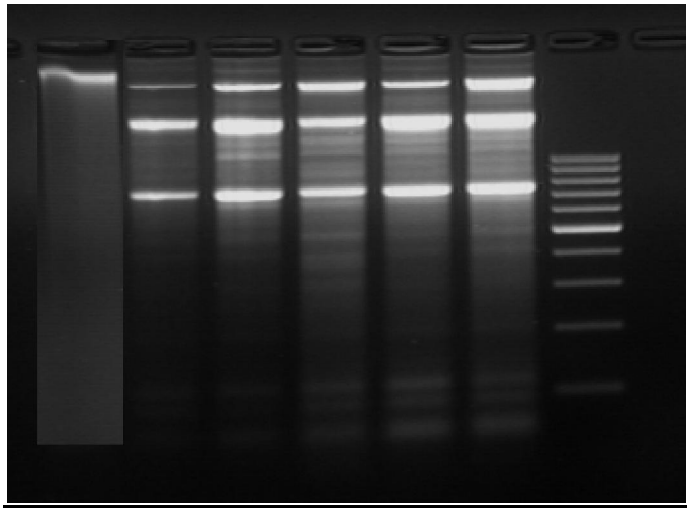

Figure (4): Untreated control, serum free control, 0.5%, 1%, 5%, 10% and ladder.

Cell cycle analysis:

To investigate further the nature of growth inhibition by WS McF7 cells, flow cytometric analysis was performed. The growing cells were treated with IC_{50} value 0.9% for intervals (24h), and then subjected to flow cytometric analysis after staining their DNA. The distribution of cells in different phases of the cycle was illustrated in **Fig. (5)**. The untreated cells showed the expected pattern for continuously growing cells, whereas the cells treated with ashwaghandha extract showed a progressive accumulation in the G2/M phase of the cell cycle correlating with decreased number of cells in the S phase.

Table (4): Cell cycle analysis of MCF7 cells.

Conc. %	%G_0-G1	%G2-M	%S-Phase	%G2/G1
0	46.37	9.37	44.26	1.94
0.9	49.53	13.74	36.74	1.95

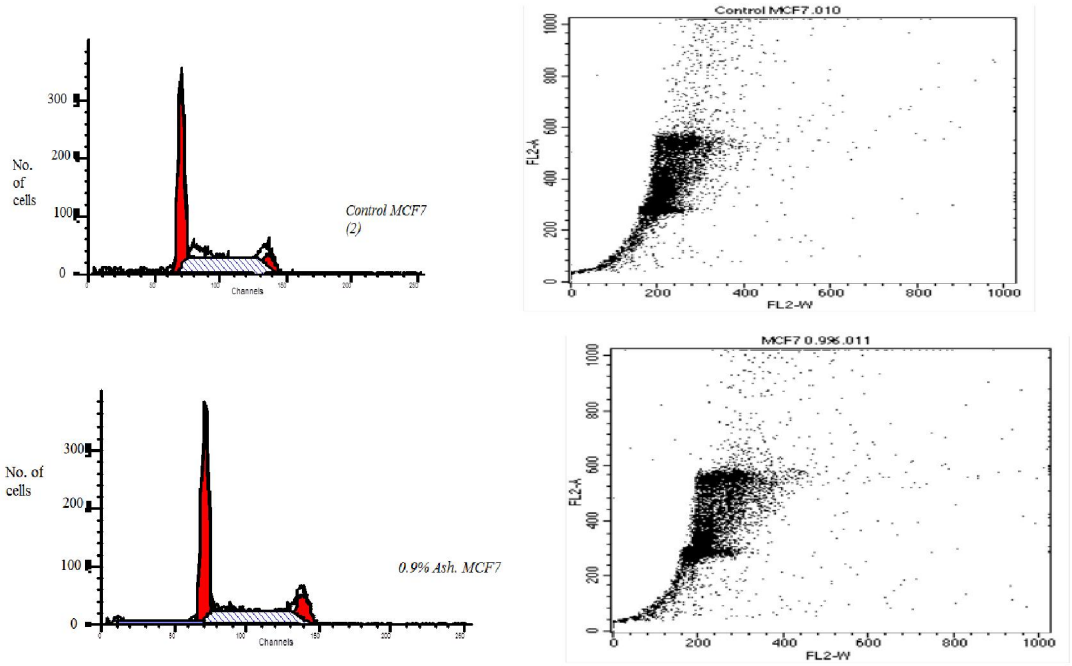

Figure (5): Stages of cell cycle analysis and dot plot of MCF7 cell.

4. Discussion:

The plant *Withania somnifera* is a well known herbal medicine used in parts of the world. It has anti-inflammatory, antioxidant and antitumor as well as neural protective properties **(Pretorius *et al.*, 2009)**. The current research evaluated the cytotoxic potential effect of WS and tries to detect its mechanism of action .The trypan blue test measures cell number and viability in the presence of 5 concentrations and the data were recorded on **(Table 1& Fig 1)**. The viability was decreased with increased concentration of compounded used in a dose dependent manner. Our result was agreed with Pretorius (2009) who reported that cell viability was decreased when using the high concentration of WS. The result of the MTT assay showed strong cytotoxic effect on McF7 cells with an IC50 of about 0.8 % **(Table 3 & Fig 3)** in a study by (**Kaur *et al.*,** 2004). Osteogenic sarcoma and breast carcinoma cell lines were treated with different concentrations of WS extract. Cells showed reduced proliferation compared with controls and assumed morphology more closely related to senescent cells, tested WS for their anti-proliferative capabilities on lung, colon, central nervous system (CNS) and breast tumor cell lines. A dose dependent anti-proliferative was observed with lung cells showing the greatest sensitivity and colon cell line showing the greatest resistance to treatment. Withania antitumor mechanisms are most likely multi-factorial. WS exhibits both antioxidant and pro-oxidant activities. It show comparable inhibition of nuclear factor kappa B (NFkB) target genes (involved in inflammation , angiogenesis, cell cycle , metastasis , apoptosis and multidrug resistance WS may also mitigate unregulated cell growth via the potent tumor suppressor gene P53,whichregulates cell cycle proliferation (**Mathur *et***

al.,) our study revealed that WS produce a progressive accumulation in the G_2/M phase of the cell cycle correlating with decreased number of cells in the S phase **(Table 4 & Fig 5)**. Similar result was reported by (**Singh *et al. 2001)*** who showed that WS root extract of down regulate the expression of P53cdc2, a cell cycle regulatory protein. This protein is expressed during cellular proliferation, and down regulation arrests the cell cycle in the G2/ M transition phase. WS has also been investigated on cell DNA damage .The result revealed that high concentration of WS extract produced DNA fragmentation in treated cells when compared with untreated control cells **(Fig 4)**. Cancer cells contain multiple signal transduction pathways whose activities are frequently increased due to cell transformation, and these pathways are often activated following cell exposure to established cytotoxic therapies, including ionizing radiation and chemical DNA- damaging agents. Many pathways activated in response to transformation or cytotoxic agents promote cell growth and invasion, which counteract the processes of cell death. As a result of these finding, many drugs with varying specifities have been developed to block the signaling by these survival pathways in the hope of killing tumor cells and sensitizing them to toxic therapy (**Kawasaki *et al., 2008***) From this strategies of different mechanisms of cancer therapy we try to identify how this natural product WS exert its effect on cancer cells and from our finding we concluded that WS may be beneficial to retard progression of tumor through different mechanisms.

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