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Ethanolic Extracts of *Ocimum sanctum*, *Azadirachta indica* and *Withania somnifera* cause apoptosis in SiHa cells

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ABSTRACT

Induction of apoptosis is considered an important step in cancer biology. It was observed that the treatment of a squamous cervical cancer cell line, SiHa with the ethanolic extracts of leaves of *Ocimum sanctum* and *Azadirachta indica* and roots of *Withania somnifera* at IC_{50} values for 48 h resulted in formation of internucleosomal fragments of DNA . The study of morphological changes also showed the formation of apoptotic bodies after treatment with these plant extracts. The IC_{50} values were determined using the cell proliferation assay, MTT assay. These results clearly show that these plant extracts, which have previously been shown to have anticarcinogenic activity, can induce apoptosis in SiHa, a cervical cancer cell line.

Keywords: Apoptosis , MTT, IC_{50}, Cervical cancer cell line.

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INTRODUCTION

Phytochemicals derived from fruits and vegetables, referred to as chemopreventive agents include genistein, diallyl sulfide, S-allyl cysteine, allicin, lycopene, curcumin etc. These chemopreventive agents are believed to have potential to be used as adjuncts to current cancer therapies [1].

Fig 1: ***(A) Ocimum sanctum (B) Azadirachta indica (C) Withania somnifera***

Ocimum sanctum, (Holy Basil or tulsi) is an aromatic plant in the family Lamiaceae which is native throughout the Old World tropics and widespread as a cultivated plant and an escaped weed [2]. It is a widely grown, sacred plant of India (Fig 1). In India, Hindus grow it as a religious plant in their homes, temples and their farms. *Azadirachta indica* (Neem) is a tree in the family Meliaceae. It is one of the two species in the genus *Azadirachta*, and is native to India and Pakistan growing in tropical and semi-tropical regions. Its fruits and seeds are the source of neem oil. It is a fast growing tropical evergreen tree with a highly branched and stout, solid stem. *Withania somnifera*, also known as Ashwagandha, Indian ginseng and Winter cherry is a plant in the *Solanaceae* or nightshade family (Fig 1). It is used as a herb in Ayurvedic medicine.The plants contain the alkaloids withanine and somniferine, which are used to treat nervous disorders, intestinal infections and leprosy. Most of the synthetic compounds used for killing cancer cells may have cytotoxic effects towards normal cells. Hence, nowadays the focus is on natural products for causing the apoptosis.

MATERIALS AND METHODS

Materials

The cervical cancer cell line, SiHa was procured from National Centre for Cell Sciences, Pune, India. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from HiMedia, India.

Culturing of cell line

SiHa cell line was cultured according to standard protocols [3]. Briefly, the cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS in 5% CO_2 at 37° C. The cells were resupplemented with fresh medium and test compounds every 48 hours.

Preparation of plant extracts

The leaves of *Azadirachta indica* and *Ocimum sanctum* and the roots of *Withania somnifera* were collected from Botanical Garden, Panjab University, Chandigarh. The air – dried and powdered plant materials (10 g) were extracted with 100 ml of ethanol by Soxhlet extraction for 8 hours. The obtained ethanolic extracts were filtered and evaporated by using a rotary evaporator and freeze dryer. The dried extracts were store at -20^{0}C till use.

Preparation of stock solutions of the test compounds and plant extracts

5-Aza-2-deoxycytidine and the plant extracts *Azadirachta indica, Ocimum sanctum and Withania somnifera* were dissolved in DMSO and filter sterilized (0.22 µm, non-pyrogenic filter). The stock solutions for the plant extracts were 10 mg/ml.

Cytotoxicity of chemopreventive agents

The cytotoxicity of plant extracts was studied on the HeLa cells by MTT method [4]. MTT assay was carried out to estimate cell viability after treatment with the plant extracts. Briefly, the cells were cultured in 96-well plates at a density of 1.0×10^4 cells per well in the presence of the above compounds. After incubation for 48 h, MTT dissolved in PBS was added to each well at a final concentration of 5 mg/ml and then incubated at 37° C and 5% CO_2 for 2 h. The water-insoluble dark blue formazan crystals that formed during MTT cleavage in actively metabolizing cells were dissolved in DMSO. The optical density was read by a microplate reader at a wavelength of 570 nm.

Morphological changes

Morphological changes in HeLa cells were observed through phase contrast microscope after 24 h of treatment with the plant extracts at IC_{50} values along with proper control.

DNA fragmentation assay

1×10^6 cells were treated with at the IC_{50} value in case of *Azadirachta indica* and 50µg/ml in case of other plant extracts for 48 h. Cellular DNA of treated cells was extracted from the cells according to Gong's modified method [5].

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RESULTS AND DISCUSSION

The IC_{50} values for *Azadirachta indica*, *Ocimum sanctum* and *Withania somnifera* were observed to be around 30, 250 and 125 µg/ml respectively (Fig. 2).The morphological studies showed that the treatment with these ethanolic extracts resulted in the formation of apoptotic bodies in SiHa cells (Fig. 3). DNA fragmentation assay was carried out in SiHa cells after treating them with the ethanolic extracts as DNA fragmentation is considered the hallmark of apoptosis. Treatment of these extracts was given at their IC_{50} values. Internucleosomal DNA fragments were observed in treated cells after 48 h of treatment (Fig. 4).

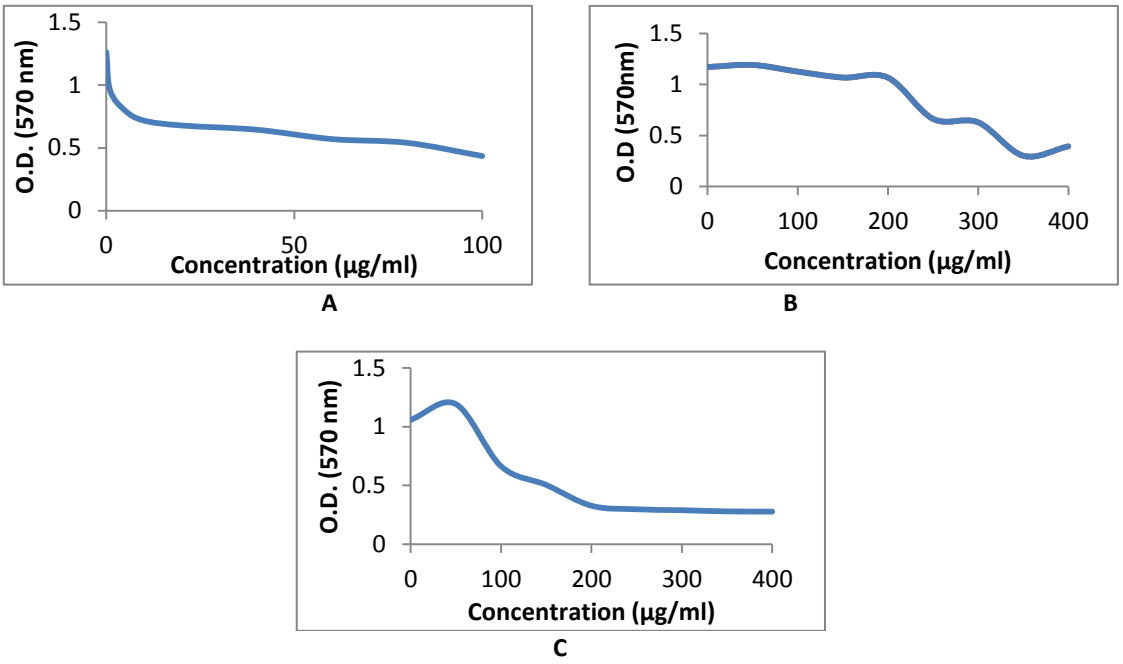
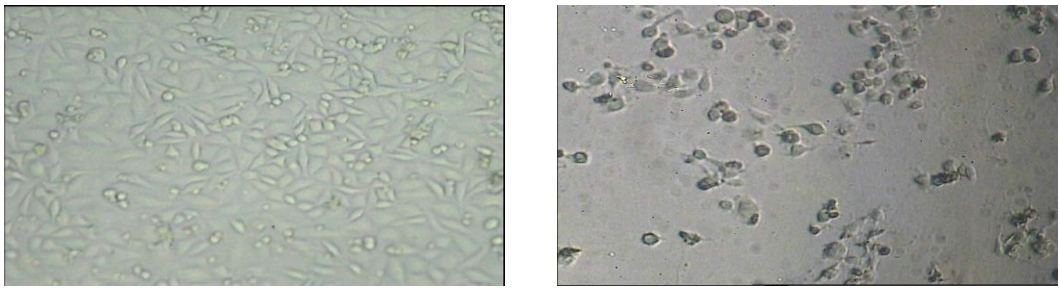


Fig 2: MTT Assay to estimate cell viability after the treatment of SiHa cells with (A) *Azadirachta indica.* (B) *Ocimum sanctum* (C) *Withania somnifera*.



(A) **(B)**

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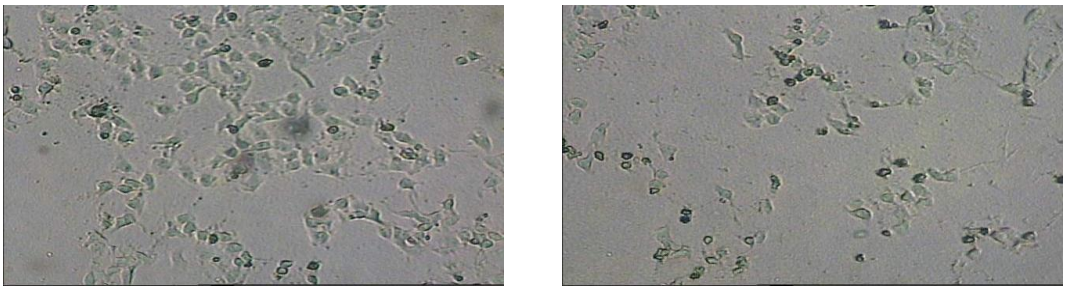

(C) (D)

Fig 3: Morphological changes in SiHa cells after 24 h of treatment with the plant extracts. (A) control (H) *Azadirachta indica* (I) *Ocimum sanctum* (J) *Withania somnifera*. Cells treated with plant extracts were observed under inverted microscope and photographed in same magnification (10X).

Fig 4: Induction of apoptosis in SiHa cells after 48 h treatment:
(1) *Withania somnifera* (2) *Ocimum sanctum*
(3) *Azadirachta indica* (4) control

The study of many types of neoplasia and their possible therapy using natural compounds forms the basis of much of the research nowadays [6]. Recently, medicinal plants have emerged as attractive candidates for cancer chemoprevention because of their safety, relative to cytotoxic synthetic agents [7]. In particular, the leaves of neem (*Azadirachta indica*) and tulsi (*Ocimum sanctum*) offer promise in chemoprevention of gastric cancer because of their antioxidant, anti-inflammatory and antiproliferative properties. The use of *Withania somnifera* as a well-tolerated, safe anti-angiogenic agent with potential in cancer chemotherapy has also been reported [8].

Ocimum sanctum has been shown to have radioprotective, anticarcinogenic and antioxidant properties [9]. Because of its tremendous therapeutic, domestic, agricultural and ethnomedicinal significance, and its proximity with human culture and civilization, *Azadirachta indica* has been called "the wonder tree" and "nature's drug store." All parts of this tree, particularly the leaves, bark, seed-oil and their purified products are widely used for treatment of cancer. Over 60 different types of biochemicals including terpenoids and steroids have been purified from this plant. Pre-clinical research work done during the last decade has fine-tuned our understanding of the anticancer properties of the crude and purified products from this plant. The anticancer properties of the plant have been studied largely in terms of its

preventive, protective, tumor-suppressive, immunomodulatory and apoptotic effects against various types of cancer and their molecular mechanisms [10]. The effect of ethanolic extract of the root of *Withania somnifera* Dunal against Dalton's Ascitic Lymphoma (DAL) has been evaluated in Swiss albino mice. A significant increase in the life span and a decrease in the cancer cell number and tumour weight were observed in the tumour-induced mice after the treatment. The hematological parameters were also corrected by this extract in tumour-induced mice. These observations are suggestive of the protective effect of *Withania somnifera* in Dalton's Ascitic Lymphoma [11].

We tried a few plant extracts like *Withania somnifera*, *Ocimum sanctum* and *Azadirachta indica* to check them for their ability to cause apoptosis in SiHa cells (a cervical cancer cell line). All of these are very important plants in the Ayurvedic system of medicine and have been shown to have anti-carcinogenic properties.

All these plant extracts caused apoptosis which was also confirmed by the formation of internucleosomal fragments after the treatment with these extracts, using DNA fragmentation. These plant extracts contain natural compounds which do not have any cytotoxic effects on normal cells.

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