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Evaluation of the effect of *Withania somnifera* root extracts on cell cycle and angiogenesis

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Abstract

In the Indian System of Medicine, the medicinal plant, *Withania somnifera* Dunal (Solanaceae) finds application for numerous ailments including cancer. This study explores the mechanism(s) underlying this property. The hydroalcoholic extract of the roots (WS) was partitioned between chloroform (WS-chloroform) and water (WS-water). Further, WS-chloroform was fractionated (A1–A12) by reverse-phase column chromatography and their withanolide content was quantified by high-performance liquid chromatography (HPLC). Preliminarily, the anti-proliferative activity of all the extracts and fractions was screened against human laryngeal carcinoma (Hep2) cells by microculture tetrazolium assay (MTT). Two extracts (WS and WS-chloroform) and three fractions (A4, A5 and A6) negatively affected Hep2 viability at the concentration of 25 μg/ml and these were further investigated pharmacologically. Flow cytometry revealed cell cycle block and accumulation of hypoploid (sub G1) cells as the mode of anti-proliferative activity of all but A4. Their anti-angiogenic potential was investigated by a chickchorio-allantoic membrane (CAM) wherein a significant inhibition ($p < 0.0001$) of vascular endothelium growth factor (VEGF), induced neovascularization was recorded. The effect was confirmed in vivo by mouse sponge implantation method. These findings suggest that the roots of *Withania somnifera* possess cell cycle disruption and anti-angiogenic activity, which may be a critical mediator for its anti-cancer action.

Keywords: Withanolides; HPLC; Anti-cancer; Flow cytometry; Anti-angiogenesis; VEGF

1. Introduction

In the Indian System of Medicine, the roots of the medicinal plant Ashwagandha (*Withania somnifera* Dunal, Solanaceae) are used in formulations that are prescribed as a remedy for cancer (Sharma and Dandiya, 1992). In our search for anti-cancer agents from traditional source, we have confirmed the efficacy of the hydroalcoholic extract of *Withania somnifera* roots in different experimental models of cancer (Prakash et al., 2001). Its activity has been tentatively explained as an adaptive reaction to normalize pathological events, although the exact mechanism remains to be elucidated (Mishra et al., 2000).

Cancer may be regarded as a series of malignant diseases characterized by abnormal growth of cells into neoplasm, ability to invade adjacent and even distant tissues and eventually the death of the patient (Park, 2002). Therefore, the cancer chemotherapeutic agents that are currently being used are cytotoxic drugs that kill malignant cells or modify their growth pattern. Natural products represent a reservoir of diverse templates and increasingly, medicinal plants are being tapped to outsource novel anti-cancer agents (Harvey, 2000). In vitro cytotoxicity assays are rapid and rational methods for screening plants with positive activity from the large number of species that exist.

It is well-established that neoplasms cannot grow beyond a certain size without adequate blood supply. Formation of

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new blood vessels from existing vasculature or 'angiogenesis' is characteristic of all cancer types and serves as a route of nutrition, oxygenation and metastasis (Folkman et al., 1989). Therefore, anti-angiogenic therapy for cancer has been considered as an attractive proposition.

The present study has been designed to explore the mechanism underlying the anti-cancer activity of *Withania somnifera* in terms of curtailing cancer cell proliferation and angiogenesis. We also report here a novel high-performance liquid chromatography (HPLC) based analytical method for identification and quantification of withanolides that are known to be biologically active constituents of *Withania somnifera*.

2. Materials and methods

2.1. Plant material and preparation of extracts

The roots of *Withania somnifera* grown in natural habitat and purchased from an authorized dealer were air-dried in shade and finely powdered. The chief botanist at Indian Agricultural Research Institute (IARI), New Delhi, India, identified the roots and a voucher specimen (accession number NISCAIR/RHM/F-3/2003/Consult/373) has been deposited at the herbarium of IARI. The root powder was exhaustively extracted with methanol:water (4:1, v/v) under reflux (WS). This extract was partitioned with chloroform and water to give WS-chloroform and WS-water, respectively. WS-chloroform was subjected to 12 successive elutions of water and acetonitrile (ACN) and were labeled from A1 to A12.

HPLC (Waters, Milford, U.S.A.) of *Withania somnifera* extracts was performed using Kromasil C8 column (4.6 mm × 25 cm, 5 μm), and the mobile phase consisted of ACN and water (1:1, v/v) at a flow rate of 1 ml/min for a run time of 30 min. The HPLC of fractions was conducted using Novapak C18 column (3.9 mm × 15 cm, 4 μm) and the mobile phase consisted of potassium-dihydrogen orthophosphate (0.05 M) and ACN (3:7, v/v) at the flow rate of 1.5 ml/min for a run time of 30 min. The photodiode array (PDA) detector was set to detect at 229 nm and scan spectral data from 190 to 400 nm. Using the standard withanolide A, and withaferin A (Natural Remedies Private Ltd., Bangalore, India), the bioactive constituents of the extracts were quantified by external calibration method.

2.2. In vitro assay for cytotoxic activity

Human laryngeal carcinoma, Hep2 cells (National Centre for Cell Science, Pune, India) were cultured in Dulbecco's modified Eagles medium (Hi Media Laboratories Pvt. Ltd., India) supplemented with fetal calf serum (10%) and ciprofloxacin (10 μg/ml). All cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C and pH of 7.4.

For the microculture Tetrazolium assay (MTT), the cells were exposed to different concentrations (100, 50, 25, 10 and 5 μg/ml) of test samples or vehicle for 24 h. Following treatment, the cells were exposed to Tetrazolium dye (5 mg/ml) for 3 h. The formation of the purple colored formazan complex was read at 560 nm using ELISA microtiter plate reader (Anthos Labtec Instruments GmbH, Austria) to determine the inhibitory concentration, IC_{50}.

2.3. Flow cytometry analysis

Hep2 cells were treated with bioactive extracts and fractions (25 μg/ml), as determined by MTT assay. The medium containing apoptotic cells was fixed with 70% ethanol. For DNA staining, 4 μl of propidium iodide (5 μg/ml, Sigma–Aldrich Co., U.S.A.) was mixed into the suspension containing 400 μl of citrate buffer (pH 7.4) and 0.1% Triton-X-100 and stored in the dark at 4 °C for 1 h. The fluorescence of the suspension was recorded at 488 nm using Coulter cytometer EPICS XL™ (Coulter, U.S.A.).

2.4. Chickchorio-allantoic membrane (CAM) assay

The chickchorio-allantoic membrane (CAM) assay for screening the effect of test samples on angiogenesis was performed according to the method given by Ribatti and co-workers (1997). Fertilized white leghorn chicken eggs on the second day of incubation were purchased from a local hatchery and incubated at 37 °C under constant humidity. On day 3, a small hole was drilled at the narrow end to withdraw 2–3 ml of albumin. On the seventh day of incubation, a small square window was opened in the shell and sterile gelfoam (Johnson & Johnson Medical Ltd., U.K.) piece (3 mm × 3 mm × 1 mm) was implanted on top of the membrane.

In the vehicle control group, the gel was impregnated with sterile PBS. The positive control group received different concentrations (2.5, 5 and 10 ng) of vascular endothelium growth factor (VEGF, Braunschweig, Germany). The test group received different concentrations (2.5, 5 and 10 ng) of extracts or fractions in combination with VEGF (10 ng). The eggs were returned to the incubator where they were incubated undisturbed till day 12.

On the 12th day of incubation, the gels were fixed in ovo with 10% formalin. The formalin fixed gels were sectioned (<4 μm) and stained with haematoxylin and eosin (H&E). Blood vessels at the boundary between sponge and surrounding CAM mesenchyme were counted in 10 consecutive fields of sections using 20× objective of the microscope (Nikon, Japan). The mean microvessel density or MVD (number of blood vessel/unit area) for each group was defined using an objective graticule (Carl Zeiss Ltd., Germany) having a least count of 0.01 mm.

2.5. Mouse sponge implantation method

Male Swiss albino mice weighing between 25 and 35 g were obtained from the Experimental Animal Facility of All India Institute of Medical Sciences, New Delhi, India and maintained under standard laboratory conditions (26 ± 1 °C, 12-h light:10-h dark cycle) with food and water ad libitum and were used in accordance with institutional guidelines for ethics. The in vivo angiogenesis assay involving subcutaneous implantation of gelfoam sponges in mice was performed according to the method proposed by McCarty et al. (2002). Absorbable gelfoam

Table 1
Percentage recovery (from dry roots) and concentration of withanolide A and withaferin A in extracts and fractions of *Withania somnifera*

Extract/fraction	Recovery from root (%, w/w)	Withanolide A (%)	Withaferin A (%)
WS	3.06	0.02	0.0007
WS-chloroform	0.78	0.0048	0.007
WS-water	0.36	ND	ND
A4	0.0019	ND	ND
A5	0.0014	0.0289	0.0015
A6	0.0035	0.0075	0.004

was cut (5 mm × 5 mm × 5 mm) and hydrated in sterile PBS and strengthened with sterile 0.4% agarose. Simultaneously, VEGF (100 ng) was impregnated for the control group. For the test group, either extracts or fractions of *Withania somnifera* (100 ng) were applied in combination with VEGF (100 ng).

Mice were anaesthetized with sodium pentobarbitone (30 mg/kg, i.p.). An incision was given along the midline and one gel piece was inserted into each subcutaneous pocket created laterally. The animals were allowed to recuperate for 14 days. On the 14th day, they were sacrificed and the gelfoam was harvested. The gels were fixed in formalin and sectioned (<4 μm), stained with H&E. The number of vessels was counted in 15 consecutive fields using a 20× objective and the mean MVD was calculated. Eight gels were evaluated per group.

2.6. Statistical analysis

In the CAM and mouse sponge implantation assay, the statistical analysis of MVD scores of control versus test group was performed by unpaired two-tailed Student's *t*-test using the Graph pad prism 4 software. The results are expressed as mean ± standard deviation (S.D.) and $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.005$, $^{*}p < 0.05$.

3. Results

3.1. Identification and quantification of Withania somnifera extracts and fractions

By an external standard method the percentage of withanolide A and withaferin A in each extract and fraction was calculated (Table 1).

3.2. Cytotoxicity and cell cycle perturbations by Withania somnifera extracts and fractions on Hep2 cell

In MTT assay, WS and WS-chloroform negatively affected the viability of Hep 2 cells. About 40% increment in killing was seen when the dose of the extracts was increased from 5 to 25 μg/ml. WS-water extract failed to show significant inhibition as compared to vehicle control. Three of the 12 fractions i.e. A4, A5 and A6 showed inhibitory effect on the viability of Hep2 at the concentration of 25 μg/ml. On this basis, WS, WS-chloroform, A4, A5 and A6 were selected for further pharmacological evaluation, viz. flow cytometry, CAM assay and mouse sponge implantation assay.

Table 2
Percentage of Hep2 cell population in different cell cycle stages (G1, S phase and G2/M) and apoptotic population after treatment with extracts and fractions of *Withania somnifera*

Treatment (μg/ml)	Apoptosis (%)	G1 (%)	SPF (%)	G2/M (%)
Normal	7.09	26.86	36.42	15.38
Cisplatin (10)	60.86	20.59	17.51	1.62
WS (25)	53.69	16.21	24.43	3.54
WS-chloroform (25)	46.33	23.21	20.78	4.75
A4 (25)	11.07	25.98	22.13	4.14
A5 (25)	44.81	23.19	19.98	5.88
A6 (25)	47.20	19.44	19.55	5.65

The effect of 25 μg/ml of bioactive extracts (WS and WS-chloroform) and fractions (A4, A5 and A6) on cell cycle was evaluated by flow cytometry. Table 2 details the apoptosis (%) and cells (%)/cell cycle stage in vehicle, positive control (cisplatin 10 μg/ml) and treated plates. The 2-D histogram obtained by flow cytometry showed a general block of all the cell cycle stages.

3.3. Anti-angiogenic effect of Withania somnifera extracts and fractions in CAM assay

In the vehicle control group, very few blood vessels from the underlying network were seen actually growing towards the gel. In the positive control group, the region immediately surrounding the gel appeared to be highly vascular with blood vessels directed towards the gel. A typical "spoke wheel" formation could be seen with the gel at the hub of the wheel (Fig. 1). Neo-

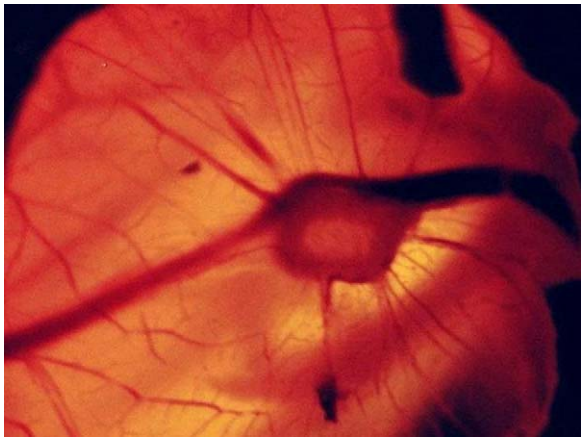

Fig. 1. In ovo photograph showing blood vessel sprouts converging towards gel impregnated with VEGF (10 ng).

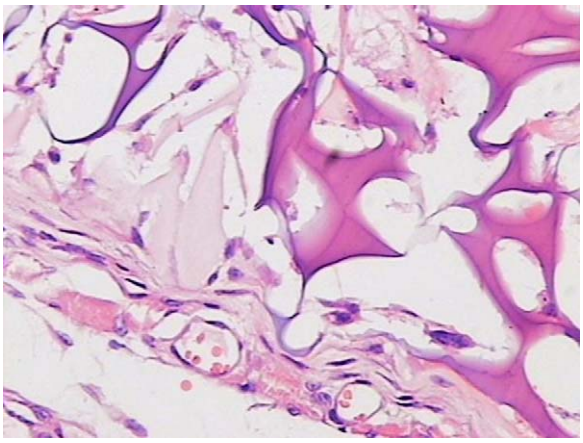

Fig. 2. Microphotograph of section showing a necrotic CAM with an occasional blood vessel around gel impregnated with A5 (10 ng) ± VEGF (10 ng) (H&E, 400×).

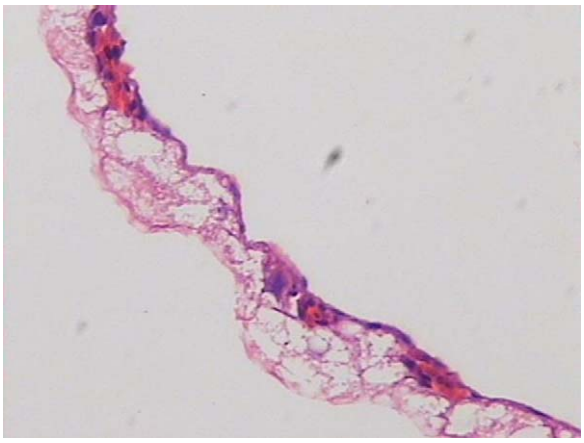

Fig. 3. Microphotograph of section from gel impregnated with A5 (100 ng) ± VEGF (100 ng) and implanted in mouse subcutis showing only an occasional blood vessel (H&E, 200×).

vascularization induced by VEGF (10 ng) was highly significant ($p < 0.0001$) as compared to the vehicle control group.

The surrounding region of CAM implanted with gel impregnated with A5 and A6 (10 ng) appeared pale-yellow in contrast to the rich blood red colored CAM in control group with a highly significant ($p < 0.0001$) inhibition of mean MVD (Fig. 2). Fraction A4 failed to inhibit VEGF induced neoangiogenesis.

3.4. Anti-angiogenic effect of Withania somnifera extracts and fractions in mouse sponge implantation model

The gels from vehicle control group appeared to be normally vascular with few blood vessels growing towards the gel. In contrast, the gels impregnated with VEGF (100 ng), appeared to be highly vascular with numerous blood vessels directed towards the gel. The mean MVD with VEGF treatment (100 ng) was 73.85 ± 0.8 and significantly greater ($p < 0.0001$) than the vehicle control group.

Histopathological assessment revealed that WS and WS-chloroform (100 ng) were highly effective in inhibiting VEGF induced neovascularization. The results with WS-water were not significantly different when compared to the control group. At the concentration of 100 ng, A5 and A6 significantly reduced the VEGF induced neovascularization ($p < 0.001$) (Fig. 3). Fraction A4 failed to produce an anti-angiogenic effect.

4. Discussion

Developing novel cancer chemotherapeutic agents that have a well-defined mechanism of action is still an emerging field of oncology where researchers in both basic and clinical sciences are facing great challenges. In this direction, plants are being actively explored as a source of new molecules that can curtail cancer growth (Dredge et al., 2003; Lee et al., 2003).

Withania somnifera has been widely regarded as the Indian Ginseng and used as an ayurvedic medicine to promote health and longevity in India for a long time. Its efficacy in many ailments has been confirmed by various in vitro and in vivo pharmacological experiments (Kulkarni, 1998). The chloroform extract of the roots of the plant contains withanolides. These compounds have been established as adaptogenics possessing anti-stress property (Singh et al., 2003). They are known to be biologically active and exhibit anti-tumour, immunopotentiating and anti-metastatic activity (Leyon and Kuttan, 2004). As no definite mechanism of action has been elucidated, the anti-tumour activity has been viewed with sceptism. Therefore, the primary focus of this study was to provide an answer to the elusive mechanism underlying the anti-cancer property.

In our study, WS, WS-chloroform, and three fractions (A4, A5 and A6) were screened for bioactivity by MTT assay at the concentration of 25 μg/ml. Further evaluation by flow cytometry revealed that the extracts (WS and WS-chloroform) and two fractions (A5 and A6) effectively inhibited the proliferation of Hep2 cancer cell line by generally blocking cell cycle at all stages. However, the activity of fraction A4, as indicated by MTT, was a false positive and could not be confirmed by flow cytometry. In addition, the fraction did not exhibit anti-angiogenic activity. Lack of known bioactive constituents (withanolides), as revealed by HPLC, may explain the pharmacological inactivity of fraction A4.

In vitro cytotoxicity assays have the limitations of low sensitivity and reliability and are prone to generate false positives (Denizot and Lang, 1986). In MTT assay, the reduction of the tetrazolium salt MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to a blue formazan product is the widely used screening method for cell viability and proliferation. The overall accuracy of this assay for clinical effects has been reported to be 83.3% (Suto et al., 1989). The assay is prone to false positive results especially when testing natural compounds with intrinsic reductive potential like phytoestrogens and antioxidants and could explain our results (Bruggisser et al., 2002).

On the other hand, analytical tools like flow cytometry prove to be useful as they reveal the mechanism of cytotoxicity at the cellular level. In principle, the DNA of the cancer cell population is intercalated with fluorescent dye (like propidium iodide) and analyzed for its distribution in cell cycle and ploidy level as it flows through the cytometer. This technique is finding wide

application especially with regard to discovery of anti-cancer medicinal plants (Van den Engh, 1993).

Angiogenesis is one of the most important factors involved in the development and progression of human tumors (Folkman et al., 1989). Angiogenesis inhibitors show promise as anti-cancer agents as they can act in both pre-invasive and invasive stages of cancer (Lee et al., 2003; Muehlbauer, 2003). Favourably, endothelial cell recruitment and proliferation is a central phenomenon of all cancer types and these cells are inherently stable and exhibit low mutagenesis rate, thereby reducing chances of multi-drug resistance (Tosetti et al., 2002; Bisacchi et al., 2003). Given the ubiquity of this pathway, experimental studies targeting it are being actively conducted (Bai et al., 2003).

For the first time our study reports the anti-angiogenic activity of the roots of *Withania somnifera*. Inhibition of VEGF induced capillary sprouting and formation by the extracts (WS and WS-chlorofom) and fractions (A5 and A6) has been recorded. VEGF represents a target for anti-angiogenic therapies in a wide spectrum of diseases, including cancer. The CAM model is proving to be useful for screening the in vivo anti-angiogenic activity of various compounds (Chen et al., 2004). The CAM model has several advantages over mammalian models. Firstly, it helps to directly study the in vivo effect of the test compound on VEGF activity (Min et al., 2004). Secondly, the vascular system of CAM is directly accessible to observation and experimentation, and there are no metabolic or hormonal influences. In addition, it is a more physiological model than in vitro models because vascularization of CAM is subject to regulations through fluxes, pressure, shear stress and growth factors (Larger et al., 2004). The results of our experiments with CAM assay show that the root extracts and fractions of *Withania somnifera* possess a significant anti-angiogenic activity.

Devi and co-workers (1992) have also demonstrated the efficacy of the alcoholic extract of *Withania somnifera* at a dose of 400 mg/kg, i.p., in controlling the tumor growth in adult BALB-c mice. At concentrations higher than this, toxic effects were recorded with no improvement in efficacy. In this study, we examined the in vivo anti-angiogenic activity of *Withania somnifera* using mouse sponge implantation method. This model circumvents numerous difficulties present in the conventional assays (Auerbach et al., 2000). It offers some distinct advantages like biocompatibility, absence of inflammatory angiogenesis, depot preparation of pro-angiogenic molecule and test sample and feasibility of long-term studies. Most importantly, the grafts can be implanted in rodents of normal immune status (McCarty et al., 2002). We have studied the effect of low concentrations of *Withania somnifera*, at which no toxic effects were recorded.

5. Conclusion

The results of this study support the development and use of *Withania somnifera* as a well-tolerated, safe anti-angiogenic agent with potential in cancer chemotherapy. An attempt has been made to provide a scientific basis for the traditional use of *Withania somnifera* roots in the treatment of cancer in human patients.

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