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Original Research Article (Experimental)

Evaluation of apoptotic activity of *Withania coagulans* methanolic extract against human breast cancer and Vero cell lines

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ABSTRACT

Background: The genus *Withania* (Family: Solanaceae) holds an important position in Ayurveda, the Indian traditional system of medicine. *Withania somnifera* Dunal and *Withania coagulans* Dunal have been documented in folklore as panaceas for various ailments since time immemorial. *W. coagulans* (WC), commonly called as Indian cheese maker is used for fermenting milk for cheese production in various parts of India.

Objectives: In the study, *in vitro* cytotoxicity of methanolic extract of dried fruits (berries) of WC was evaluated in a dose dependent manner using trypan blue dye exclusion method against human breast cancer cell line MDA-MB-231 and normal kidney epithelial cell line Vero in the range 20–200 μg/ml.

Material and methods: The percentage viability of the cell lines was determined by using MTT assay and cytometry.

Results: Methanolic extract of WC showed significant anticancer activity against MDA-MB-231 cell line. Cell viability was reduced to about 50% at 40 μg/ml of methanolic extract in 50% DMSO. Cytotoxicity of the extract was lower in 10% and 1% DMSO. On the other hand, methanolic extract of WC did not exhibit any significant *in vitro* activity against Vero cells at 170 and 200 μg/ml. AGE of isolated DNA from treated cancer cells revealed characteristic ladder like fragmentation, a hallmark of apoptosis. HPLC profiling was carried out for identification of the active components, which demonstrated the presence of Withaferin A in the methanolic extract.

Conclusion: Methanolic extract of WC possesses apoptotic activity against human breast cancer cells *in vitro* albeit lower in comparison to *W. somnifera* and warrants further investigation.

1. Introduction

Medicines derived from natural products have generated widespread interest and attention globally on account of their safety, efficacy and relatively fewer side effects. This has caused a paradigm shift towards opting for safer plant-based remedies. The plant, *Withania coagulans* Dunal is well-documented in Ayurveda as a cure for a plethora of diseases and conditions [1]. Fruits and leaves of *W. coagulans* Dunal (Fig. 1) contain an enzyme called Withanin and are commonly used to coagulate milk [1,2].

Various parts of this plant are known to possess and exhibit a wide spectrum of biological activities [1]. The fruit is a berry having a sweet taste with reported sedative, emetic and diuretic properties [2]. The shrub has a widespread distribution in India and is found in Punjab, Rajasthan, Simla, Kumaun and Garhwal regions [1]. The dry fruits are commonly used for treatment and management of diabetes in northern parts of India [1]. No wonder, the fruit is also commonly referred to as 'tukhm-e-hayat' (fruit of life). The plant has also been found to possess a range of activities *viz.* hypoglycemic [3,4], hypolipidemic, free radical scavenging, cardiovascular, hepatoprotective, anti-inflammatory, wound healing, antitumor, immuno-suppressive, cytotoxic, antifungal and antibacterial properties [1].

The most active chemical components of genus *Withania* are withanolides, which are known anticancer agents, as well as flavonoids [2]. The detailed mechanism underlying the anti-cancer effect of WC is still largely unknown. The anti-mutagenic activity of

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Fig. 1. (a). *Withania coagulans* (L.) Dunal Habit (b) Fruits (Image courtesy Gupta, 2012).

WC fruit extracts has been evaluated in bone marrow cells of mice. The results showed that a single i.p. dose of 500–1500 mg/kg body weight of WC fruit extract before 24 h, significantly reduced micronucleus formation in mouse bone marrow cells as compared to cyclophosphamide group [5].

Withaferin A, Withanolide A and Withanone are the major withanolides present in genus *Withania* [6,7] out of which Withaferin A is the most important in terms of its concentration and spectrum of activity [1]. It possesses significant antibiotic activity and its anti-tumor effect has been studied against malignant nasopharyngeal (KB) cells *in vitro* [1]. It interferes in cell division by arresting mitosis at metaphase. *In vivo* studies have also demonstrated the growth-inhibitory and radio-sensitizing effects of Withaferin A in mouse Ehrlich ascites carcinoma [8,9]. It has also been found to arrest cell division in embryonal chicken fibroblast cells. Anti-cancer and apoptotic activities of WC aqueous and methanolic fruit extracts have also been studied on DMBA induced skin papillomagenesis *in vivo* [5] as well as *in vitro* [10,11]. In the present study, the apoptotic activity of WC has been tested against human breast cancer as well as normal epithelial cell lines. The results revealed that the methanolic extract of fruits possesses significant activity against breast cancer cell line but no appreciable activity against the normal cell line. The genus *Withania* can, as such, be used as an adjunct or complementary medicine in patients undergoing aggressive treatment in clinical settings against a number of chronic and debilitating diseases like cancer.

2. Materials and methods

2.1. Reagents

PBS (pH = 7.2, 1x), 0.25% trypsin–EDTA (1x), Dulbecco's Modified Eagle's Medium DMEM/F-12 (1x), 0.4% trypan blue, and antibiotic/antimycotic solution (100×) were obtained from Gibco, Life Technologies; whereas fetal bovine serum (FBS) and MTT were obtained from Himedia. Doxorubicin hydrochloride solution was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dimethyl sulfoxide (DMSO) from Calbiochem. Withaferin A standard was from Natural Remedies Ltd. Veerasandra, Bangalore-100. All HPLC grade reagents were used in HPLC. All other chemicals were of analytical grade.

2.2. Collection of plant material

The plant WC was identified by a competent botanist from National Botanical Research Institute (NBRI), Lucknow. The dry fruits of *W. coagulans* were purchased from the local market of Lucknow, India, and were shade dried further followed by grinding.

2.3. Sample preparation

All procedures were carried out as per our previous study reporting the cytotoxic activity of methanolic and ethanolic extracts of *Withania somnifera* [12]. Briefly, 50% methanolic extract was prepared by extracting 25 g of powder of WC berries thrice. All three extracts were pooled and filtered through Whatman No. 1 filter paper (125 mm) and concentrated in a water bath till formation of a semi-solid paste. The paste was dried in a desiccator until a powdered form was attained and stored in an air-tight container till further use. For comparing better dissolution and activity, 20 mg/ml each of the powdered extract was dissolved in 1%, 10%, and 50% DMSO. All extracts were filtered through sterile syringe filter units (0.22-μm, Millipore, Fisher Scientific) before addition to cell culture medium.

2.4. Biological evaluation

2.4.1. Cell lines

Two cell lines viz. MDA-MB-231 (human breast carcinoma) and Vero (normal African green monkey kidney epithelial cells; ATCCCCL-81), were obtained from the National Centre for Cell Science (NCCS), Pune, India, and as such, were maintained in Tissue and Cell Culture Lab, Era's Lucknow Medical College, Lucknow, as per previously established protocol [12].

2.5. Cell culture

For the experiments, cells were trypsinized and seeded at a density of 0.5×10^5 cells/well for 24 h in 6-well plates (Linbro, MP Biomedicals) for adherence. Cells were exposed to 20–200 μg/ml of methanolic extract of WC in DMSO for the next 48 h. Suitable untreated controls were also included. All experiments were done in triplicates. Results were plotted as cell viability *versus* time period graph.

2.6. Morphological study

Cells were observed and photographed for morphological characteristics under a phase contrast microscope (Nikon Eclipse Ti, Japan).