Chelonian Conservation And Biology

Vol. 19 No. 1 (2024) | https://www.acgpublishing.com/ | ISSN - 1071-8443

DOI: doi.org/10.18011/2024.01(1).632.643

ASSESS THE APOPTOTIC EFFECT OF CRUDE EXTRACT OF ROOT POWDER OF *WITHANIA SOMNIFERA* IN BREAST CANCER CELL LINE

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Abstract

Background: Cancer is one of the greatest health challenges in the developing countries. About half (51%) of cancers occurred in developing countries in 1975. In 2018, this proportion was 55% in 2018 and will projected to reach 61% in 2050. Notably, breast cancer alone contributes to 30% of female cancer cases. It was evident that bioactive component (withaferin –A) of root of *Withania somnifera* has capacity to work against the breast cancer. In this present study, the crude methanolic extract of root of *Withania somnifera* screened for in vitro apoptotic activity on MCF-7 and MDA-MB-231 cell line. In Ayurveda, *Withania somnifera* is commonly known as Ashwagandha, its roots are specifically used in medicinal and clinical applications. It possesses numerous therapeutic actions which include anti-inflammatory, anticancer, sedative, hypnotic and narcotic.

Objectives: To Assess the apoptotic activity of crude extract of root of *Withania somnifera* on breast cancer cell line.

Materials and Methods: MDA-MB-231 and MCF-7 breast cancer cell lines, DMEM complete media, 10% fetal bovine serum (FBS), MEM nonessential amino acids, gentamicin and 10µg/mL insulin. MCF-7 and MDA-MB-231 cells were treated with different concentration of sample (crude extract of root powder of *Withania somnifera* for 24 hrs incubation. Cytotoxicity was measured by MTT assay. The morphological change of untreated (Control) and treated cells were observed under digital inverted microscope and photographed.

Results: The lower concentration of sample show less cytotoxic activity on both cell line MCF-7 and MDA-MB-231. Higher concentration of sample show more cytotoxic activity on both cell line MCF-7 and MDA-MB-231.The IC_{50} value of crude extract of root powder of *Withania somnifera* against both MCF-7 and MDA-MB-231 was determined.

Conclusions: The crude methanolic extract of root of *Withania somnifera* have anticancer potential but additional studies required.

Keywords: Apoptosis, *Withania somnifer*, cancer, MCF-7, MDA-MB-231, withaferin A.

1. Introduction

Cancer **c**ounts modelled at various levels, including national and state, were combined yearly, and a time series projection technique (vector auto regression) was utilized across the 15-year span to forecast cancer cases for the year 2020. Projected cancer-related fatalities in 2020 were determined by estimating the annual percent change in reported cancer deaths from 2003 to 2017, utilizing join point analysis at state and national levels, as reported to the National Center for Health Statistics (NCHS). For a comprehensive understanding of this methodology, please consult [1].

Among women, the three most prevalent cancers are breast, lung, and colorectal, collectively representing half of all new diagnoses. Notably, breast cancer alone contributes to 30% of female cancer cases [2]. The gender gap in cancer incidence exhibits age-dependent variations. In childhood (ages birth -14 years), incidence is about 10% higher in males compared to females (18.2 vs. 16.4 per 100,000 population). Conversely, during early adulthood (ages 20-49 years), the incidence is notably 77% higher in females (203.4 vs. 114.9 per 100,000 population), primarily attributable to the incidence of breast cancer in young women [2]. The marginal increase in breast cancer incidence rates (around 0.3% per year) since 2004 is linked, in part, to ongoing declines in fertility rates and rising obesity. These factors are also associated with the sustained rise in incidence for uterine corpus cancer, showing a yearly increase of 1.3% from 2007 to 2016 [2]. Breast cancer stands as a significant global health concern, impacting the lives of numerous women across the world. In 2020, over 40,000 women in the United States were anticipated to succumb to the challenges posed by breast cancer [3].There remains a crucial need for innovative therapeutic and preventive approaches to reduce both mortality and the burden of suffering associated with this disease. Ongoing research explores extract from medicinal plants or their small- molecule constituents as potential novel strategies for the therapy and/or chemoprevention of breast cancer.

Withania somnifera, commonly known as winter cherry or ashwagandha and belonging to the solanaceae family, is a compelling medicinal plant currently undergoing rigorous investigation for its potential impact on cancer and various other health conditions. The root/leaf extract of *Withania somnifera* remains a key component in the formulation of Ayurveda, Siddha and Unani medicine practices, prevalent in India and neighbouring countries[4][5][6][7](Tandon N, 2020)(Saggam A, Tillu G, Dixit S, Chavan-Gautam P, Borse S, Joshi K, 2020) [10]. ClinicalTrials.gov lists over 15 ongoing clinical trials utilising *Withania somnifera* extract for diverse medical conditions[3].

Studies have explored the clinical effects of *Withania somnifera* extract, examining its potential in managing male reproductive functions, neuroprotection, alleviation of stress and anxiety, enhancement of memory and cognitive functions, muscle strength, and recovery, among other areas[10][11] [12][13](Shohat B, Gitter S, Abraham A,1967). *Withania somnifera* extract is accessible without a prescription in the United States, offered as a dietary supplement over the counter. The phytochemical composition of *Withania somnifera* extract is notably diverse, evident in the presence of withanolides, alkaloids, and sitoindosides [15].While the anticancer potential of each identified chemical component in *Withania somnifera* extract is still under investigation, withaferin A, member of the withanolide family, has been the subject of extensive research for its anti- cancer effects across various types, notably in breast cancer (Shohat B, Gitter S, Abraham A, 1967)[17][18][19][20][21][22][23].

2. Material and methods

2.1 Reagents

MDA-MB-231 and MCF-7 cells were obtained from NCCS, Pune. DMEM complete media supplemented with 10% foetal bovine serum (FBS), MEM nonessential amino acids, gentamicin Gibco, Life Technologies. 10µg/mL insulin obtained from Sigma-Aldrich. 0.25% (w/v) Trypsin-0.53 mM EDTA solution were obtained from GeminiBio. MTT were obtained from Himedia. withaferin A was obtained from Cayman chemicals.

2.2 Plant materials

The dried root of ashwagandha (*Withania somnifera*) plant was collected from Harberium (Government Ayurvedic college and hospital), Patna, Bihar. Dried root of ashwagandha was minced and grinded with a mechanical grinder (Hanil Co. Seoul, South Korea) into a mesh size 120 mm and stored at 4 °C, until further analysis. The samples were authenticated for their correct botanical identity by the professor of the Govt. ayurvedic college and hospital, patna, bihar.

2.3. Sample preparation

50 g of powdered ashwagandha sample extracted with 500 ml of 80% methanol for 5–10 h using Soxhlet extraction apparatus, at 60°C. An extraction time of 7 h was taken as optimum by mass yield. Further, the obtained extract was filtered and concentrated to dryness with subsequent evaporation of alcoholic content using Rota vapour (temperature, 50 °C), further the obtained extract was lyophilized until a constant weight was obtained and stored at 4 °C until used for further studies[24].

2.4. Biological evaluation

2.4.1. Cell lines

The two cell lines MDA-MB-231 and MCF-7 were obtained from National Centre for Cell Science, Pune, India. These two cell lines were maintained in Genetic toxicology laboratory, Whizbang bioresearch pvt Ltd., Avadi, Chennai as per the standard protocol.

2.4.2. Cell line culture

MDA-MB-231 and MCF-7 breast cancer cell lines were grown adherently and maintained in DMEM complete media supplemented with 10% fetal bovine serum (FBS), MEM nonessential amino acids, gentamicin and 10µg/mL insulin in a 5% CO_2 and 95% air incubator at 37°C. Media was changed every 2–3 days and cells were passaged at 65–80% reached confluency.

2.4.3. Trypsinization

Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. Add 1.0 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 minutes). Cells that are difficult to detach can be placed at 37°C to facilitate dispersal. Add 2.0-3.0 mL of complete growth medium and aspirate cells by gently pipetting. Transfer the cell suspension to the centrifuge tube with the medium and centrifuge at 1000 rpm for 5-10 minutes. Discard the supernatant. Resuspend the cell pellet in the fresh growth medium. Add appropriate aliquots of the cell suspension to well plates.

2.4.4. Morphological study

Cells were observed and photographed for morphological characteristics under digital inverted microscope.

3. Cytotoxicity assays

The cytotoxicity effect of the sample was tested against MCF-7 cell line and MDA-MB-231 cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

3.1. MTT Assay

The cells were seeded in 96-well microplates ($1x10^6$ cells/well) and incubated at 37^0C for 48 hrs. in 5% CO_2 incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the calls were treated with different concentration of samples and incubated for 24hrs. The morphological changes of untreated (Control) and the treated cell were observed under digital inverted microscope (40X magnification) after 24 hrs. and photographed. The cell was then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 ul of (MTT) solution (5mg/mL in PBS) was added to each well. The plates were then stand at 37^0C in dark for 2 hrs. The formazan crystals were dissolved in 100ul DMSO and the absorbance was read spectrophotometrically at 570 nm.

3.2. Evaluation of cytotoxicity and cell viability

Treated and control wells were subjected to MTT assay. MDA-MB-231 cells and MCF-7 cell were seeded at concentration $1x10^6$cells/100ul were seeded in 96-well microtiter tissue culture plates. Make different concentration of sample (crude extract of root powder of *Withania somnifera*) 31.25, 62.5, 125, 250, 500 ug/ml. The well-used as blank contain only medium. The well-used as positive control contain standard withaferin A (500ug/ml). The both cells MDA-MB-231 and MCF-7 were incubated at 37ºC for 48 hrs. in 5% CO2 incubator and observe till confluence reached 70-80%. Replace the medium and treat the both cells with different concentration of sample. Again incubate for 24hrs. The cells were then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 ul of (MTT) solution (5mg/mL in PBS) was added to each well. The plates were then stand at

Concentration (ug/ml)	**Absorbance**		**Average**	**Cell Viability (%)**	**Inhibition (%)**
	I	II			
Control	0.745	0.761	0.753	100	0
31.25	0.685	0.697	0.691	91.766	8.234
62.5	0.58	0.561	0.571	75.830	24.170
125	0.31	0.301	0.306	40.637	59.363
250	0.183	0.196	0.190	25.232	74.768
500	0.097	0.082	0.090	11.886	88.114
Withaferin A 500ug/ml	0.074	0.069	0.072	9.562	90.438

Table 1. % of cell viability and % of inhibition on MCF-7 according to the different dose of sample.

Concentration (ug/ml)	**Absorbance**		**Average**	**Cell Viability (%)**	**Inhibition (%)**
	I	II			
Control	0.745	0.758	0.752	100	0
31.25	0.722	0.731	0.727	96.676	3.324
62.5	0.686	0.692	0.689	91.622	8.378
125	0.616	0.627	0.622	82.713	17.287
250	0.520	0.503	0.512	68.085	31.915
500	0.260	0.271	0.266	35.372	64.628
Withaferin A	0.200	0.205	0.203	26.995	73.005

500ug/ml					

Table 2. % of cell viability and % of inhibition on MDA-MB-231 according to the different dose of sample.

37^0C in dark for 2 hrs. The formazan crystals were dissolved in 100ul DMSO and the absorbance was read spectrophotometrically at 570 nm. Results were interpreted as percentage cell viability obtained by plotting absorbance against the different concentration of the extract in ug/ml as depicted in table 1 and table 2.

The percentage of cell viability was calculated as –

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100

The IC_{50} values of the extracts were determined from the plot as the concentration which decreased the cell viability by 50%.

3.3 Comparison of the cytotoxic activity of sample on two breast cancer cell lines

In dose dependent manner different concentration of sample (crude extract of root powder of *Withania somnifera*) were treated on breast cancer cell lines MDA-MB-231 and MCF-7 using MTT assay as described above. Compare the result of sample on both breast cancer cell line. MDA-MB-231 cells and MCF-7 cells were seeded at concentration $1x10^6$cells/100ul in 96-well microtiter tissue culture plates. Total five test concentration of sample 31.25, 62.5, 125, 250, 500 ug/ml were treated with both cell lines (MDA-MB-231 and MCF-7) found to be cytotoxic on the breast cancer cell lines (MDA-MB-231 and MCF-7) by MTT assay. Results were observed, recorded and interpreted as discussed.

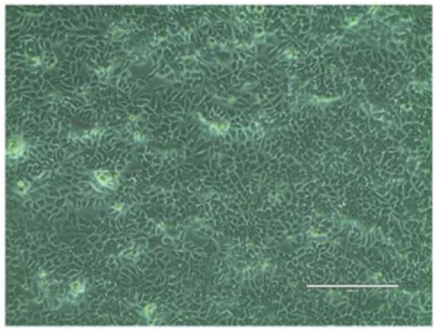
4. Result

Methanolic crude extract of root powder of *Withania somnifera* treated on the different concentration of breast cancer cells. Different concentration of sample 31.25, 62.5, 125, 250, 500 ug/ml treated with MCF-7 cell lines. The same concentration of sample was treated with MDA-MB-231 cell lines. It was found that at low concentration of sample the percentage viability on breast cancer cell lines was high and inhibition percentage was low. At highest concentration of sample, the percentage viability on breast cancer cell lines was low and percentage of inhibition was high. In control cell viability was 100% and inhibition was 0% in both MCF-7 and MDA-MB-231 breast cancer cell line. At concentration of 31.25, 62.5, 125, 250, 500 ug/ml of sample the percentage cell viability was 91.766, 75.830, 40.637, 25.232, 11.886 on MCF-7 cell lines (table 1). At the same concentration the percentage cell viability was 96.676, 91.622, 82.713, 68.085, 35.372 on MDA-MB-231 cell lines (table 2). At concentration of 31.25, 62.5, 125, 250, 500 ug/ml of sample the percentage inhibition was 8.234, 24.170, 59.363, 74.768, 88.114 on MCF-7 cell lines

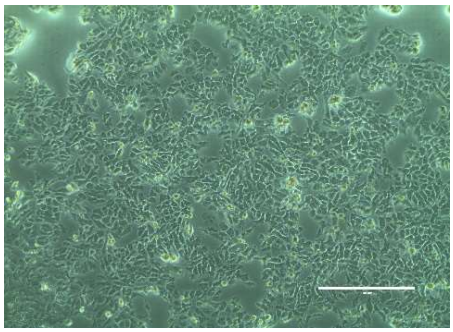
(table 1). At the same concentration the percentage inhibition was 3.324, 8.378, 17.287, 31.915,64.628 respectively on MDA-MB-231 cell lines (table 2). IC_{50} value 79.361 on MDA-MB-231 as compared to IC_{50} value 187.801 on MCF-7 was found.

After completion of reaction of MTT assay absorbance were taken at 570nm. The mean absorbance reading for all concentration of (31.25, 62.5, 125, 250, 500) ug/ml of the sample was 0.691, 0.571, 0.306, 0.190, 0.090 respectively on MCF-7 cell lines (table 1). The mean absorbance reading for all concentration of (31.25, 62.5, 125, 250, 500) ug/ml of the sample was 0.727, 0.689, 0.622,0.512,0.266 respectively on MDA-MB-231 cell lines (table 2). The absorbance value of control on MCF-7(table 1) and MDA-MB-231(table 2) cell lines was 0.753 and 0.752 respectively.

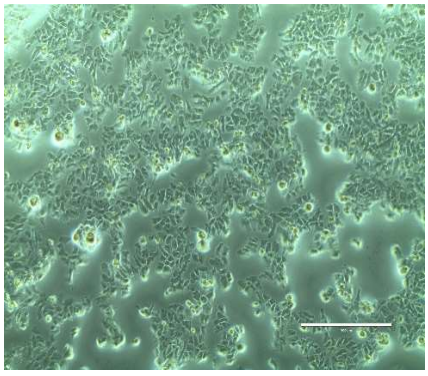
Morphological analysis of different concentration (31.25, 62.5, 125, 250, 500) ug/ml of the sample (crude extract of root powder of *Withania somnifera*) on MCF-7 has been depicted in figure 1 and on MDA-MB-231 has been depicted in figure 2.



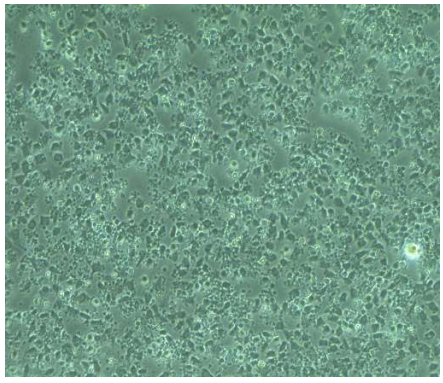
A. Morphological image of control cells



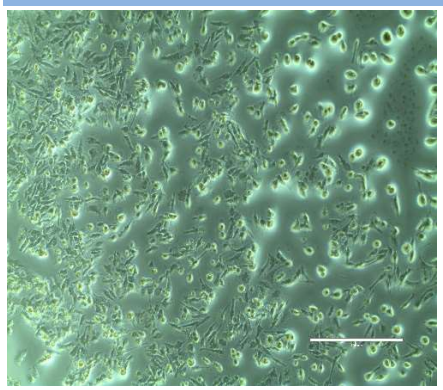
A. Morphological change at concentration 31.25 ug/ml



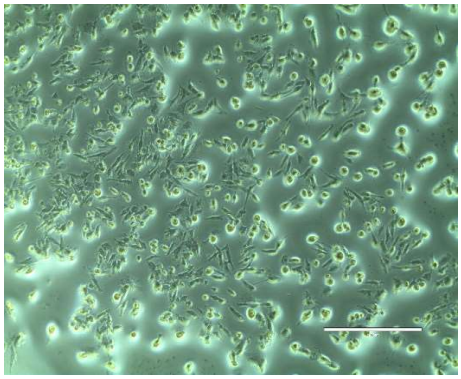
B. Morphological change at concentration 62.5 ug/ml



C. Morphological change at concentration 125 ug/ml

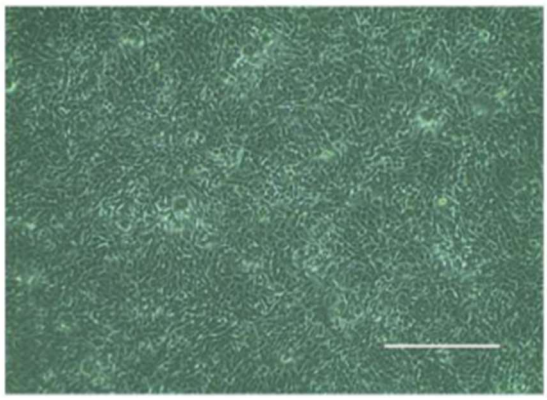


D. Morphological change at concentration 250 ug/ml

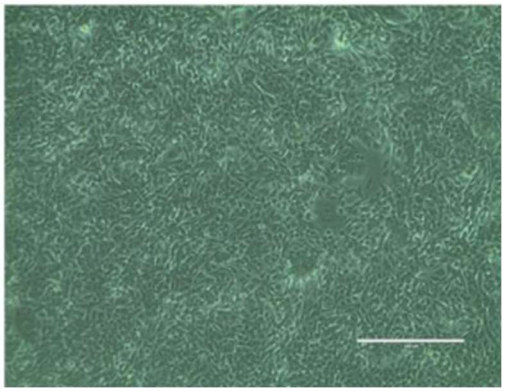


E. Morphological change at concentration 500 ug/ml

Figure 1. Morphological change of MCF-7 cells at different concentration of methanol extracted root powder of Withania somnifera. A. Morphological change at concentration of plant extract at 13.25, B.Morphological change at concentration of plant extract at 62.5, C.Morphological change at concentration of plant extract at 125, D.Morphological change at concentration of plant extract at 250, E.Morphological change at concentration of plant extract at 500, F.Morphological change at concentration of 500 withaferin A (standard).



A. Morphology of control cell



B. Morphological change at concentration 31.25

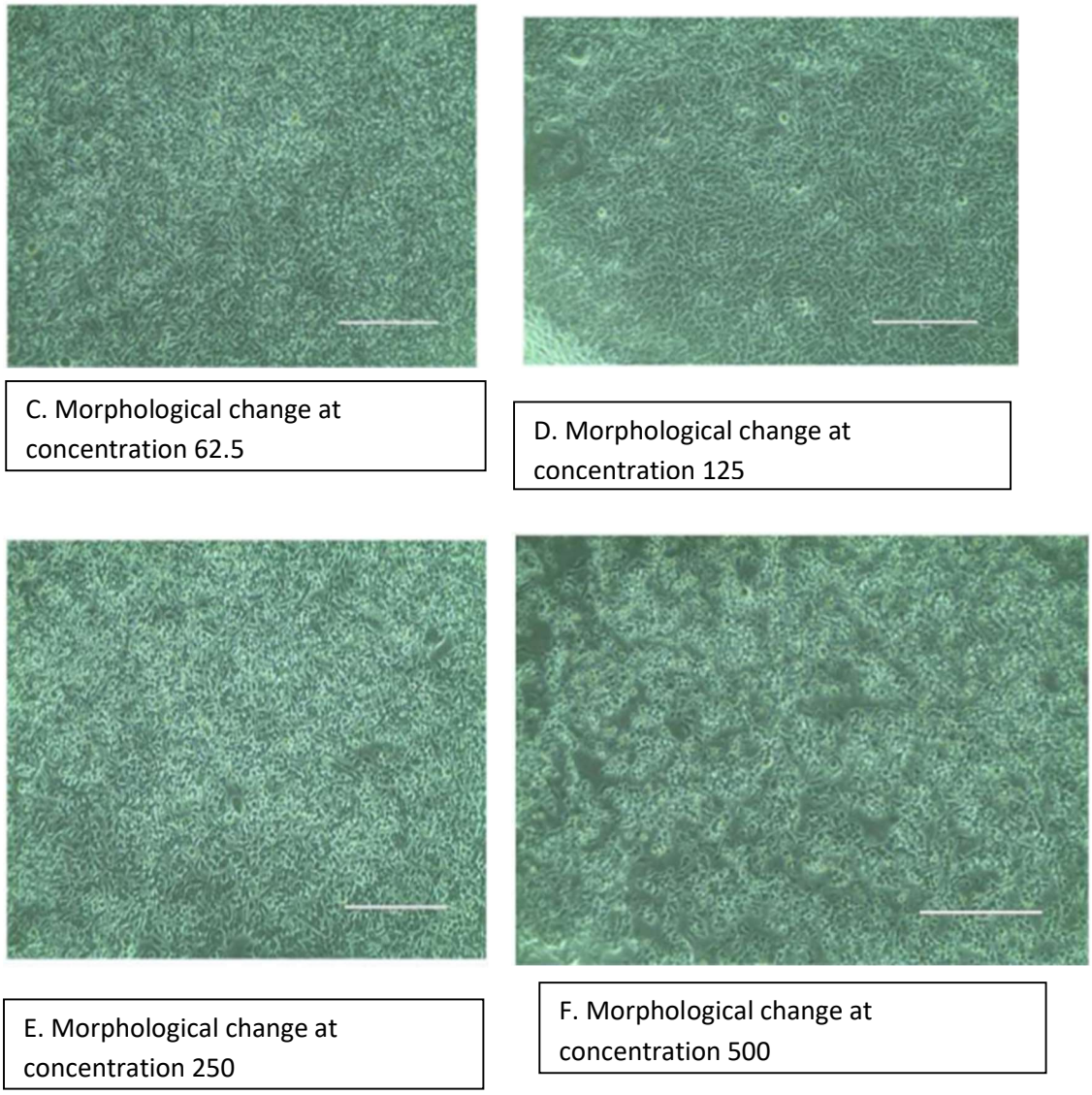


Figure 2. Morphological change of MDAMB-231 cells at different concentration of methanol extracted root powder of *Withania somnifera*. A. Morphological image of control cells. B. Morphological change at concentration of plant extract at 31.25ug/ml, C. Morphological change at concentration of plant extract at 62.5ug/ml. D. Morphological change at concentration of plant extract at 125ug/ml, E. Morphological change at concentration of plant extract at 250ug/ml, F. Morphological change at concentration of plant extract at 500ug/ml.

5. Discussion

Herbal medicines have several benefits over synthetic chemotherapeutic drugs, especially since the former seriously impair normal cellular homeostasis while the latter frequently provide therapeutic interventions with little to no clinical significance. Any therapeutic approach should therefore try to selectively target cancerous or tumour cells while causing the least amount of

damage to healthy cells. Because of their safety, potency, and usefulness, compounds derived from both natural and synthetic products have gained popularity and preference in the field of cancer therapy in recent years [23]. These compounds also show good promise as anticancer agents.

Numerous epidemiological studies have demonstrated that the majority of vegans and vegetarians have lower cancer incidence rates [24]. Therefore, the primary method of treating tumours is now evaluating plant extracts for the existence and identification of anticancer chemicals based on their potency and therapeutic index. Due to the fact that plant-based medicines have few or no negative effects, about 80% of medicines used today for a variety of health conditions are derived from plants. Cytotoxic activity of methanolic extract of *Withania somnifera*, was found to possess an IC_{50} value 79.361 on MDA-MB-231 as compared to IC_{50} value 187.801 on MCF-7. It means the methanolic extract of root powder of *Withania somnifera* kills MDA-MB-237 cells at low concentration as compare to MCF-7 because the IC_{50} value of MCF-7 was found to be high.

6. Conclusion

The methanolic extract of root powder of *Withania somnifera* can be used to formulate novel drugs in future or might be used as adjunct/complementary therapy given along with the main line of treatment. The results from the MTT assay indicate that 24hr incubation with methanolic extract of root powder of *Withania somnifera* is toxic to the both cell lines MDA-MB-231 and MCF-7. The level of damage is concentration dependent. Advanced studies on this plant would unravel the detailed mechanism behind its anticancer and apoptotic activity.

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