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Cytotoxic Effects of Alcoholic Extract of Dorema Glabrum Seed on Cancerous Cells Viability

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ABSTRACT

Purpose*:** In the present study cytotoxic effects of the alcoholic extract of Dorema Glabrum seed on viability of WEHI-164 cells, mouse Fibrosarcoma cell line and L929 normal cells were compared with the cytotoxic effects of Taxol (anticancer and apoptosis inducer drug). ***Methods: To find out the plant extract cytotoxic effects, MTT test and DNA fragmentation assay, the biochemical hallmark of apoptosis were performed on cultured and treated cells. ***Results:*** According to the findings the alcoholic extract of Dorema Glabrum seed can alter cells morphology and because of chromatin condensation and other changes they shrink and take a spherical shape, and lose their attachment too. So the plant extract inhibits cell growth albeit in a time and dose dependent manner and results in degradation of chromosomal DNA. ***Conclusion***: Our data well established the anti-proliferative effect of methanolic extract of Dorema Glabrum seed and clearly showed that the plant extract can induce apoptosis and not necrosis in vitro, but the mechanism of its activities remained unknown. These results demonstrated that Dorema Glabrum seed might be a novel and attractive therapeutic candidate for tumor treatment in clinical practices.

Introduction

Cancer with high death rate, second only to cardiac arrest comprises at least 100 different diseases. All cancer cells share one important characteristic; they are abnormal cells in which the processes regulating normal cell division are aberrant. Cell cycle and growth control are profoundly relevant to biological regulation of development and tissue renewal. Apoptosis (programmed cell death) was a term introduced in 1972 to distinguish a mode of cell death with characteristic morphology and apparently regulated, endogenously driven mechanism.[1,2] Defective apoptosis represents a major causative factor in the development and progression of cancer. Our understanding of the complexities of apoptosis and the mechanisms evolved by tumor cells to resist engagement of cell death have focused research efforts into the development of strategies designed to selectively induce apoptosis in cancer cells.[3-5]

There are considerable efforts to identify naturally occurring substances as new drugs in cancer therapy.[6-9] A number of chemotherapeutic agents, with properties including apoptosis induction and anti-angiogenesis, have been isolated from natural products and characterized to prevent the development of malignancies, such as curcumin from Curcuma longa, epicatechin gallate from tea, paclitaxel from Pacific yew[10] Emodin, a natural anthraquinone derivative from Rheum palmatum L[11] and Honokiol, a biphenyl extract from Magnolia obovata bark.[10] Understanding the modes of action of these compounds should provide useful information for their possible applications in cancer prevention and perhaps in cancer therapy.[12-13] Approximately half of the drugs currently in clinical use are of natural origin.[7,14,15] Although herbal therapies are becoming increasingly popular worldwide, we know little about the molecular mechanisms and active ingredients in many of those therapeutic herbs.[7,16] Some of them tend to possess functional groups (providing hydrogen bond acceptor/donors, etc).[7]

Dorema glabrum is a species that grows in Transcaucasia (Nakhichevan and Armenia zone) and North West of Iran. The genus Dorema from Apiaceae family is represented by seven species in Iranian flora,

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among them Dorema glabrum Fisch. C.A. Mey, D. aucheri Boiss and D. ammonicum D. Don are endemic.[17] Dorema glabrum which grows in loamy or rocky slopes is a perennial herb. It is useful. as an herbal remedy or food additive in mentioned regions.[18] According to the common folk believes of Armenian and Azeri people, D glabrum can suppress different kinds of cancer. We aimed to study this matter by a scientific work; hence the effects of alcoholic extract of D. Glabrum seed on WEHI-164 cell line viability were investigated. Of course it should be mentioned in a preliminary work, antioxidant activity and anti-lipidemic effects were seen in the crude extract of the plant.[19]

In the present study cytotoxic effects of the extract of Dorema Glabrum seed on WEHI-164 cells were compared with its effects on L929 normal cells in contrast with the effects of Taxol as a positive control. Taxol which contains Paclitaxel as the main active compound is used in chemotherapy of cancer. Paclitaxel ($C_{47}H_{51}NO_{14}$, MW=853.9 Da) is yielded from Yew tree and its anticancer effects was known since 1971.[20,21]

Materials and Methods

Plant Material

Seeds of Dorema glabrum Fisch. C.A. Mey were collected during the fruiting stage from slopes of Aras River bank; Jolfa, Eastern Azerbaijan (38 30' 9.2", 45 27'36.2"; 1590 m, 15 km from Jolfa to St. Stephanus Church), Iran. Air dried and finely powdered seeds were subjected to extraction by refluxing Methanol in a soxhlet in order to obtain its ooze. Then the extract was dried using a Rotary Evaporator (Heidolph, Germany). 20 mg of dried extract were dissolved in 100 µl DMSO and diluted with 3.90 ml RPMI-1640 to give a concentration of 5000 µg/ml. The cells were treated with different concentrations (10, 30, 50, 100, 200, 300, and 400 µg/ml) of the extract.

Cell culture

WEHI-164 cells, mouse Fibrosarcoma cell line (NCBI Code: C200) and L929 cells, mouse normal adipose tissue cell line (NCBI Code: C161) were obtained from National Cell Bank of Iran (Pasteur Institute, Iran-Tehran). WEHI-164 cell line was originally established by M Rollinghoff and NL Warner from a fibrosarcoma induced by subcutaneous injections of 3-methylcholanthrene to Balb/c mice[22,23] and L929 cells, one of the first to be established in continuous culture, subclone of parental strain L, established by W R Earle in 1940. The L strain was derived from normal subcutaneous areolar and adipose tissue of a 100 day old male C3H/An mouse. These cells are $APRT^+$ (Adenine Phodphoribosyl Transferase) and $HPRT^+$ (Hypoxanthine-Guanine Phosphoribosyl Transferase).[24] The both cell lines were cultured in RPMI-1640 (Sigma, Germany, pH=7.2) containing 10% FCS (Fetal Calf Serum) and antibiotic (100 U/ml Penicillin, 100 µg/ml Streptomycine, Gibco), placed in 37 °C and 5% CO_2 in an incubator (Memert, Germany) overnight.

MTT Test

MTT assay is one of the most useful tests for investigating cells viability and cytotoxic effects of drugs, cosmetics and food additives. MTT (3-[4, 5-dimethyl-2-thiazolyl]-2, 5 diphenyl tetrazolium bromide) which is yellow and soluble in water, can be reduced by mitochondrial dehydrogenases of live cells to give a bluish purple and insoluble salt called Formosan that can easily and rapidly be quantitated by an ELISA plate reader at 570 nm.[25,26]

WEHI-164 and L929 cells were separately seeded, in a triplicate manner, in 96-well microplates (5000 cells/well) with RPMI-1640 containing 10% FCS (Fetal Calf Serum) and antibiotic (total volume of 200 µl), placed in 37 °C and 5% CO_2 in the incubator. After 6 hours both cells were treated with different concentrations (10, 30, 50 and 100, 200, 300, and 400 µg/ml) of alcoholic extract of Dorema glabrum seeds with different time periods (6, 24 and 36 hours). No plant extract was added to negative controls, but the same amount of DMSO was added to eliminate its intervening effects, if any. Positive control cells were treated with Taxol (Onco-time, Australia) as the same concentrations of plant extract in test cells. Of course prior to treatment the cells viability was determined by counting on a Neubauer slide (Hemocytometer) with the aid of Trypan blue. Trypan blue can penetrate into dead cells' membrane and colour them purple.

After desired time the supernatants of all wells were discarded and washed with PBS, then 100 µl of RPMI and 50 µl of MTT solution (2 mg/ml) were added to each well. Following incubation at 37 °C for 4 hours the liquid phase of wells were discarded again. After adding 200 µl DMSO and 25 µl Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl, pH=10.5) the plates were incubated at 37 °C in the dark for another half an hour. At the end absorbencies of wells were determined at 570 nm wavelength using a microplate reader (Awareness technology, USA).

DNA fragmentation Assay

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability (prelytic DNA fragmentation). In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+} dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono and oligonucleosomal DNA fragments.[25] The DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis. This assay involves extraction of DNA from a lysed cell homogenate followed by agarose gel electrophoresis. This, results in a characteristic "DNA

ladder" with each band in the ladder separated in size by approximately 180 base pairs. This methodology which is easy to perform, has a sensitivity of 1×10^6 cells (i.e., level of detection is as few as 1,000,000 cells), and is useful for tissues and cell cultures with high numbers of apoptotic cells per tissue mass or volume, respectively.[4]

DNA Extraction

Both cell lines, WEHI-164 and L929 were separately cultured in 6-well plates (1200000 cells/well) with RPMI-1640 supplemented by 10% FCS and antibiotic, placed in 37 °C and 5% CO_2 in the incubator. After 6 hours the cells were treated with different concentrations of plant extract (0, 30, 50 and 100 µg/ml) for different time periods (24 and 36 hours). Then the wells were washed with PBS buffer and cells were detached from the plates with the aid of Trypsin-EDTA (Gibco, Germany). The cells pellets were removed to falcon tube and 500 µl of lysis buffer were added. 10 µl of proteinase K (Fermentas, Life Sciences), 20 mg/ml were added, followed by incubation at 56 °C overnight. The next day 40 µl of saturated NaCl (5 M) were added and mixed completely and incubated at 4 °C for 10 minutes. After centrifugation in 12000 RPM for 20 minutes, their upper liquids were transferred to a fresh microtube and 1 ml of cold ethanol 100 % (stored in -20 °C) was added. The procedure was continued by incubation at -20 °C for 10 minutes, followed by centrifugation for 15 minutes in 12000 RPM. Then ethanol in upper phase was removed completely and 1 ml of ethanol 70 (kept in 4 °C) was added and mixed well by pipetting up and down. Next the samples were centrifuged again for 10 minutes in 12000 RPM, followed by removeing ethanol completely. After drying the samples in room temprature or 37 °C for 10-20 minutes, the pellets were dissolved in 100 µl distilled, deionised and sterile water or TE (Tris/EDTA). The samples concentrations were determined using a nanodrop UV spectrophotometer and equivalent amount of DNA samples diluted with the 6X DNA loading dye (supplied with the ladder) were subjected to 1.5 % agarose submarine electrophoresis in company with DNA ladder marker (Fermentas, Life Sciences, 1 kb DNA Ladder). Finally the fragmented DNAs bands were visualized by UV transilluminator (UVP, USA) following ethidium bromide staining.

Statistical analysis

All the data represented in this study are means±SEM of three identical experiments made in triplicate. Statistical significance was determined by independent T-test and p value ≤0.05 was considered significant. All analyses were conducted using the SPSS 20.

Results

Natural and live WEHI-164 cells are fusiform or spindle like (Figure 1A). But after treatment with Taxol or methanolic extract of Dorema glabrum seed they undergo morphological changes and because of chromatin condensation and other changes they shrinke and take a spherical shape (Figure 1B), characteristics of apoptotic cells.

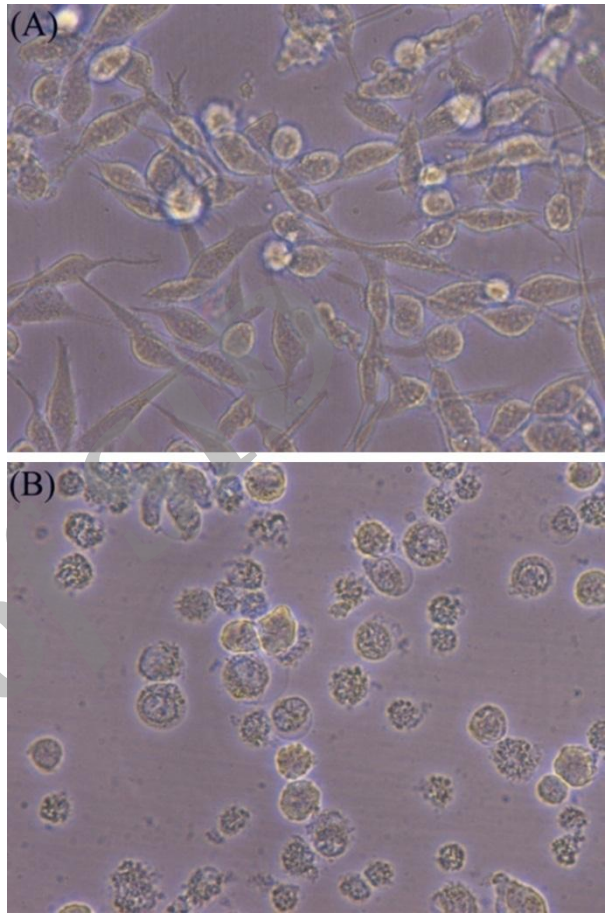

Figure 1. Panel A, Spindle like natural and live WEHI-164, 40X Panel B, Spherical apoptotic WEHI-164 cells, 40X.

Cells viability

Using Neubauer slide (Hemocytometer) and with the aid of Trypan blue the cells viability before treatment was determined >94%.

MTT Test

The antiproliferative effect of plant extract was determined by MTT method which showed a time and dose-dependent inhibition of the cell growth. Also effecivity of plant extract on both cell lines viabilities follows the same pattern as Taxol.

As the Figure 2 shows IC_{50} value, the concentration that causes 50% loss of cell viability, in WEHI-164 cell line is about 50 µg/ml in 36 hours for the plant extract. By contrast the plant extract had higher IC_{50} value (about 100 µg/ml in 36 hours) for normal L929 cells, meaning it is toxic to the normal cells in higher concentrations than WEHI-164 cells.

Statistical analysis using independent T-test was performed to show significant differences of

cytotoxicity effects of 50 µg/ml plant extract in 36 hours to WEHI-164 and L929 cells. The test resulted in P<0.0001, meaning that 50 µg/ml plant extract affects cancerous cells viability more than normal cells.

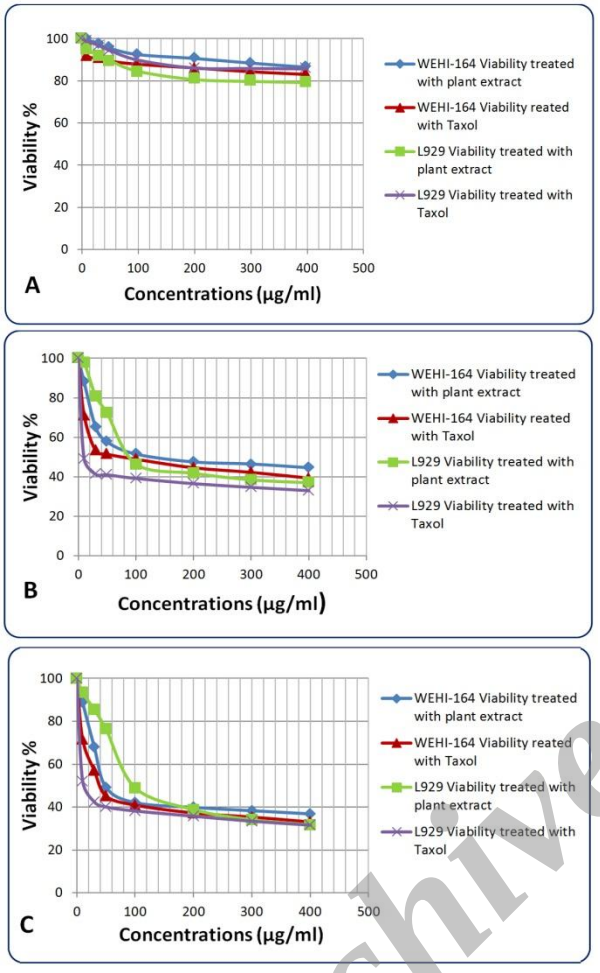

Figure 2. The viability of WEHI-164 and L929 cells treated with the different concentrations of plant extract in the different time periods in contrast with that of the cells treated with Taxol. A: Viability of cells after 6 hours, B: Viability of cells after 24 hours, C: Viability of cells after 36 hours

DNA fragmentation

DNA fragmentation can be analysed by the typical ‘‘DNA ladder’’ formation, for which DNA is extracted from the apoptotic cells and separated in an agarose gel. As shown in Figure 3 treatment with Dorema Glabrum seed extract resulted in degradation of chromosomal DNA into small internucleosomal fragments, a biochemical hallmark of cells undergoing apoptosis.

Discussion

Despite a period in which pharmaceutical companies cut back on their use of natural products in drug discovery, there are many promising drug candidates in the current development pipeline that are of herbal origin. After all, traditional cytotoxic chemotherapy although kills cancer cells by indirectly inducing apoptosis unfortunately, side effects are brutal, and most tumors become resistant.[27-29]

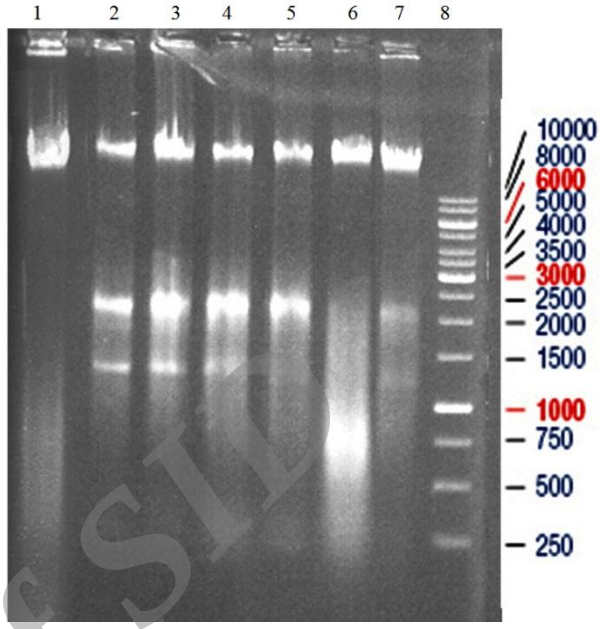

Figure 3. DNA ladder formation. From left Lane 1: negative control, Lane 2-4 treated cells with different concentrations (30, 50, 100 µg/ml) of plant extract in 24 hours, Lane 5-7 treated cells in 36 hours, and Lane 8 Ladder (1 kb).

To evaluate the effects of Dorema Glabrum seed extract on cell proliferation and identify its therapeutic potential we demonstrated, for the first time, the potent cytotoxic activity of different concentrations of methanolic extract of Dorema Glabrum seed against WEHI-164 Mouse Fibrosarcoma cell line and L929 normal cell line. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damages to normal cells, meaning minimum side effects. This ideal situation is reachable by inducing apoptosis in cancer cells. Cell cycle modulation by various natural and synthetic agents is gaining widespread attention in recent years.[13]

In the present study prior to treatment, first we determined cells viability by counting on a Neubauer slide (Hemocytometer) with the aid of Trypan blue. Trypan blue can penetrate into dead cells’ membrane and colour them purple. The outcome was >94%. Then MTT assay was performed which showed that the methanolic extract of Dorema Glabrum seed causes growth inhibition in the WEHI-164 Mouse Fibrosarcoma cells in a dose and time dependent manner. But it appeared less toxic in low concentrations to normal or nonmalignant cells in vitro, because IC_{50} value of the plant extract for WEHI-164 cells is 50 µg/ml but for L929 cells is 100 µg/ml in 36 hours. So higher doses of plant extract were effective in L929 normal cells than in tumor cells. This claim was confirmed by statistical analysis using independent T-test that resulted in P<0.0001, meaning that the mean

differences of cytotoxicity effects of 50 µg/ml plant extract in 36 hours to WEHI-164 and L929 cells are significant. 36 hours treatment was selected because in shorter times higher concentrations of plant extract were needed to cause 50% loss of cell viability. Since concentrations more than 50 µg/ml affect L929 cells viabilty too, it is preference to choose 36 hours treatment with 50 µg/ml plant extract in order to avoid massive damages to normal cells. Also we compared the effects of plant extract with the effects of Taxol, an anticancer and apoptosis inducer drug and it should be mentioned here that effects of plant extract on both cell lines followed the same pattern as Taxol effects on the cells (Figure 3).

Microscopic studies showed morphological changes of the cells too. Chromatin condensation, cell shrinkage and other alterations, characteristics of apoptotic cells, cause the morphology of treated cells with the plant extract, change from spindle like to spherical shape and also make them to lose their attachment (Figure 2). In conclusion the plant extract induced apoptosis and not necrosis in treated cells.

Also apoptosis induction was confirmed by DNA ladder technique. Treatment with the plant extract resulted in degradation of chromosomal DNA into smaller fragments (Figure 3), a biochemical hallmark of cells undergoing apoptosis.[4] Once more induction of apoptosis and not necrosis, by plant extract was confirmed, because electrophoresis of necrotic cells' DNA results in smear not ladder.

Conclusion

In conclusion our data, well established the anti-proliferative effects of methanolic extract of Dorema Glabrum seed and clearly showed that the plant extract can induce apoptosis and not necrosis in vitro, but its activities in vivo and mechanisms of its actions remained unknown. These results demonstrated that Dorema Glabrum seed with anti-proliferative properties, especially with IC_{50} value for cancerous cells lower than that of normal cells, might be a novel and attractive therapeutic candidate for tumor treatment in clinical practice.

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Conflict of Interest

The authors report no conflicts of interest.

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