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# Demethylation of *RARβ2* Gene Promoter by *Withania somnifera* in HeLa Cell Line

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***Authors' contributions***

*Author AKJ carried out all the experiments and drafted the manuscript. Author MN helped in MTT assay and MSP. Authors NC and JK conceived the study, drafted and edited the manuscript.*

***Original Research Article***

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## ABSTRACT

An important molecular target for cancer therapy is the possible reactivation of tumor suppressor genes that have been silenced by promoter methylation. It was observed that the treatment of an adenocarcinoma cervical cancer cell line, HeLa with 20 µg/ml of the ethanolic extract of *Withania somnifera* for 6 days resulted in demethylation of promoter of *RARβ2* gene. However, treatment with *Ocimum sanctum* and *Azadirachta indica* (20µg/ml) did not cause the reversal of hypermethylation after 6 days of treatment. This is the first report to show the reversal of hypermethylation of *RARβ2* gene by *Withania somnifera* extract in a cervical cancer cell line.

*Keywords: Methylation; adenocarcinoma; RARβ2; cervical cancer cell line.*

## 1. INTRODUCTION

Reactivation of tumor suppressor genes that have been silenced by promoter methylation is a very attractive molecular target for therapy against cancer [1]. DNA methyltransferase (DNMT) inhibitors like 5-azacytidine and 5-aza-2'-deoxycytidine have recently been

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approved by FDA for treatment of myelodysplastic syndrome. Some other drugs like procainamide and hydralazine are also in different stages of trial [1].

Most of the synthetic compounds may have cytotoxic effects towards normal cells. Hence, the focus is on natural products for causing the epigenetic reversal. 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 possess potential to be used as adjuncts to current cancer therapies [2]. Epigallocatechin-3-gallate (EGCG) has been shown to cause demethylation of CpG islands in the promoters and reactivation of methylation-silenced genes such as *p16$^{INK4a}$,* retinoic acid receptor *ß* and O6-methylguanine- DNA methyltransferase in human cancer cell lines [3,4,5].

Genistein, from soy, and lycopene, from tomato, have been shown to alter gene expression in ways that can either promote or potentially inhibit the carcinogenic processes in breast cancer cell lines. Both genistein and lycopene, at very low, dietarily relevant concentrations can potentially mitigate tumorigenic processes via promoter methylation modulation of gene expression [6]. We had already shown that curcumin and genistein cause reversal of hypermethylation and reactivation of *RARβ2* gene in SiHa (a squamous cervical cancer cell line) after 6 days of treatment [7]. Curcumin has also been shown by our group to cause reversal of hypermethylation of the same gene in HeLa cells [7].

Recently, medicinal plants have emerged as attractive candidates for cancer chemoprevention because of their safety, relative to cytotoxic synthetic agents [8]. 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 [9]. Hence, the present study was undertaken to check these extracts for their ability to cause demethylation of *RARβ2* gene promoter.

## 2. MATERIALS AND METHODS

### 2.1 Materials

The cervical cancer cell line, HeLa, was procured from National Centre for Cell Science, Pune, India. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from HiMedia, India. 5-aza-2-deoxycytidine was obtained from Sigma Chemicals Pvt. Ltd, USA.

#### 2.1.1 Culturing of cell line

HeLa cells were cultured according to standard protocols [10]. Briefly, the cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS in 5% $CO_2$ at 37ºC. The cells were resupplemented with fresh medium and test compounds every 48 hours.

#### 2.1.2 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 ethanolic extracts thus obtained were filtered and evaporated by using a rotary evaporator and freeze dryer. The dried extracts were store at -20ºC till use.

### 2.1.3 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 plant extracts were 10 mg/ml.

### 2.1.4 Cytotoxicity of chemopreventive agents

The cytotoxicity of plant extracts was studied on HeLa cells adopting MTT method [11]. MTT assay was carried out to determine cell viability after treatment with the plant extracts. Briefly, the cells were cultured in 96-well plates at a density of $1.0 \times 10^4$ 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 $37^o$ 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 in a microplate reader at 570 nm and the $IC_{50}$ values were determined (Fig. 1).

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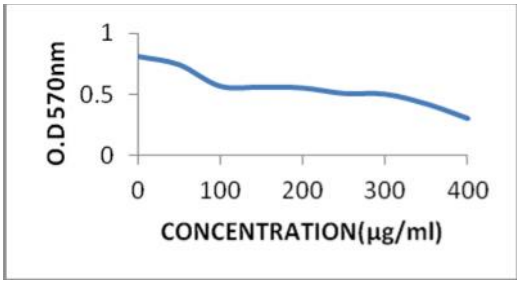
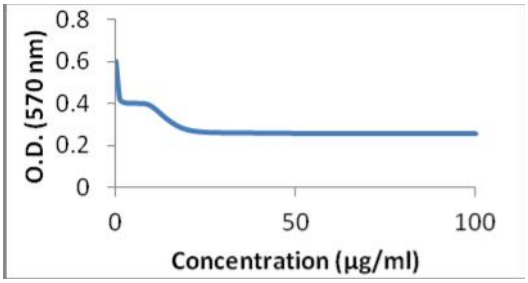
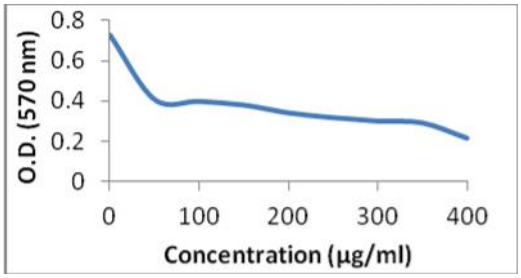



***Withania somnifera***




***Azadirachta indica***




***Ocimum sanctum***

**Fig. 1. MTT Assay was carried out to determine cell viability after the treatment with *Withania somnifera, Azadirachta indica* and *Ocimum sanctum* for 48 h.**

### 2.1.5 Morphological changes

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

### 2.1.6 DNA fragmentation assay

$1 \times 10^6$ cells were treated with the $IC_{50}$ value in the case of *Azadirachta indica* and 50 µg/ml in case of other plant extracts for 48 h. DNA of treated cells was extracted from the cells according to Gong's modi?ed method [12].

### 2.1.7 Methylation –specific PCR (MSP)

The effect of the extracts of *Withania somnifera* and *Ocimum sanctum* on the promoter hypermethylation of RARβ2 gene in the HeLa cells was monitored by MSP [13] after treating the cells with the ethanolic extracts at 20 µg/ml concentration for different time intervals i.e., 48 h, 72 h and 6 days. In case of *Azadirachta indica,* similar study was carried out after the treatment was given at 10 µg/ml as the $IC_{50}$ value was 18 µg/ml. DNA extracted from cells [14] was modified with sodium bisulfite, and MSP was carried out using specific primers for methylation and unmethylation for *RARβ2* gene [3, 7].

## 3. RESULTS

The $IC_{50}$ values of *Withania somnifera, Azadirachta indica* and *Ocimum sanctum* extracts in HeLa cells was found to be 350, 18 and 200 µg/ml, respectively (Fig. 1).

The morphological studies showed that the treatment with these ethanolic extracts resulted in the formation of apoptotic bodies in HeLa cells (Fig. 2). Since DNA fragmentation is the hallmark of apoptosis, DNA fragmentation assay was carried out in HeLa cells after treating them with the ethanolic extracts. Treatment of *Azadirachta indica* was given at its $IC_{50}$ value. In case of other plant extracts the treatment was given at 50µg/ml since their $IC_{50}$ values were higher. Internucleosomal DNA fragments were not observed in treated cells except after treatment with *A. indica* (Fig. 3).

Treatment with the plant extracts (20 µg/ml in case of *Withania somnifera* and *Ocimum sanctum,* but 10 µg/ml in case of *Azadirachta indica*) did not result in reversal of methylation status of *RARβ2* gene after 48 h (data not shown) or 72 h of treatment in HeLa cells (Fig 4). But demethylation was observed after the time period of treatment with *Withania somnifera* extract was increased to 6 days (Fig. 5).

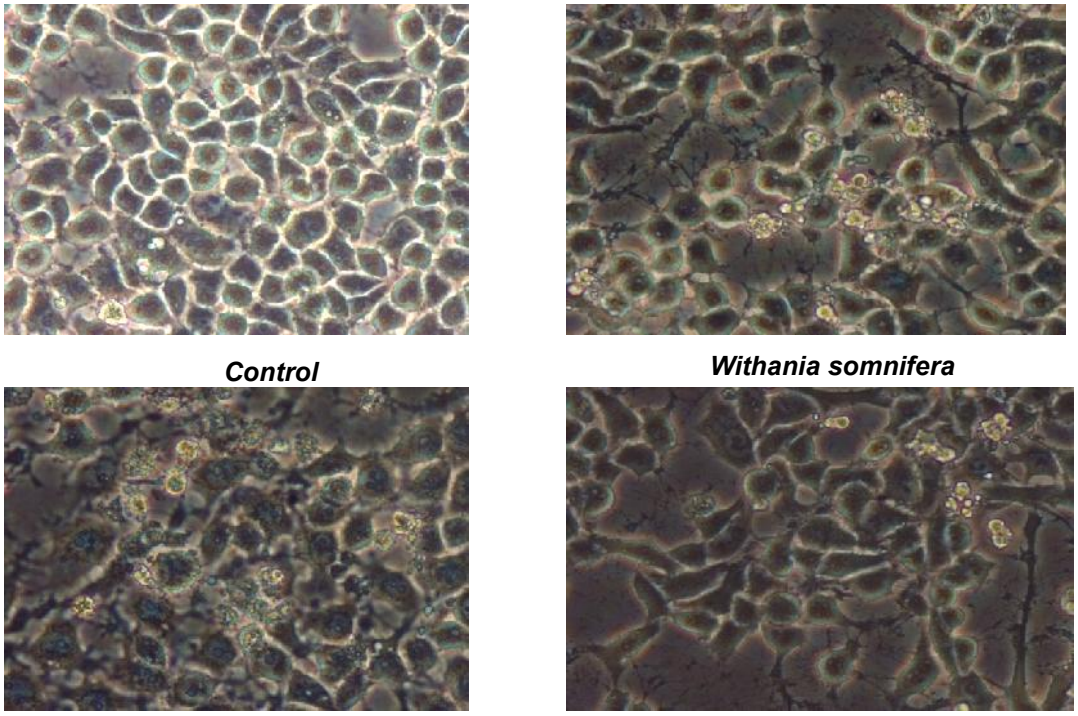



**Fig. 2. Morphological changes in HeLa cells after 24 h of treatment with *Withania somnifera, Azadirachta indica* and *Ocimum sanctum* at $IC_{50}$ values. Control or treated cells were observed under phase contrast microscope and photographed in same magnification (40x)**

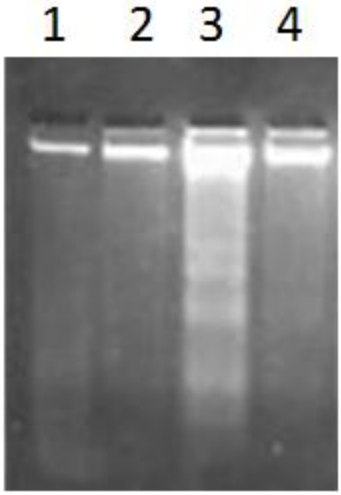



**Fig. 3. Induction of apoptosis in HeLa cells after 48 h treatment with plant extracts (1) *Ocimum sanctum* (50 µg/ml) (2) *Withania somnifera* (50 µg/ml) (3) *Azadirachta indica* (18 µg/ml) (4) control.**


**Fig. 4. MSP of *RARβ2* gene after 72 h of treatment with the plant extracts in HeLa cells. (1) 100 bp ladder (2) control (3) *Ocimum sanctum* (4) *Withania somnifera* (5) *Azadirachta indica***

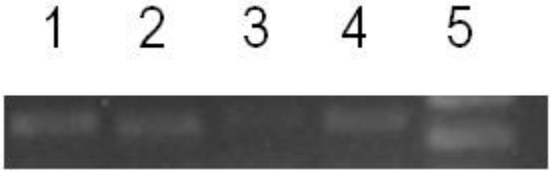



**Fig. 5. MSP of *RARβ2* gene after 6 days of treatment with the plant extracts in HeLa cells. (1) control (2) *Ocimum sanctum* (3) *Withania somnifera* (4) *Azadirachta indica* (5) 100 bp ladder**

## 4. DISCUSSION

The study of epigenetic changes resulting in many types of neoplasia and their possible reversal using natural compounds as unlike the genetic changes, forms the basis of much of the recent research as the epigenetic changes like DNA methylation can be reversed [7, 15]. MSP was carried out using methylation–specific primers to check the ability of the selected plant extracts to cause reversal of hypermethylation [13]. After calculating the $IC_{50}$ values for the compounds, the treatment was given at 20 µg/ml (below the $IC_{50}$ value) in case of *Withania somnifera* and *Ocimum sanctum* as some reports have shown demethylating activity of EGCG, curcumin and genistein at 20 µM concentration in some oesophageal, prostate and cervical cancer cell lines [3,4,16,7]. In case of *Azadirachta indica*, the treatment was given at 10 µg/ml to obtain a sufficient amount of cells so that sufficient DNA could be isolated for study on reversal of promoter hypermethylation.

According to a recent study, several phytochemicals inhibit the DNA methyltransferase activity with betanin being the weakest while rosmarinic and ellagic acids the most potent modulators (up to 88% inhibition) among the compounds selected for the study [17]. Our group has already shown that curcumin and genistein cause reversal of hypermethylation and reactivation of *RARβ2* gene in SiHa (a squamous cervical cancer cell line) after 6 days of treatment [7]. The MSB (Methylation– specific band-) showed a time dependent decrease in intensity after treatment with the genistein and curcumin. The expression of mRNA increased approximately proportional to the appearance of unmethylated DNA [7]. Curcumin has also been shown by our group to cause reversal of hypermethylation of the same gene in HeLa cells.

In further extension of our work, we tried a few plant extracts viz., *Withania somnifera* (Ashwagandha), *Ocimum sanctum* (Tulsi ) and *Azadirachta indica* (Neem) to check them for their ability to cause reversal of hypermethylation. All of these are very renowned plants in the Indian system of medicine and have been shown to have anti-carcinogenic properties.

*Withania somnifera* extract caused apoptosis as well as reversal of promoter hypermethylation of *RARβ2* gene. The other two extracts caused apoptosis but could not cause reversal of hypermethylation. These plant extracts contain natural compounds which do not have any cytotoxic effects on normal cells unlike the demethylating chemicals.

## 5. CONCLUSION

The present study could prove to be an important step in the direction of therapy against cancer. *Withania somnifera* proved to be the most effective amongst all the three plant extracts as it caused both apotosis as well as reversal of promoter hypermethylation.

## CONSENT

Not applicable.

## ETHICAL APPROVAL

Not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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