JBUON 2015; 20(2): 625-630
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

In vitro antiproliferative activity of partially purified *Withania* somnifera fruit extract on different cancer cell lines

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Summary

Purpose: *Cancer is a major health problem worldwide. There is a continuous need to search for safer and more effective alternatives to overcome the side effects and resistance of the chemotherapeutic agents. Therefore, in this study we investigated the antiproliferative activity and the apoptotic potential of Withania somnifera (W. somnifera).*

Methods: *W. somnifera was extracted with methanol and then solvent partitioned by sequential extractions with hexane, dichloromethane and ethyl acetate. Each extract was assayed for antiproliferative activity against different cancer cell lines using MTT assay. The nuclear morphology of HepG2 cells was investigated by DNA-binding fluorescent dye (Hoechst 33342 stain). The percentage of viability, death and apoptosis were evaluated by the Tali™ Image-based cytometer using annexin-V/PI (propidium iodide). A chromatographic fingerprint was constructed using high performance liquid chromatography (HPLC).*

Results: *The most potent anticancer activity of the crude extract was against HepG2 cell line (LC_{50}=164.7μg/ml). Dichloromethane fraction showed remarkable changes in the chromatin structure i.e., fragmentation, uniform condensation. Of the HepG2 cells 43.6% were apoptotic when treated with dichloromethane fraction for 24 hrs at 95μg/ml concentration. HPLC showed the presence of a major peak at 11.85 min.*

Conclusion: *W. somnifera may have the potential to serve as a template for future anticancer drug development. However, further investigation is required to identify the active compound/s.*

Key words: *apoptosis, cell lines, cytotoxicity, Withania somnifera*

Introduction

Cancer is a major health problem both in developed and developing countries. Malignancies are one of the largest single-cause of death claiming over six million lives every year [1,2]. A total of 95,183 cancer cases were diagnosed among the Gulf Cooperation Council GCC) States between January 1998 and 2007 [3]. The top cancer deaths among men and women in Saudi Arabia (2002) were due to breast and liver [4].

Chemotherapy is one of the common methods used for the treatment of different types of cancers, however, long-term use of chemotherapy can lead to drug resistance via gene mutation, DNA methylation and histone modification [5]. Accordingly, cancer patients are gradually developing resistance to commonly used anticancer agents, such as paclitaxel, 5-fluorouracil, cisplatin, doxorubicin, and campothecins [6]. Owing to this resistance, it is imperative to find new anticancer agents to circumvent the resistance mechanisms. Plant natural products have become an interesting source for this purpose. *W. somnifera* a well-known traditional herb that belongs to the Solanaceae family. It is found in the Arabian peninsula and locally known as Sum-ul-Firakh [7]. The major active compounds of *W. somnifera* is are an-

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Received: 18/10/2014; Accepted: 06/11/2014

olides that are isolated from its root and leaves. Several reports have demonstrated that this plant possesses antibacterial, antiinflammatory, antioxidant, antitumor and immunodulatory properties [8-10].

In this study, an effort was attempted to partially purify the crude extract of *W. somnifera* fruit using a bioassay-guided isolation procedure and exploring the anticancer potential of the unripe fruit crude extract and the fractions obtained against different cancer cells lines in terms of cell viability and apoptosis.

Methods

Plant material

W. somnifera fruits were collected from Riyadh in January 2014. The voucher specimen was deposited, botanically identified and authenticated in the Department of Botany and Microbiology, College of Science, King Saud University. 100 g of fresh unripe fruits were blended with 500 ml methanol and left to stir overnight at 150 rpm and 30°C. This procedure was repeated three times, adding fresh methanol each time to left over residues. The extract was centrifuged at 5000 rpm for 10 min and the supernatant was collected and evaporated under reduced pressure using rotary evaporator.

Partial purification of the crude methanolic extract by solvent partitioning with different polarity solvents

The methanolic extract of *W. somnifera* fruit was dissolved in 20% (v/v) methanol and then an equal volume of hexane was added and stirred for 5 min. The hexane phase containing the non-polar compounds was harvested. This process was repeated two more times and the hexane extracts were pooled, combined and the solvent was evaporated in a rotary evaporator at 45°C. The methanol phase was then mixed with an equal volume of dichloromethane and the same process was repeated as per hexane above. The process was repeated with ethyl acetate following the same procedure. The residual methanol phase was separately evaporated to dryness in a rotary evaporator at 45°C. All the fractions were dissolved in methanol and used for the tests.

Proliferation and cytotoxicity assays

All cell lines were obtained from the Department of Zoology, Faculty of Science, King Saud University.

The selected cancer cell lines for the in vitro screening of antiproliferative bioactivity were Colo20, HCT116 (human colorectal adenocarcinoma) DLD (human colorectal adenocarcinoma), MCF7 (human breast cancer), Jurkat (human T cell lymphoma), HepG2 (human hepatocarcinoma), and L929 (mouse fibrosarcoma cells). The cells were grown in a 24-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, USA). One ml cell suspension (10^5 cells/mL) was seeded in each well and incubated at 37 °C for 24 hrs in 5% CO_2. Extracts were tested for cytotoxic activity using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test. The cells were treated with extract for 24 hrs and then were replaced with fresh culture medium containing MTT (0.1 mg/ml). After 2-h incubation at 37°C, this solution was removed, and the resulting blue formazan was solubilized in 100 µl of 0.1% HCl-MeOH and the optical density was read at 595 nm using microplate reader (Biochem Ltd, England).

Hoescht 33258 staining

The cells were fixed with 3% paraformaldehyde for 20 min at room temperature, washed twice with phosphate buffered saline (PBS) and permeabilized in methanol. The cells were washed and stained with 0.1 µg/ml Hoescht dye for 15 min in the dark at room temperature. The plates were then rinsed with PBS 5 times and observed under a fluorescent microscope at 340-380 nm.

Tali™ analysis

Cells were plated in T25 flask and treated with 95 µg/ml of the extract. Cells were harvested 24 hrs post-treatment and stained using annexin-V Alexa Fluor® 488/PI (propidium iodide), according to the specifications of Tali™ apoptosis kit (Invitrogen, Carlsbad, CA, USA). Cell viability, death and apoptosis were evaluated using the Tali™ Image-based Cytometer (Life Technologies, USA). The annexin-V positive/PI negative cells were recognized as apoptotic cells by the cytometer software whereas the annexin V positive/PI positive cells were identified as dead cells. Similarly, the annexin V-negative/PI negative cells were identified as viable cells. The tests were performed in triplicate.

High performance liquid chromatography (HPLC) analysis

Samples were filtered through 0.2 µm polyvinylidene difluoride (PVDF) filter (Millipore) prior to HPLC analysis. Analysis was performed using a HPLC (Perkin Elmer Series 200, Norwalk, CT, USA) equipped with ultraviolet/visible detectors. Ten µl of the sample were injected onto the column and analyzed. The column used was C18 (250x4.6 mm) and was maintained at room temperature. A mobile phase consisting of water (A) containing 1% acetic acid and acetonitrile (B) was used as solvent system. The gradient program was organized as shown in Table 1.

Statistics

The results are presented as means ± standard deviation of 3 independent experiments. Statistical differences among means were determined by two-tailed Student's t-test. Differences were considered significant at $p< 0.05$ and $p<0.001$.

Table 1. The gradient program of HPLC mobile phase in profiling of dichloromethane fraction

Step	*Time (min)*	*Flow (ml/min)*	*Solvent A*	*Solvent B*	*Curve*
0	5	0.5	80	20	0
1	15	1	20	80	1
2	5	1	0	100	0
3	2	1	80	20	0

Results

In present study, methanol was used as the extraction solvent to provide crude *W. somnifera* extract. Several purification steps were performed by solvent partitioning with different polarity solvents on the methanol extract in order to enrich the selective antiproliferative bioactive extract. The yield of fruit total extract (weight of dried crude extract / weight of fresh fruit extract, g/g x 100%) was 1.54%. The yields of the hexane, dichloromethane, ethyl acetate, and methanol fraction (weight of dried fraction / weight of dried total extract from which the fraction is partitioned, g/g x 100%) were 1.46%, 3.2%, 2.75% and 37.0%, respectively.

In the present study, the anticancer properties of methanolic extract of unripe fruit of *W. somnifera* (L) were found cytotoxic against human and mouse cell lines Colo20, HCT116, MCF7, Jurkat, HepG2, and L929 but they were moderately active against DLD. The LC_{50} values of the methanolic extract of unripe fruit part of W. somnifera against Colo20, HCT116, MCF7 , Jurkat, HepG2 , and L929 were 287.8, 410.2, 226.3,356.4, 164.7,236.2 and 409.2 µg/mL, respectively. Figure 1 shows the cytotoxic activities of *W. somnifera* against the mentioned cell lines. The most potent anticancer activity of the crude extract was against HepG2 and MCF7 cell lines with LC_{50} values of 164.7 and 226 µg/mL, respectively. Solvent partitioning based on different solvent polarities was used for further purification. HepG2 cell line was chosen to test the anticancer activity using hexane, dichloromethane, ethyl acetate and methanol fractions. Dichloromethane fraction isolated from *W. somnifera* crude extract indicated anticancer properties on HepG2 cells with an IC_{50}= 95µg/mL, whereas the rest of the fractions did not show cytotoxic effects (Figure 1).

The morphology of the HepG2 cells was altered by dichloromethane fraction whereas the untreated cells have normal cellular morpholo-

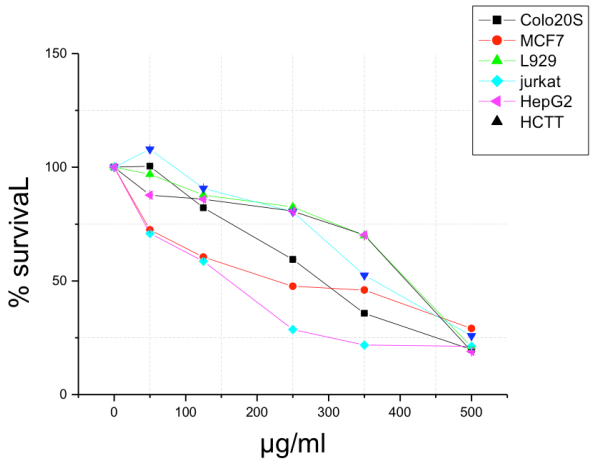

Figure 1. Cytotoxicity of crude methanol fruit extract of *W. Somnifera* on different cancer cell lines. Values are means of three experiments.

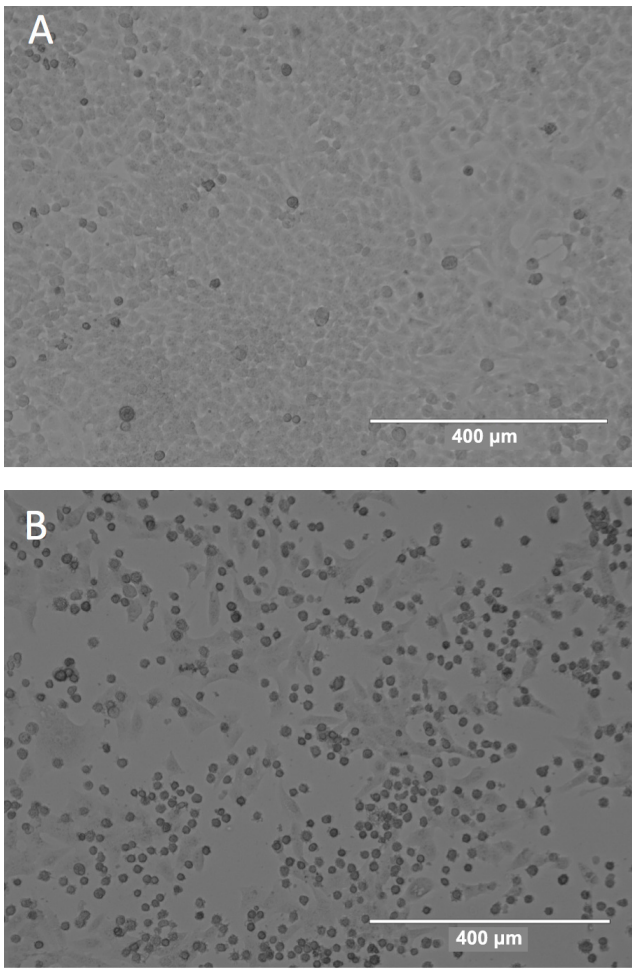

Figure 2. Inverted light photomicrographs (x10) of control and treated HepG2 cells with active dichloromethane fraction of *W. somnifera* (95µg/ml). **(A)** Control; **(B)** Treated culture showing shrinkage and detachment of the cells (24 h).

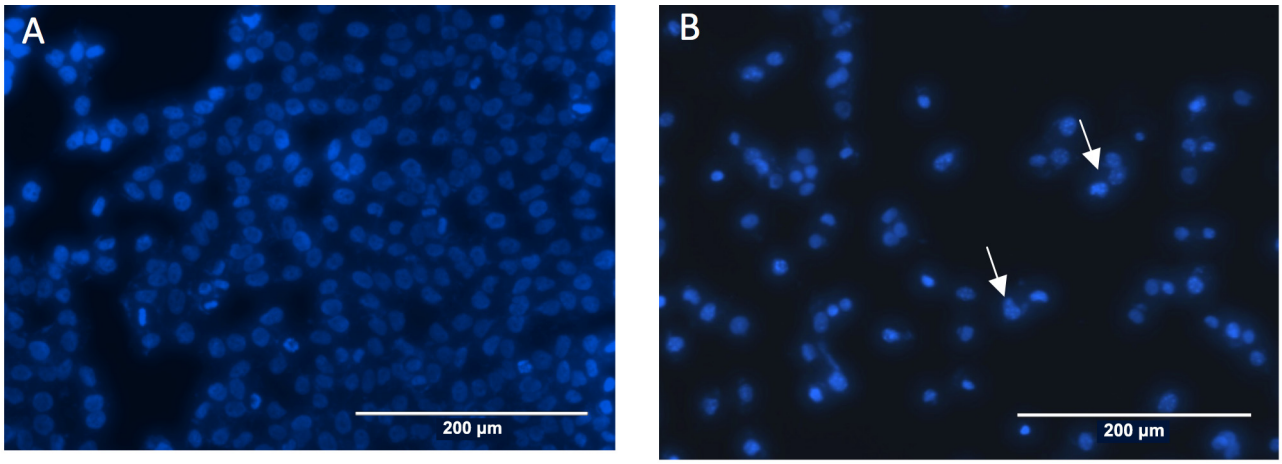

Figure 3. Fluorescent photomicrographs (x20) of cells stained with Hoechst 33342 and treated with active dichloromethane fraction of *W. somnifera* (95µg/ml). **(A)** Untreated controlled cells. **(B)** Treated cells. Arrows indicate cells with fragmented nuclei.

gies. Shrinkage and contraction of cytoplasmic materials were observed in HepG2 cells along with the detachment and complete loss of cellular integrity by dichloromethane fraction (Figure 2)

The effect of dichloromethane fraction on the nuclear morphology of HepG2 cells was investigated by DNA-binding fluorescent dye (Hoechst 33342 stain). As shown in Figure 3B, cells treated for 24 hrs with dichloromethane fraction at the concentration of 95µg/ml showed remarkable changes in the chromatin structure i.e., fragmentation, uniform condensation and formation of clusters against the nuclear periphery with brighter fluorescence. On the other hand, the untreated cells remained uniformly stained (Figure 3A).

Statistically significant induction of apoptosis appeared at 24 hrs with 95 µg/mL as compared with the control group ($p<0.01$). In HepG2, 43.6% of the cells were positive for annexin V following 24 hrs of treatment with the extract, compared with only 4.6% of the cells in the control group (Figure 4).

HPLC fingerprint (Figure 5) of *W. somnifera* dichloromethane fraction showed presence of various constituents as evidenced by the chromatogram obtained at various retention times (2.46, 3.34, 9.87, 10.57, 10.87, 11.59, 11.85 and 12.38 min).

Discussion

To the best of our knowledge and according to internet survey this is the first report on the antiproliferative activity of unripe fruit extract of *W. somnifera*. However, the cytotoxic potential of *W. somnifera* stem, leaves and roots are already established in various cancer cell lines including MCF7, MDA MB 231 (breast cancer), HeLa (cervical cancer), and HaCaT (human immortalized, but 'non-cancerous' cells) [11], HL-60 (human leukemia cells) [12], PC-3 and DU-145 (Prostate cancer), HCT-15 (colon cancer), A-549 (lung cancer) and IMR-32 (neuroblastoma) [13], and Panc-1, MiaPaCa2 and BxPc3 (pancreatic cancer cell lines) [14]. In the present study, the anticancer properties of methanolic extract of unripe fruit of *W. somnifera* (L) was found cytotoxic against all the cell lines tested. The most potent anticancer activity of the crude extract was against HepG2 and MCF7 cell lines with LC_{50} value of 164.7 and 226 µg/mL, respectively, but it was moderately active against human colorectal adenocarcinoma (DLD). Solvent partitioning based on different solvent polarities was used for further purification. HepG2 cell line was chosen to test the anticancer activity using hexane, dichloromethane, ethyl acetate and methanol fractions. Dichloromethane fraction isolated from *W. somnifera* crude extract indicated anticancer properties on HepG2 with an IC_{50}= 95µg/mL. The proliferation of cell lines tested for crude extract and the dichloromethane fraction were found to be reduced in a dose-dependent manner. The antiproliferative active fraction was probably due to the moderately polar fraction since the antiproliferative activity was only in the dichloromethane fraction at the tested concentration (Figure 1).

Induction of apoptosis has been recognized as a sign for the identification of an anticancer

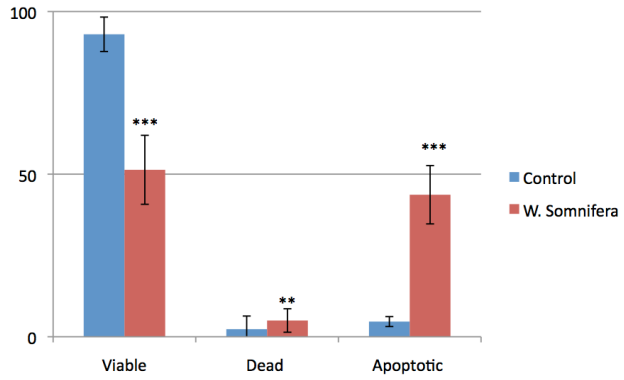

Figure 4. Percents of viable, dead and apoptotic cells after treatment with dichloromethane fraction of *W. somnifera* at the concentration of 95µg/ml for 24 hrs. All experiments were performed in triplicate and values are means of the 3 experiments ± standard deviation; *** p<0.001 in comparison to the untreated control group; **p<0.05 in comparison to the untreated control group.

agent [15]. Chemotherapeutic agents act primarily by inducing cancer cell death through apoptosis [16]. One of the interesting findings of this study was that the dichloromethane fraction induced cell death through apoptosis. Our results also showed that the dichloromethane fraction of *W. somnifera* seeds also induced apoptosis when analyzed by Annexin V/PI double parameter assay. This data correlated with the morphological studies. The morphology of the HepG2 cells was altered by dichloromethane fraction, whereas the untreated cells showed normal cellular morphologies. Shrinkage and contraction of cytoplasmic materials were observed in HepG2 cells along with the detachment and complete loss of cellular integrity by dichloromethane fraction (Figure 2). Additionally, cells undergoing apoptosis exhibit a very similar pattern of morphological changes. Nuclear changes such as chromatin condensation and DNA fragmentation are characteristic marks of apoptotic cells. As shown in Figure 3B, cells treated for 24 hrs with dichloromethane fraction at 95µg/ml concentration showed remarkable changes in the chromatin structure i.e., fragmentation, uniform condensation and formation of clusters against the nuclear periphery with brighter fluorescence. On the other hand, the untreated cells remained uniformly stained (Figure 3A).

According to Springfield et al. [17] the HPLC fingerprinting is the best way for chemical characterization. Although the fraction may contain several compounds, it certainly has potent anticancer compound/s which could be developed as a plant drug in cancer patients before it is systematically analyzed for the isolation, characterization and testing of anticancer compounds.

Acknowledgement

This Project was supported by King Saud University, Deanship of Scientific Research, College of Sciences Research Center.

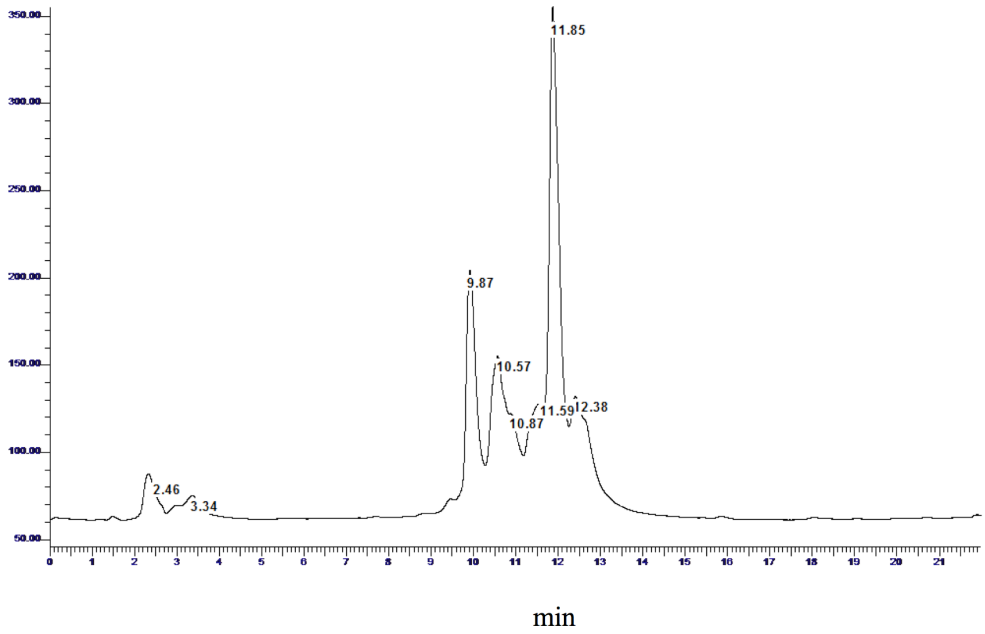

Figure 4. HPLC profile showing the main components present in the dichloromethane fraction at 254 nm.

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