RESEARCH ARTICLE

Antiproliferative Activity of the Methanolic Extract of *Withania Somnifera* Leaves from Faifa Mountains, Southwest Saudi Arabia, against Several Human Cancer Cell Lines

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Abstract

Cancer represent one of the most serious health problems and major causes of death around the world. Many anticancer drugs in clinical use today are natural products or derived from natural sources. *Withania somnifera* (L.) Dunal is a small shrub widely distributed in many parts of the world including Saudi Arabia. The antiproliferative activities of the methanolic extract of *W. somnifera* leaves collected from Faifa mountains, southwest Saudi Arabia against MCF-7, HCT116 and HepH2 cell lines were investigated. The extract showed a strong antiproliferative activity against all cell lines with IC_{50} values of 3.35, 2.19 and 1.89 μg/ml, respectively. Flow cytometry results showed that the extract arrested the cell cycle at S phase, and the increase in the caspase 3 activity suggested that the extract could induce cell apoptosis by a caspase mediated pathway. These results demonstrated that the methanolic extract of *W. somnifera* leaves collected from Faifa mountains has comparable strong antiproliferative activities to samples collected from different locations.

Keywords: *Withania somnifera* – MCF-7 – HCT116 – HepG2 – Caspase 3 – Antiproliferative

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Introduction

Cancer represents one of the most serious health problems and major cause of death around the world (Ouyang et al., 2014; Sultana et al., 2014). Plants were and still important source for new anticancer drugs (HemaIswarya and Doble, 2006; Itokawa et al., 2008; Ouyang et al., 2014). Many anticancer drugs in clinical use today are natural products or derived from natural sources. It has been reported that 69% of the anticancer drugs approved between 1980 and 2002 are natural products or designed based on compounds isolated from plants (Kumar et al., 2011). Patients favor natural products because of their lower side effects and chemical balance comparing to synthetic chemicals (Sultana et al., 2014).

Withania somnifera (L.) Dunal (*W. somnifera*) is a small shrub belonging to the family Solanaceae. It is widely distributed in many areas including Africa, Mediterranean, Middle East, India and China (Alwadi and Baka, 2001; Dar et al., 2015). In Saudi Arabia, it is widely spread in south western region, eastern Najd and Hijaz (Rahman et al., 2004). *W. somnifera* has been used since ancient times as an abortifacient, anthelmintic, diuretic, deobstruent, aphrodisiac, narcotic and febrifuge (Rahman et al., 2004; Kumar and Kushwaha, 2006; John, 2014). The plant's different parts are source of many withanolides and special alkaloids that have been shown to act as antioxidants and steroidal hormones with auspicious impacts on human health (Shah et al., 2009; Samadi, 2013). Anticancer and antiproliferative activity of the plant's parts collected from different locations have been reported in many studies (Shah et al., 2009; Yadav et al., 2010; Yang et al., 2013; Halder et al., 2015). In a study designed to elucidate the effect of the environment on the antiproliferative activity of *W. somnifera*, (Kaur et al., 2004) reported that the leaves extract of field raised *W. somnifera* has a significant antiproliferative activity against breast carcinoma (MCF-7) cells and Osteogenic sarcoma (U2OS) while extract of laboratory raised *W. somnifera* failed to arrest cancer cell growth at the same concentrations. The present study investigated the antiproliferative activity of the methanolic extract of *W. somnifera* leaves collected from Faifa mountains, southwest region, Saudi Arabia against different human cancer cell lines.

Materials and Methods

Reagents

SulphoRhodamine-B (SRB), ethanol and methanol

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were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Culture media and growth supplements were purchased from Gibco-Life Technologies Co, (Carlsbad, CA, USA).

Preparation of plant materials

W. somnifera fresh leaves were collected from Faifa mountains, southwest region, Saudi Arabia, on March 2015. Leaves were washed with distilled water and dried in the air. Dried leaves were grounded into fine powder using laboratory grinder and soaked in methanol for 24 hours at room temperature. The extract was filtered and the solvent was removed under reduced pressure. The dry extract was stored as aliquots at -20 until further use (Paydar et al., 2013; Alhazmi et al., 2014).

Cell culture

Human breast cancer (MCF-7), Human colorectal carcinoma (HCT116) and Human hepatocellular carcinoma (HepG2) were obtained from Vacsera (Giza, Egypt). Cells were grown in RPMI media supplemented with 100 μg/mL streptomycin, 100 units/ml penicillin and 10% heat-inactivated fetal bovine serum in a humidified, 5% (v/v) CO_2 atmosphere at 37ºC.

In vitro cytotoxicity screening

The Sulforhodamine B colorimetric assay (SRB assay) (Skehan et al., 1990; Vichai and Kirtikara, 2006) was used to determine the inhibitory effect of *W. somnifera* methanolic extract against MCF-7, HCT116 and HEPG-2 cells. Exponentially growing cells were detached from dishes using 0.25% trypsin-EDTA and plated in 96-well plates at 1000 cells/well. After 24 hours of incubation, cells were exposed to various concentrations of extract for 48 hours. At the end of treatment time, cells were fixed with TCA (10%) for 1h at 4°C, washed several times with distilled water, stained with 0.4% SRB solution for 10 min in a dark place and washed with 1% glacial acetic acid. After drying overnight, Tris-HCl was used to dissolve the SRB-stained cells and the color intensity was measured at 450nm using microplate reader (Anthos Zenyth-200RT, Cambridge, England). The half maximal inhibitory concentration (IC_{50}) was determined by the trend line equation using SigmaPlot version 12.0.

Cell cycle distribution using DNA flow-cytometry

The cells were treated with IC_{50} of *W. somnifera* methanolic extract for 48 hours and collected by trypsinization, washed with ice-cold PBS, and re-suspended in 0.5 ml of PBS. 10 ml of 70% ice-cold ethanol was added while vortexing, and cells were kept at +4 ˚C for 1 hour and stored at-20 ˚C until analysis. Fixed cells were washed and re-suspended in 1 ml of PBS containing 50 μg/ml RNase A and 10 μg/ml propidium iodide (PI). After 20 min incubation at 37˚C, cells were analyzed for DNA contents by FACSVantageTM (Becton Dickinson Immunocytometry Systems, San Jose, CA). For each sample, 10,000 events were acquired. Cell cycle distribution was calculated using CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) (Al-Abd et al., 2013).

Determination of caspase-3 activity

Caspase-3 activity in cells treated with IC_{50} of *W. somnifera* methanolic extract for 48 hours was determined using Human caspase-3 ELIZA kit (SunRed, Biotechnology Company) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis of the difference between treated and untreated groups was performed with Student's t-test. P-values less than 0.05 was considered statistically significant.

Results

In vitro cytotoxicity

SRB assay was used to assess the anti-proliferative effect of *W. somnifera* methanolic extract against MCF-7, HCT116 and HepG2 cells. The extract exerted high cytotoxic effect against all three cell lines as shown in table 1 and Figure 1. The highest toxic effect was found in the HepG2 cells with IC_{50} of 1.89 μg/ml and the minimum toxic effect was shown in the MCF-7 cells with IC_{50} of 3.35 μg/ml.

Caspase 3 activity

Caspase 3 activity was assessed in the treated cell lines in attempt to determine the effect of the methanolic extract of *W. somnifera* on the proteolytic phase of apoptosis. The methanolic extract of *W. somnifera* increased the concentration of caspase 3 in the HTC116 and the HepG2 cells by 112.8 % and 129.41% respectively. On the other

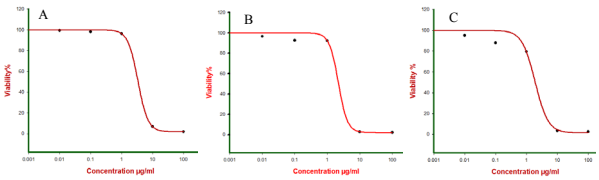

Figure 1. Dose response curve of W. somnifera leaves methanolic extract against: A: MCF-7, B: HCT116 and C: HepG2. Cells were incubated with different concentrations of the extract for 48 hours

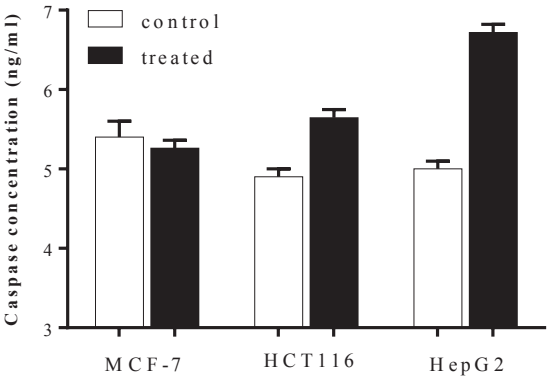

Figure 2. Effect of *W. somnifera* Methanolic Extract on Caspase 3 Concentration in Different Human Cell Lines. Cells were incubated with IC_{50} of the extract for 48 hours

Table 1. The IC_{50} (μg/ml) of *W. Somnifera* Methanolic Extract Against Human Cancer Cell Lines

Cell line	*W. somnifera* IC_{50}
MCF-7	3.35
HCT116	2.19
HepG2	1.89

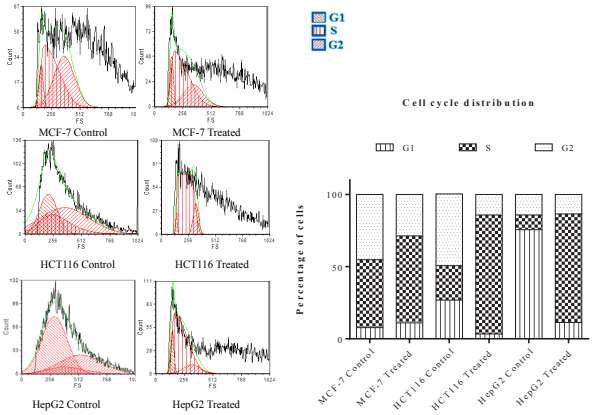

Figure 3. Effect of *W. somnifera* methamolic extract on cell cycle distribution in treated human cell line. Cells were incubated with IC_{50} of the extract for 48 hours.

hand, caspase 3 concentration didn't change markedly in the MCF-7 cells (Figure 2).

Cell cycle distribution

To assess the effect of *W. somnifera* methanolic extract on the cell cycle distribution, we used the DNA flow cytometry. The methanolic extract of *W. somnifera* caused cell cycle arrest at S phase in all cell lines after 48 hours of incubation. The treatment of the cell lines with the extract also decreased the percentages of G2 in MCF-7 and HCT116 cells and decreased of G1 fraction in HepG2 cells (figure 3).

Discussion

Withania somnifera (L.) Dunal, also known as Indian ginseng and Ashwagandha, is a medicinal plant with wide range of biological activities including anti-inflammatory, immuno-modulatory, anti-arthritis, anti-bacterial, anti-oxidant, anti-diabetic, anti-tumor and anti-inflammatory (Mishra et al., 2000; Kumar and Kushwaha, 2006; Winters, 2006; Widodo et al., 2008). In the present study we investigated the cytotoxic effects of the methanolic extract of *W. somnifera* leaves collected from Faifa mountains, southwest region, Saudi Arabia against Human breast cancer (MCF-7), Human colorectal carcinoma (HCT116) and Human hepatocellular carcinoma (HepG2). The Sulforhodamine B colorimetric assay (SRB assay) showed that the methanolic extract of *W. somnifera* has a strong cytotoxic effect on the three cell lines used in this study. The HepG2 cells was the most sensitive cells to the extract toxic effect with IC_{50} of 1.89 μg/ml. These results are in line with other studies. (Yadav et al., 2010) evaluated in vitro cytotoxic effect of 50% ethanol extract of leaves, stem and roots of *W. somnifera* against different human cancer cell lines. They have reported that leaves extract showed antiproliferative activity against HCT-116 (colon), NCI-H460 (lung), MCF-7 (breast) and NCI-H460 (lung) human tumor cell lines. In another study, antitumor activity of leaf extract from field raised *W. somnifera* plants was tested against breast carcinoma (MCF-7) cells and Osteogenic sarcoma (U2OS), and the extract arrested growth of both cell lines (Kaur et al., 2004). Water crude extract of *W. somnifera* root reduced viable cell count in dose and time dependent manner when tested against human malignant melanoma A375 cells (Halder et al., 2015).

In the present study, the DNA flow cytometry showed that the methanolic extract of *W. somnifera* leaves arrest the cell cycle in all three treated cell lines at S phase. On the other hand, caspase 3 activity was increased in the HTC116 and the HepG2 cells by 112.8 % and 129.41% respectively. Caspases are a family of cysteine proteases that plays a crucial role in the apoptotic response. They expressed in cells as inactive pro enzymes. The activation of caspases leads to initiation and execution of apoptosis. Caspase 3 is the most important key executioner of apoptosis which activated by other caspases, such as caspase 8 and caspase 9. Activation of caspase 3 causes cleavage of many cellular proteins that eventually leads to cell death (Earnshaw et al., 1999; Hengartner, 2000; Kim et al., 2002). Our study showed that the concentration of caspase 3 was elevated in the treated HTC116 and the HepG2 which indicates that methanolic extract of *W. somnifera* induced cell apoptosis by caspase mediated pathway. The lack of caspase activity increasing in MCF-7 cells suggests that there could be different mechanism of apoptosis other than caspase mediated pathway.

In conclusion, the present investigation demonstrated that the methanolic extract of *W. somnifera* leaves collected from Faifa mountains has comparable strong antiproliferative activities to those collected from different locations.

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