WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 6.647
Volume 6, Issue 9, 933-940 Research Article ISSN 2278 – 4357

IN-VITRO ANTICANCER ACTIVITY EVALUATION OF *WITHANIA COAGULANS* ON BREAST CANCER CELL LINES

Gopinath S. M.[1], Ismail Shareef M.[2*], Sonia Gupta[3] and Ayushi Gupta[3]

[1]Professor & Head of Department, Department of Biotechnology, Acharya Institute of Technology, Bangalore.

[2]Assistant Professor, Department of Biotechnology, Acharya Institute of Technology, Bangalore.

[3]Research Scholar, Department of Biotechnology, Acharya Institute of Technology, Bangalore.

Article Received on
22 June 2017,
Revised on 13 July 2017,
Accepted on 03 August 2017
DOI: 10.20959/wjpps20179-9899

***Corresponding Author**
Ismail Shareef M.
Assistant Professor, Department of Biotechnology, Acharya Institute of Technology, Bangalore.

ABSTRACT

Breast cancer has become issue of major concern because of its increasing prevalence worldwide. Synthetic chemotherapeutic agents fail to treat it as tumor cells tend to develop resistance against the Plant derived chemotherapeutic agents offers a high potential to combat the situation. Though much research is done on this field, but still many plants are yet to be explored for their anti carcinogenic properties against breast cancer. In the present study we analyzed the ethanol extract of *Withania coagulans* for its cytotoxic and hemolytic properties. Cell cycle studies shows that MCF7 cells when subjected to 320µg/ml of plant extract was able to arrest 25.99% of MCF7 cells at G2M phase. Also, the plant extract is non hemolytic and thus, can be prove to of high potential against breast cancer.

KEYWORDS: Adenocarcinoma, withnaolides, caspase cascade, FACS.

INTRODUCTION

Unlike normal cells, cancer cells lose their control on proliferation and apoptosis. Hence, tend to multiply invariably. Uncontrolled proliferation of cells of ducts or glandular lobules of breast leads to development of breast cancer. According to American cancer society, breast

cancer will be detected in approximately 252,710 American women in the year 2017. (American Cancer Society, 2017).

Synthetic chemotherapeutic agents not only cause several side effects but fail to kill cancer cells when these cells develop resistance against them. Since time memorial ayurveda has blessed us with the numerous number of plant derived therapeutic agents to heal several diseases. Several plants derived anti cancer agents like taxol(Sparano *et al.,* 2008), vinblastine, vincristine and the camptothecin (Hsiang *et al.,* 1985) derivatives are actively used clinically worldwide. But still the many of the potential plants have been left unexplored. Much research is being conducted to find the promising phytomedicine that not only lacks the toxic side effects but also highly efficient.

Withania coagulans which is commonly known as Indian rennet is branched shrub that belongs to solanaceae family and is popularly known for its milk coagulating properties (Naz *et al.,* 2009). Along with steroids like dihydrostigmasterol and β-sitosterol and sugars like maltose, galactose and arabinose (Gupta & Kesari, 2013), it holds steroidal lactones called withanolides like coagulin F, Coagulin G, coagulanolides, withacoagulin. (Gupta, 2012) The berry extract of the plant has hypoglycemic effect and is also used to treat high blood pressure due to its diuretic nature. Withacoagulins are reported to induce apoptosis in tumor B-cell, HL-60 leukemia cells by mitochondria mediated cyctochrome c release and thereby activating caspase cascade. (Senthil *et al.,* 2007).

Though, this plant shows its cytotoxic effect on the B-cells, we have very less information about their effect on the human breast adenocarcinoma. In the present study, we have extracted metabolites from the berry of *Withania coagulans* and have evaluated its cytotoxic and apoptogenic effect on breast cancer cell lines, MCF7 by various biochemical and flow cytometry assays. Also, we have analyzed if the plant extract also induces lysis of erythrocytes by hemolysis assay.

MATERIAL AND METHODS

Preparation of Plant Extract

Beeries of Withania coagulans were collected from Amruth Kesari, Bangalore. It was then shade dried for almost 5 days and milled into fine powder. For ethanol extraction, 10 g of powdered material was dissolved in 50 ml of ethanol and kept on hot water bath at 50º C for 4 hours. The extract was filtered through Whatman No.1 filter paper and the filtrate was kept

in water bath at 80 ºC for few hours until it get into semisolid. The stock solution of extract was prepared in DMSO at 320 mg/ml concentration and was kept on hot water bath at 60 ºC for 1 hour for proper dissolution of the pellet.

Cell Culture

Michigan Cancer Foundation-7 (MCF7) cell line obtained from the American Tissue Culture Collection (ATCC) was cultured in the Dulbecco's Modified Eagle's Medium (DMEM) media (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS), 1000 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a 5% CO_2 humidified incubator.

Thawing and Revival

Cryo-vials containing the frozen cells from liquid nitrogen storage were quickly thawed (< 1 minute) by gently swirling the vial in the 37°C water bath. Thawed cells were transferred to a sterile tube containing required amount of medium corresponding to the cell lines and inverted for uniform distribution. The cell suspension was centrifuged at 1200g for 5 minutes. Clear supernatant was checked for visibility of the complete pellet, re suspended in complete growth medium and transferred to T-25 flaskunder the recommended culture environment (5% CO2 at 37o C). Growth was monitored and cells were trypsinized and sub cultured once they reached a confluence of 70-80%.

Anti carcinogenic Activity of Plant Extract

MTT Assay

MCF7 cells (5.0 X 10^4) were plated in 96 well plates with different concentrations of plant extracts made in complete DMEM media (0, 10, 20, 40, 80, 160 and 320 µg/ml) and incubated for 24 hours at 37˚C in a 5% CO_2 incubator. Then, media was removed and 100 µl of MTT reagent was added to each well and incubated again for 4 hours. MTT reagent was removed and 100 µL DMSO was added to each well for solubilizing the formazan product. The plate was shaken well and absorbance reading was taken at 570nm using microplate reader. The percentage inhibition was determined using a formula [% Inhibition = 100-(optical density of sample/optical density of control) × 100]. IC50 values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell.

Hemolysis Assay

5ml blood was collected from the healthy person in a vial containing 5.4 mg of EDTA. Erythrocytes were collected by centrifuging the blood at 1000 rpm for 10 mins at 4°C. Plasma and white buffy layer was discarded and erythrocytes were washed with 1×PBS thrice, pH 7.4. In 50µl of the 10 times diluted RBCs, 100 µl of 160 and 320 µg/ml dilution of each plant extract was added in separate eppendrof tubes. For positive and negative control, 1% SDS solution and 1×PBS was added respectively. They were then incubated for 1 hour in incubated shaker at 37°C. After incubation, the volumes of reaction mixture were adjusted to 1 ml using 1XPBS. Finally, centrifuged at 3000 rpm for 3 min and the resulting haemoglobin in supernatant was measured at 540 nm by Tecan micro plate reader and determined the concentration of haemoglobin using Magellan- data analysis software. The haemolysis caused by 100 µl of 1% SDS was taken as 100 % haemolysis and the percentage haemolysis was calculated [% Haemolysis = [(control – sample)/ control] * 100].

G2M phase cell cycle study

MCF7 cells (1×10^6) were seeded in 6 well plate with 160 µg/ml and 320 µg/ml concentration of each plant extract and one well as control with 1% DMSO. The plate was incubated for 24 hours at 37˚C in a 5% CO^2 incubator. Next day cells were collected in individual vial by trpsinization and centrifuged to get cell pellet at 1500rpm for 5 minutes. After washing the cell pellet with 1×PBS, 1 ml of 70% ethanol was added slowly while continuous stirring and another 1 ml was added directly to it. The cells were kept for fixing at 4°C overnight. Next day pellet was collected by centrifugation and washed twice by cold 1× PBS. To cell pellet 500µl of propidium iodide solution (0.05 mg/ml PI and 0.05 mg/ml RNase A in PBS) was added and cell at various stages were recorded using FACS Caliber (BD Biosciences, San Jose, CA).

Apoptotic study

After seeding about 1×10^6 cells in 6 well plate, next day cells were treated with 160 and 320µg/ml concentration of each plant extract and incubated at 37°C in CO2 incubator overnight. Cells were harvested by trypsinization and cells was washed twice by cold 1×PBS. Cell pellet was resuspended in 1× PBS at a concentration of ~1 x 10^6 cells/mL. To the 100 µL of cells (~1 x 10^5 cells) transferred to a 5-mL FACS tube, 5 µL Annexin V and 5 µL PI was added, gently mixed and incubated for 15 minutes at RT in the dark. 400 µL of 1X Binding Buffer to each tube and analyze by using FACS Caliber (BD Biosciences, San Jose, CA).

RESULTS AND DISCUSSION

The cell cytotoxic activity of Withania coagulans berry extract was analyzed by MTT Assay whose % inhibition reading are mentioned in Table 1.

Table 1: Cell Viability on MCF7 cell lines.

Conc. (µg/mL)	Dilution	OD	% Inhibition
0	-	1.190	0
10	1:32	1.113	7.97
20	1:16	0.953	13.89
40	1:8	0.866	26.26
80	1:4	0.733	33.00
160	1:2	0.631	43.29
320	Neat	1.190	51.16

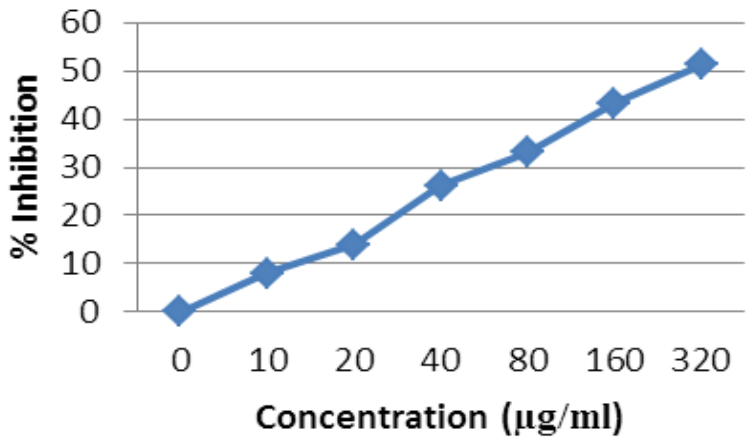


Figure 1:% inhibition of berry extract on MCF7 cell lines.

Based on the analysis of % inhibition by treatment with the different concentration of *Withania coagulans* berry extract, Inhibitory concentration 50 value was calculated as 296.4168µg/ml.

In order to check whether berry extract induce lysis of normal erythrocyctes, hemolysis assay was performed whose % hemolysis reading were plotted against the different concentration the plant extract.

Conc.(µg/mL)	Absorbance	% Haemolysis
PBS	0.79	0.00
1%SDS	0.16	80.26
160	0.79	1.01
320	0.70	11.85

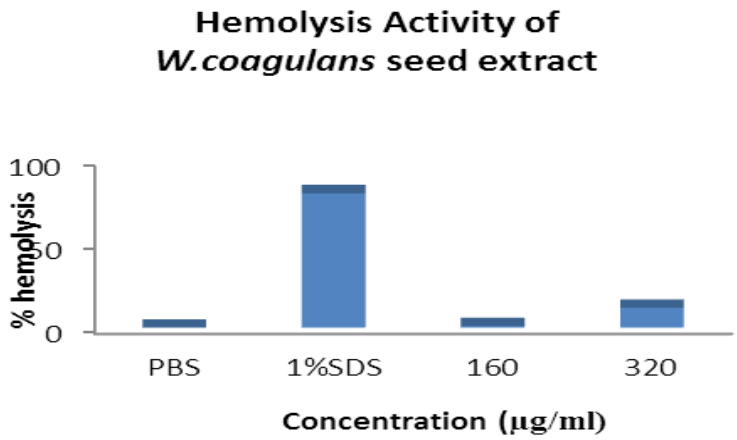
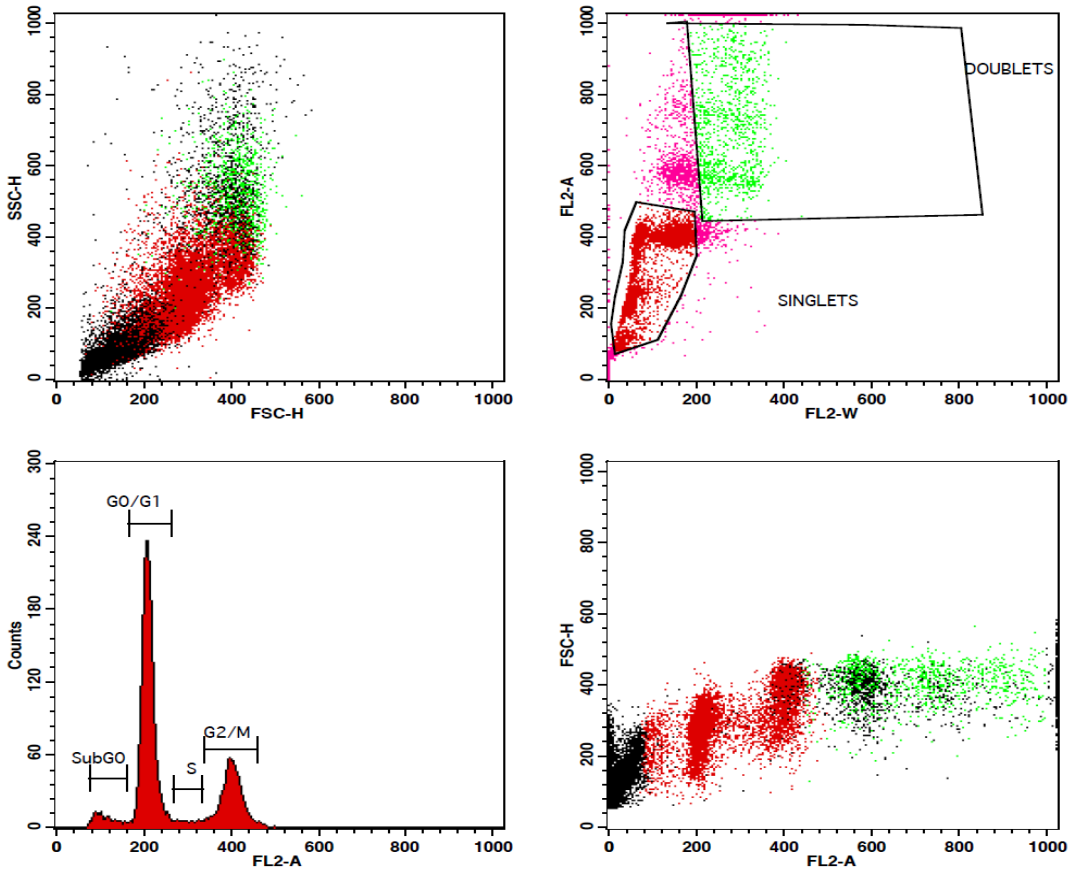


Figure 2: Hemolysis Assay OD.

Based on the results of MTT Assay and Hemolysis assay, it was found that the plant extract is cytotoxic to MCF7 cancer cell lines and does not affect the normal erythrocytes.

Thus, G2M phase cell cycle studies and Annexin V based apoptotic assay were performed whose results are shown in Figure 1 and 2 respectively.

Fig 1 shows incubation of MCF-7 cells with 320 µg/ml of berry extract for 24 h significantly arrest 25.99% of cells at G2M phase of cell cycle. 65.63% of cells were in G0/G1 phase while 78.10% of cells were in the same phase in the control cells. Fig 2 shows the annexin V based apoptotic studies of the MCF7 cells treated with *W. coagulans* berry extract. At concentration of 160 and 320 µg/ml, W. coagulans has induced apoptosis in 3.64% and 9.36% of the MCF7 cells respectively as compared to untreated MCF7 control cells of only 0.24% cells.



Histogram Statistics

Marker	Left, Right	Events	%Gated	%Total	Mean	CV
All	0, 1023	10000	100.00	44.33	257.93	35.35
SubG0	77, 163	496	4.96	2.20	111.72	19.71
G0/G1	165, 264	6563	65.63	29.10	209.96	6.62
S	268, 335	267	2.67	1.18	301.56	6.74
G2/M	340, 460	2599	25.99	11.52	399.39	5.51

Figure 1: Flow cytometry plots of MCF7 cells treated with 320 µg/ml of *W.coagulans* berry extract.

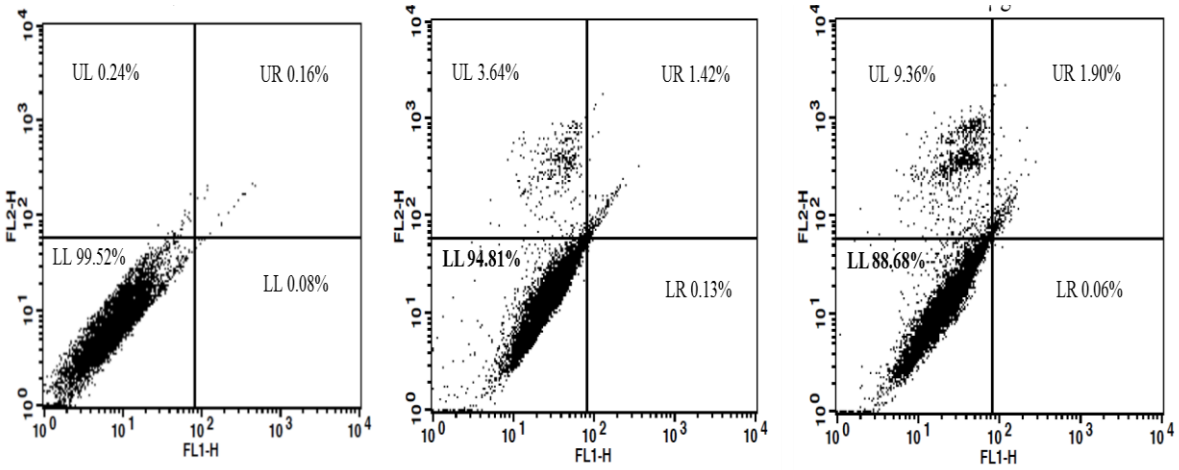


Figure 3: Control plot of MCF-7 cells and plots of cells treated with 160µg/mL and 320µg/mL of sample W. coagulans.

CONCLUSION

The IC 50 value calculated by MTT assay is found to be 296.4µg/ml which is high. Also, the G2M phase cell cycle studies and the apoptotic studies do not show significant cancer inhibition activity. Thus, it does not show any potential to treat breast cancer.

ACKNOWLEDGEMENT

We thank our colleagues from Skanda Life Sciences Pvt. Ltd. who provided insight and expertise that greatly assisted the research.

REFERENCES

1. American Cancer Society. (2017). *Cancer Facts & Figures, 2017.* Atlanta: American Cancer Society.
2. Gupta Vandana, Bipin Bihari Keshari. (2013). Withania coagulans (Stocks) Dunal (Paneer Doda): A review. *International Journal Of Ayurvedic And Herbal Medicine, 3*(5): 1330-36.
3. Gupta, Prakash Chandra. (2012). Withania Coagulans Dunal- An Overview. *International Journal of Pharmaceutical Sciences Review and Research, 12*(2): 68-71.
4. Hsiang YH, Hertzberg R, Hecht S, Liu LF. (1985). Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *The Journal of Biological Chemistry, 260*(27): 14873-8.
5. Naz S, Masud T, Nawaz MA. (2009). Characterization of milk coagulating properties from the extract of Withania coagulans. *International Journal of Dairy Technology, 62*(3): 315-20.
6. Senthil V, Ramadevi S, Venkatakrishnan V, Giridharan P, Lakshmi BS, Vishwakarma RA, Balakrishnan A. (2007). Withanolide induces apoptosis in HL-60 leukemia cells via mitochondria mediated cytochrome c release and caspase activation, *167*(1): 19-30.
7. Sparano JA, Wang M, Martino S, Jones V, Perez EA, Saphner T, Wolff AC, Sledge GW Jr, Wood WC, Davidson NE. (2008). Weekly paclitaxel in the adjuvant treatment of breast cancer. *The New England Journal of Medicine, 358*(16): 1663-71.