

Research Article

Determination of Combined Effect of pH and *Withania somnifera* on Human Breast Cancer Cell Line MDA-MB-231

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Background: The intratumor microenvironment is intrinsically acidic due to accumulation of lactic acid as a result of increased aerobic and anaerobic glycolysis by the tumor cells. Generally, the extracellular pH (pH_e) in human tumors is below 7.0, whereas the intracellular pH (pH_i) is maintained at neutral range, i.e., >7.0 , by powerful pH_i control mechanisms. The low pH_e and the significant gradients between pH_e and pH_i affect markedly the response of tumors to various treatments such as chemotherapy, radiotherapy and hyperthermia.

Methods: In the present study, the effect of alkaline pH on human breast cancer cell line MDA-MB-231 was investigated in presence of methanolic extract of *Withania somnifera* (ashwagandha) in 10% DMSO at the rate of 50 μ g/ml.

Results: It was found that alkaline pH caused an increase in the cytotoxic effect of *Withania somnifera* on MDA-MB-231 cells by approximately ten folds.

Conclusion: The alkaline pH_e caused an increase in the cellular uptake of *Withania* extract and thus increased its overall cytotoxicity. Better understanding of the control mechanisms of pH_e and pH_i in tumors may lead to device effective treatment strategy of human tumors.

Keywords: pH; Cancer therapy; *in-vitro*; MDA-MB-231; Breast cancer; *Withania somnifera*

Introduction

The extracellular pH (pH_e) of malignant solid tumors is acidic, in the range of 6.5 to 6.9, whereas the pH_e of normal tissues is significantly more alkaline, in the range of 7.2 to 7.5 [1-3]. Mathematical models of the tumor-host interface [4] and *in vivo* measurements have shown that solid tumors export acid into the surrounding parenchyma [5,6]. Previous *in vitro* studies have shown that tumor cell invasion can be stimulated by acidic conditions and that this may involve lysosomal proteases [7-9]. These observations have led to the "acid-mediated invasion hypothesis", wherein tumor-derived acid facilitates tumor invasion by promoting normal cell death and extracellular matrix degradation of the parenchyma surrounding growing tumors. Furthermore, pretreatment of tumor cells with acid before injection leads to increased experimental metastases [10,11] and these observations suggest that low pH upregulates pro-invasive and survival pathways. It has been argued that metastatic cancers are selected for their ability to export acid [12]. Acid is a by-product of glucose metabolism and notably, elevated consumption of fluorodeoxyglucose by more aggressive cancers has been observed with fluorodeoxyglucose positron emission tomography [13]. It has been reported previously by our group [14] that alkaline pH of medium inhibits the growth of cancer cells *in vitro*. The present study attempts to investigate whether alkalinity increment of the medium can improve the effect of cytotoxic drugs (*W. somnifera*) on cancer cells *in vitro*. Cytotoxic effect of methanolic extract of

stem of *W. somnifera*, a medicinal plant commonly used in herbal pharmaceuticals and nutraceuticals, was evaluated in presence of varying alkaline pH using cytometry and cytotoxic assays against MDA-MB-231 cell line.

Materials and Methods

Collection of plant material

Fresh stems of *Withania somnifera* Dunal were collected and dried under the shade and then blended into fine powder with a grinder.

Sample preparation

In separate experiments, 25g of powder was extracted with 50% methanol (1:8). After 24 hrs, the upper layer of solvent was collected in a beaker and the procedure was repeated thrice in the interval of 24 hrs continuously till the color of solvent disappeared. All extracts from a single source were pooled together and filtered using Whatman No.1 filter paper (125 mm). The filtered extracts were concentrated at 100°C in water bath. The semi solid paste formed was transferred to a Petri plate and kept in hot air oven till it attained a powdered form. The total weight of powder was measured and stored in air tight container for further use. For biological studies, 20 mg of methanolic extract was dissolved in 10% DMSO (Calbiochem) at a concentration of 20 mg/ml. The extracts were passed through 0.22 μ m sterile Millipore syringe filter units (Fisher Scientific) prior to being used in cell culture studies.

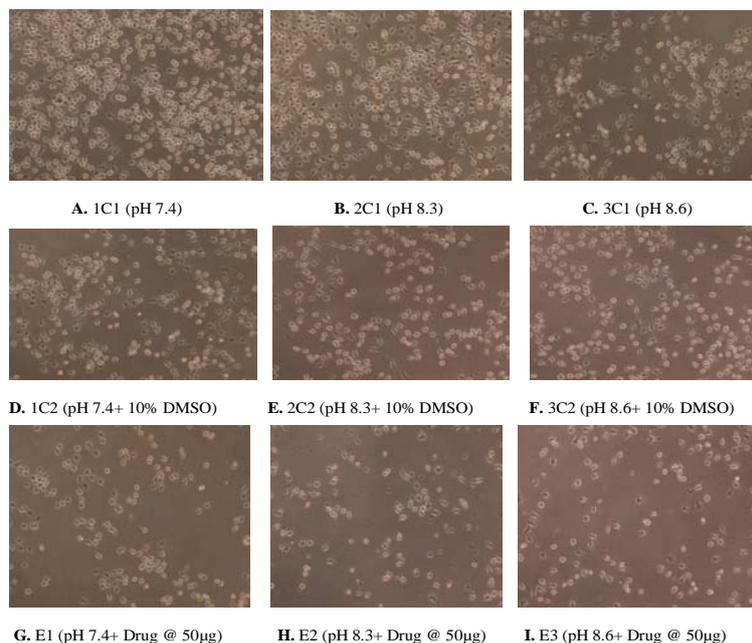


Figure 1: (A) Control showing untreated MDA human breast cancer cells maintained at pH 7.4, (B) pH 8.3 (C) pH 8.6 (D) Control showing untreated MDA human breast cancer cells in presence of 10% DMSO at pH 7.4 (E) 10% DMSO at pH 8.3 (F) 10% DMSO at pH 8.6 (G) Cytotoxic activity of *W. somnifera* methanolic extract at 50 µg/ml at pH 7.4 (H) pH 8.3 (I) pH 8.6 after 24 h (Magnification 10X).

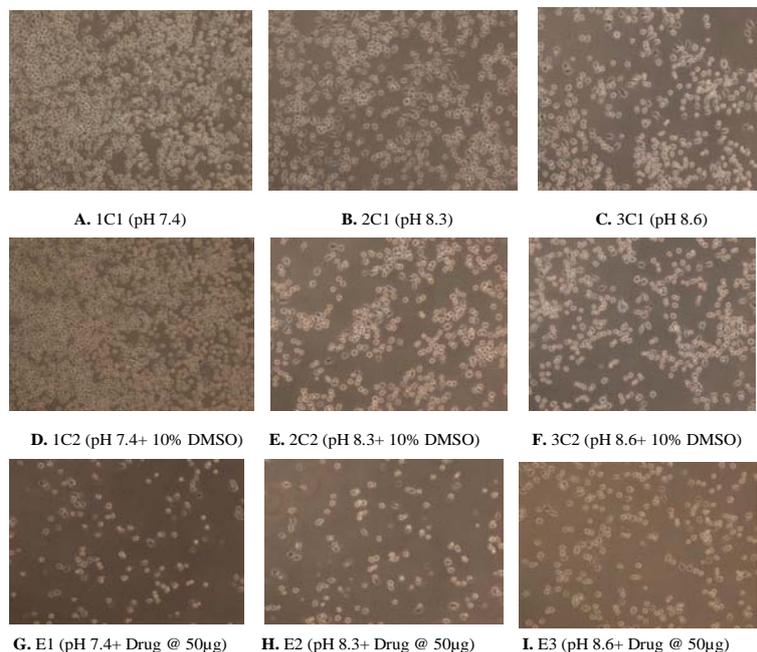


Figure 2: (A) Control showing untreated MDA human breast cancer cells maintained at pH 7.4, (B) pH 8.3 (C) pH 8.6 (D) Control showing untreated MDA human breast cancer cells in presence of 10% DMSO at pH 7.4 (E) 10% DMSO at pH 8.3 (F) 10% DMSO at pH 8.6 (G) Cytotoxic activity of *W. somnifera* methanolic extract at 50 µg/ml at pH 7.4 (H) pH 8.3 (I) pH 8.6 after 48 h (Magnification 10X).

Biological evaluation

Cell line

MDA-MB-231 (human breast carcinoma, ER⁻, tumorigenic and invasive cell line) was obtained from the National Centre for Cell Science (NCCS), Pune, India, and as such, was maintained by sub-culturing and passaging as monolayers in 25 and 75 cm² cell

culture flasks (Nest, Tarsons) at 37°C in Tissue and Cell Culture Lab, Era's Medical College, Lucknow, in an incubator gassed with an atmosphere of 5% CO₂ at 95% humidity, in advanced Dulbecco's Minimum Essential Medium (DMEM) containing phenol red as a pH indicator and supplemented with 5% FBS. The medium, prior to be used in cell culture experiments was vacuum filtered using a Corning filtration system. The medium requires an atmosphere of 5% CO₂ to

produce HCO₃ buffering capacity to maintain pH at 7.4 for normal cell growth.

Cell Culture

For experiments, MDA cells were trypsinized and cultured in 6-well plates (Linbro, MP Biomedicals) at a density of 0.5 x 10⁵ cells/well initially for 24 h, so as to allow the cells to attach. After 24h, two sets of controls were taken. One set (1C1, 2C1, 3C1) each contained 0.5 x 10⁵ MDA cells/ml maintained in medium having pH 7.4, 8.3 and 8.6 respectively. Second set (1C2, 2C2, 3C2) each contained 0.5 x 10⁵ MDA cells/well maintained in medium having pH 7.4, 8.3 and 8.6 respectively and each containing drug vehicle (10% DMSO) only. The experimental set (E1, E2 and E3) each contained 0.5 x 10⁵ MDA cells/well maintained in medium having pH 7.4, 8.3 and 8.6 respectively and each containing methanolic extract of *W. somnifera* (ashwagandha) in 10% DMSO at the rate of 50 µg/ml. The plates were kept in 5% CO₂ (incubator) and observed at an interval of 24, 48 and 72 and 96 h. After 96h, cells from each well were trypsinized, passaged and counted in a Tali Image-Based cytometer, Life Technologies (Invitrogen), to determine cytotoxicity and *in vitro* anticancer activity. Results were interpreted as cell viability versus time period graph.

Morphological Study

For morphological analysis, cells in 6-well plate were observed under phase contrast microscope & photographed (Nikon Eclipse Ti, Japan).

Cytotoxicity assays

Trypan blue dye exclusion assay

A cell suspension was made at a suitable dilution (1.0 x10⁵ cells/ml) in PBS. 50 µl of cell suspension was taken and mixed with an equal

volume of 0.4% trypan blue. The solution was mixed thoroughly and allowed to stand for 5 min at room temperature. 50 µl of the solution was transferred to a hemocytometer and viable cells were counted as clear cells and dead cells as blue ones. The number of live cells per ml was calculated using the following formula: % viability = (live cell count/total cell count)*100.

(Methyl tetrazolium-MTT assay)

Determination of optimal cell number for assay: In order to determine optimal cell number required for the assay, serial dilutions of MDA-MB-231 (2,000, 4,000, 6,000, 8,000, 12,000, 14,000, 16,000 and 18,000 cells/100 l) were made in cell culture media and seeded in 96-well microtiter tissue culture plates (Linbro, MP Biomedicals). Cells were cultured, in a humidified 5% CO₂ incubator at 37°C for 24 h. At the end of the incubation period, 20 µl of MTT solution (Stock concentration, 5.0 mg/ml in PBS) was added to each well and incubated for 4h under the same conditions. Thereafter, medium containing MTT was gently replaced by 200 µl DMSO to dissolve formazan crystals and the absorbance values were read in an ELISA plate reader at (Biorad PW41) at 550 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 density of cell line corresponding to absorbance values of 0.9 to 1.0 in the assay was selected for MDA-MB-231 to facilitate measurement of both stimulation and inhibition of cell proliferation within the linear range.

Evaluation of cytotoxicity and cell viability: Briefly, MDA-MB-231 cells were trypsinized and resuspended in the culture medium to get a defined cell number for MDA MB 231 (16,000/100 l) in a 96-well microtiter tissue culture plate and cultured in a humidified 5% CO₂ incubator at 37°C for 24h. Defined concentration of methanolic extracts of *W. somnifera* in 10% DMSO was freshly prepared in culture media by serial dilution to get a final concentration of 50 µg/ml. Serial

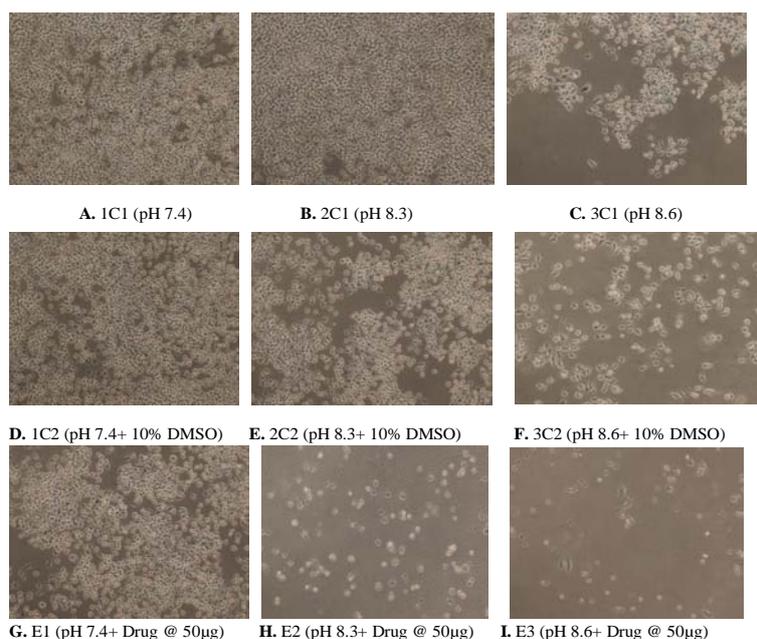


Figure 3: (A) Control showing untreated MDA human breast cancer cells maintained at pH 7.4, (B) pH 8.3 (C) pH 8.6 (D) Control showing untreated MDA human breast cancer cells in presence of 10% DMSO at pH 7.4 (E) 10% DMSO at pH 8.3 (F) 10% DMSO at pH8.6 (G) Cytotoxic activity of *W. somnifera* methanolic extract at 50 µg/ml at pH 7.4 (H) pH 8.3 (I) pH 8.6 after 72 h (Magnification 10X).

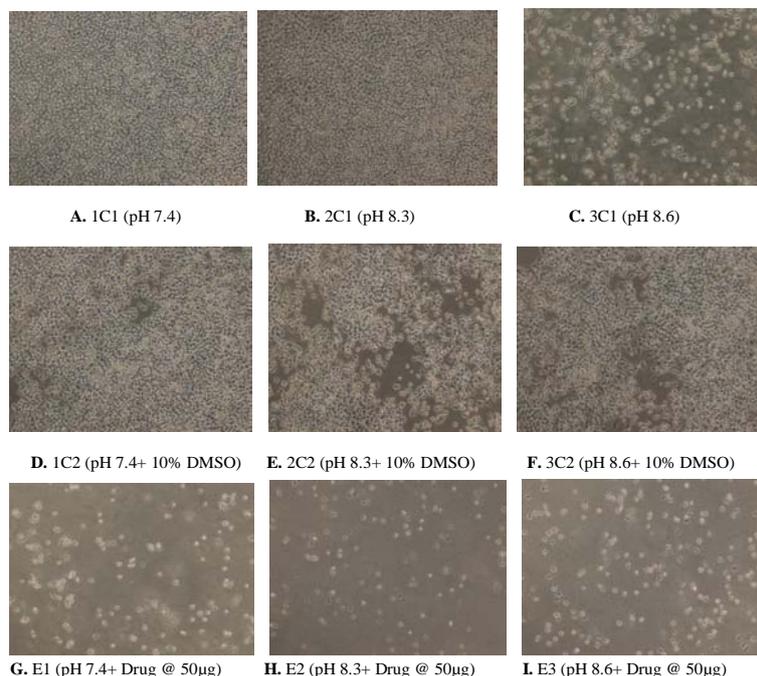


Figure 4: (A) Control showing untreated MDA human breast cancer cells maintained at pH 7.4, (B) pH 8.3 (C) pH 8.6 (D) Control showing untreated MDA human breast cancer cells in presence of 10% DMSO at pH 7.4 (E) 10% DMSO at pH 8.3 (F) 10% DMSO at pH 8.6 (G) Cytotoxic activity of *W. somnifera* methanolic extract at 50 µg/ml at pH 7.4 (H) pH 8.3 (I) pH 8.6 after 96 h (Magnification 10X).

dilution was carried out in cell culture media in such a way that the final concentration of DMSO in the well did not exceed 0.5% (v/v). Three control wells containing medium alone were also included to serve as blanks. After 24h of incubation, cells were treated with the above-mentioned concentration of *W. somnifera* methanolic extract in triplicates for 96 hours. Tamoxifen, 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 made in PBS at 5.0 mg/ml) reagent was added to each well and incubated for further 4h. Thereafter, the culture medium was removed and formazan crystals were dissolved in 200µl of DMSO. The plates were read in an ELISA plate reader (Biorad PW41) at 550 nm with a reference wavelength of 630nm. Percentage cell viability (Y-axis) was calculated from absorbance and plotted against concentration in µg/ml (X-axis). % cell survival was calculated as $\{(A_T - A_B) / (A_C - A_B)\} \times 100$ where,

A_T = Absorbance of treatment well

A_B = Absorbance of blank

A_C = Absorbance of control well

% cell inhibition = 100 - Cell Survival

Cytometer based analysis

For cell viability analysis, MDA cells from treated and control wells were trypsinized and resuspended in culture medium. Propidium iodide (20 g/ml) was then added and cells were incubated at RT for 5 min and read in a Tali Image-Based Cytometer, Life Technologies (Invitrogen). The number of live and dead cells in treated and control wells were determined. For comparison purpose, the cytotoxicity of the standard anti-breast cancer drug Tamoxifen (positive control) was also evaluated under the same experimental conditions. The

number of live cells in both treated and control wells was used for calculating the percentage cytotoxicity as % Cytotoxicity = $\{ \text{Live Cell No. in Treated Wells} - \text{Live Cell No. in Control Wells} / \text{Live Cell No. in Control Wells} \} \times 100$.

Statistical analysis

Results were expressed as mean ± SD of experiments done in triplicates.

Results and Discussion

As can be seen from Figures 1-4, pH 8.3 and pH 8.6 alone were responsible for considerable cytotoxicity, proving that alkaline pH has an inhibitory effect on cancer cells. However, the presence of methanolic extract of Ashwagandha (*W. somnifera*), augmented the cytotoxic effect caused due to alkaline pH by approximately ten folds (Table 1).

The external pH of solid tumors is acidic as a consequence of increased metabolism of glucose and poor perfusion. Acidic pH has been shown to stimulate tumor cell invasion and metastasis *in vitro* and *in vivo* [15]. The present study investigates whether inhibition of this tumor acidity (by increasing pH) could reduce cancer cell division *in vitro*. It is clear from the above study that alkaline pH has an inhibitory effect on human breast cancer cells. In future, the mechanism of drug uptake by cancer cells in presence of alkaline pH as well as the effect of ethanolic extract of *W. somnifera* on cancer cells in presence of alkaline pH would be studied. Medically, oral administration of buffers such as bicarbonate (that would increase the physiological pH) prior to drug administration can be an attractive idea for the treatment of cancer in future.

Table 1: Effect of alkaline pH and *W. somnifera* dose on MDA-MB-231 cells *in vitro*.

No. of Live Cells								
1C2 (pH=7.4)	E1 (pH 7.4+ Drug @ 50µg)	% Cytotoxicity	2C2 (pH=8.3)	E2 (pH 8.3+ Drug @ 50µg)	% Cytotoxicity	3C2 (pH=8.6)	E3 (pH 8.6+ Drug @ 50µg)	% Cytotoxicity
1.09x10 ⁶ ±22.12	1.48x10 ⁶ ±10.30	86.4±8.90	1.44x 10 ⁶ ±18.25	4.15x10 ⁴ ±12.56	97±15.42	1.31x10 ⁶ ±16.32	3.10x10 ⁴ ±26.10	97.6±10.25

Conclusion

Alkaline pH significantly augments/increases the cytotoxic effect of methanolic extract of *W. somnifera*, probably by affecting its uptake by cancer cells.

Acknowledgement

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